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Review

A brief introduction to cell-penetrating peptides

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Cell membranes act as protective walls to exclude most molecules that are not actively imported by living cells. This is an efficient way for a cell to prevent uncontrolled influx or efflux of solutes, which otherwise would be harmful to it. Only compounds within a narrow range of molecular size, polarity and net charge are able to diffuse effectively through cell membranes. In order to overcome this barrier for effective delivery of membrane-impermeable molecules, several chemical and physical methods have been developed. These methods, e.g. electroporation, and more recent methods as cationic lipids/liposomes, have been shown to be effective for delivering hydrophobic macromolecules. The drawbacks of these harsh methods are, primarily, the unwanted cellular effects exerted by them, and, secondly, their limitation to *in vitro* applications. The last decade's discovery of cell-penetrating peptides translocating themselves across cell membranes of various cell lines, along with a cargo 100-fold their own size, via a seemingly energy-independent process, opens up the possibility for efficient delivery of DNA, antisense peptide nucleic acids, oligonucleotides, proteins and small molecules into cells both *in vitro* and *in vivo*.

Keywords: **cell-penetrating peptides; translocation; delivery vector; cytotoxicity**

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INTRODUCTION

Cell-penetrating peptides (CPPs) are defined as peptides with a maximum of 30 amino acids, which are able to enter cells in a seemingly energy-independent manner, thus being able to translocate across membranes in a non-endocytotic fashion (Langel, 2002). Depending on author and origin of the peptide, different names for these peptides have been proposed in the literature. CPPs derived from proteins are often referred to as protein transduction domains (PTDs) (Schwarze *et al.*, 1999; Mi *et al.*, 2000), and are, as the name indicates, the domains from a protein responsible for its transduction. Membrane translocation sequence (MTS) is another name frequently used to describe the ability of these peptides to cross plasma membranes. This discrepancy in notation can be very confusing for readers not familiar with the field of the research.

The first indication that proteins might contain sequences responsible for their translocation across cell membranes derived from the observation that living cells internalized an 86 amino acid-long fragment from the HIV-1 Tat protein, Tat-86 (Green and Loewenstein, 1988). Some years later it was discovered that the 60 amino acid homeodomain of the Antennapedia protein of *Drosophila* was also able to translocate over cell membranes (Joliot *et al.*, 1991). In order to understand the driving force for the internalization, the homeodomain was modified by site-directed mutagenesis, leading to the discovery that its third helix was necessary and also sufficient for membrane translocation, which resulted in the development of a 16 amino acid-long CPP called penetratin, now often referred to as pAntp (Table 1; Derossi *et al.*, 1994). It was soon followed by other protein-derived CPPs, e.g. the basic sequence of the HIV-1 Tat protein (Vivès *et al.*, 1997), chimeric peptides like transportan (Pooga *et al.*, 1998a) and also by synthetic peptides such as the amphipathic model peptide (Oehlke *et al.*, 1998).

Further evaluation of these newly described peptides as delivery vectors led to the successful down-regulation of the amyloid precursor protein, by linking its antisense DNA to penetratin (Allinquant *et al.*, 1995), and successful down-regulation of the galanin receptor *in vivo* by a transportan–peptide nucleic acid (PNA) conjugate (Pooga *et al.*, 1998b). These works opened up a whole new window of opportunities for delivery of hydrophobic macromolecules without disturbing the stability of the cell membrane and with seemingly low toxic effects. Since then numerous experiments have been performed with improvement of cargo delivery by CPP attachment to the bioactive molecules, ranging from small peptides to liposomes (Torchilin *et al.*, 2001).

What makes it possible for these specific peptide sequences to tranclocate across biological membranes is still much debated. Endocytosis does not seem to be required for translocation (Silhol *et al.*, 2002); however, some recent reports state that the uptake of both Tat and penetratin is mainly endocytotic (Koppelhus *et al.*, 2002). Another recent

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Abbreviations used: CPP, cell-penetrating peptide; MAP, model amphipathic peptide; MTS, membrane translocation signal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMR, nuclear magnetic resonance; PFA, paraformaldehyde; PNA, peptide nucleic acid; PrP, prion protein; PTD, protein transduction domain.

Table 1. Selection of cell-penetrating peptides used for cargo delivery

Name	Sequence	Reference
Penetratin	RQIKIWFQNRRMKWKK[a]	Derossi *et al.* (1994)
Tat	YGRKKRRQRRR[b]	Vivès *et al.* (1997)
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD amide	Oehlke *et al.* (1997)
MAP	KLALKLALKALKAALKLA amide	Oehlke *et al.* (1998)
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL amide	Pooga *et al.* (1998a)
Transportan-10	AGYLLGKINLKALAALAKKIL amide	Soomets *et al.* (2000)
pVEC	LLIILRRRIRKQAHAHSK amide	Elmquist *et al.* (2001)
pISL	RVIRVWFQNKRCKDKK amide	Kilk *et al.* (2001)
$(R)_7$	RRRRRRR[c]	Rothbard *et al.* (2000)
Pep-1	KETWWETWWTEWSQPKKKRKV[c]	Morris *et al.* (2001)
Mouse PrP^C (1–28)	MANLGYWLLALFVTMWTDVGLCKKRPKP amide	Lundberg *et al.* (2002)

[a] Originally synthesized as free acid C-terminally, but later also shown to have CPP properties when C-terminally amidated (Hällbrink, 2001).
[b] Synthesized as free acid and prolonged C-terminally with cysteine.
[c] C-terminally modefied with a cysteamide group.

report claims that the observed internalization of the peptides Tat and $(Arg)_9$, might simply be cell-fixation artefacts (Richard *et al.*, 2003). This would also explain the observed internalization of these peptides at 4 °C. Even though the mechanism of CPP uptake is much debated, the importance of CPPs as delivery vectors is still significant.

MECHANISMS OF MEMBRANE TRANSLOCATION

One might hypothesize that the amino acid sequences responsible for the translocation were developed by nature in order to facilitate transport of certain proteins and peptides. In favor of this theory is the finding that antimicrobial peptides, such as buforin, are able to rapidly translocate across membranes (Takeshima *et al.*, 2003). It is also possible that translocation sequences of proteins might be used for infection in diseases with unknown mechanism of infection, such as prion protein related disorders (Lundberg *et al.*, 2002).

Independence of endocytotic and receptor mediated uptake

Uptake of CPPs is often unaffected by endocytosis inhibitors, confirming a non-endocytotic pathway of internalization, and no reduced uptake can be observed at 4 °C compared with 37 °C, showing an energy-independent mode of entry. What also rules out the receptor-mediated uptake is the fact that substitution of a CPP sequence to its D-enantiomer shows no decrease in internalization, for penetratin, pVEC and Tat, respectively, and the D-enantiomer of $(Arg)_9$ showed even higher uptake (Brugidou *et al.*, 1995; Wender *et al.*, 2000; Elmquist *et al.*, 2001).

Recently it was suggested that heparan sulphate proteoglycans, which are expressed in most cell types, could be responsible for the internalization of the full-length Tat protein (Tyagi *et al.*, 2001). This process was also confirmed to be important for the Tat peptide, as well as for $(Arg)_8$ (Suzuki *et al.,* 2002). Contradictory to the non-endocytotic theory is a recent paper by Koppelhus *et al*. (2002) claiming that the uptake of penetratin and Tat is endocytotic, and varies depending on cargo. It might be that these controversial reports are just reflecting two sides of a coin, showing that there are different competing mechanisms contributing to the uptake of the peptides. There is also a possibility that, considering the physico-chemical diversity of the known CPPs, different mechanisms of internalization occur for different CPPs.

Important peptide features needed for membrane translocation

In order to understand what makes these specific sequences cell-penetrating, one can compare physico-chemical characteristics from different CPPs. The only consistently found feature present in all CPPs is the high content of basic amino acids, resulting in a positive net charge. Rothbard *et al.* (2000) showed that cyclosporin A was efficiently delivered into dermal T lymphocytes and inhibited inflammation by linking to a hepta-arginine segment, suggesting that positive charge is the required feature for cellular translocation. Futaki *et al.* (2001) showed that charge itself is not sufficient for translocation, showing that an octamer of Arg, $(Arg)_8$ was successfully internalized, whereas $(Arg)_{16}$ did not show any significant signs of translocation. The importance of Arg was further demonstrated by Wender *et al.* (2000) by showing the critical role of the guanidine headgroup, as homopolymers of citrullin showed no CPP properties. However, increasing the distance between the guanidine group and the peptide backbone increased the uptake. The importance of the secondary structure has also been investigated and, generally, an α-helical structure has been predicted for CPPs. This assumption was verified for some cases, although for penetratin the original α-helical structure maintained in the parental Antennapedia homeodomain can adopt a β-sheet structure in the presence of a charged lipid monolayer (Bellet-Amalric *et al.*, 2000). Furthermore,

Oehlke *et al.* (1997) showed that an amphipathic β-sheet peptide was able to enter cells efficiently.

Recent reports claim that the α-helicity can be retained in the presence of SDS micelles, which are often used as phospholipid mimics. This is the case for transportan, which forms a random coil in water, but it is mainly α-helical in the presence of SDS (Lindberg and Gräslund, 2001; Magzoub *et al.,* 2003).

Suggested mechanisms of membrane translocation

The most popular mechanisms suggested for the translocation of CPPs are: the inverted micelle model (Fig. 1), the carpet model [Fig. 2(A)] and the pore formation model [Fig. 2(B)]. It should be noted that there may be other mechanisms of uptake, and also that different CPPs might utilize different mechanisms.

The inverted micelle model of internalization was proposed by Alain Prochiantz's group, based on NMR studies of the interaction of penetratin with phospholipid membranes (Derossi *et al.*, 1996; Fig. 1). According to the hypothesized mechanism the positive charge of the peptide interacts with the negative charge of the phospholipid membrane, followed by a membrane shuttling with the aid of the interaction of the hydrophobic amino acids with the membrane. Although this model can explain the translocation for some CPPs, it is not sufficient to explain the uptake

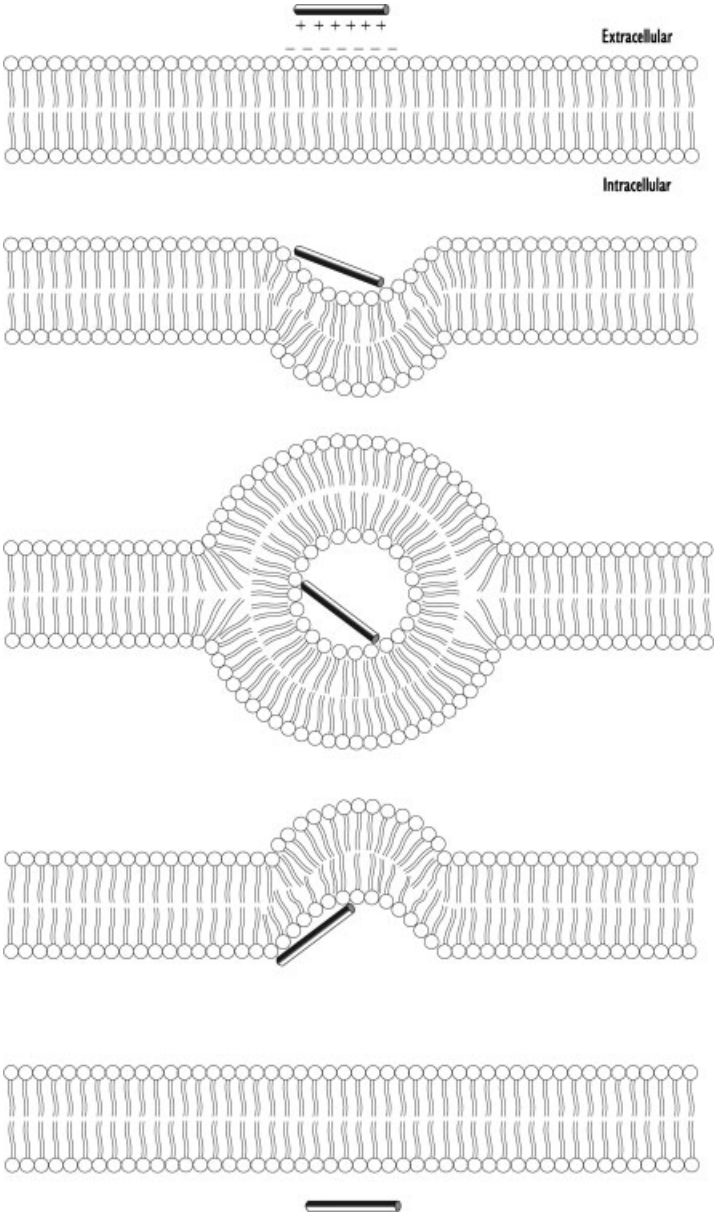

Figure 1. The inverted micelle model for CPP translocation (Derossi *et al.*, 1996). The positively charged part of the peptide interacts with the phospholipids in the membrane, followed by the interaction of the hydrophobic part of the peptide with the membrane, creating the inverted micelle. (Picture adapted from Elmquist 2003, with permission.)

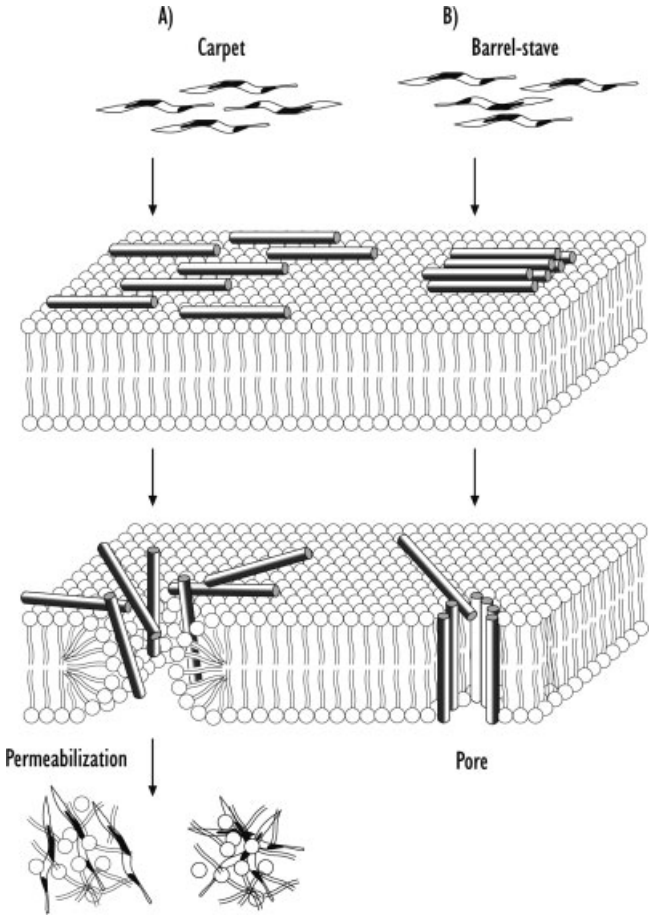

Figure 2. Illustration of the possible interactions of the peptides with the phospholipid membranes, and different mechanisms for translocation. Before the contact with the membrane, the peptides have no distinct secondary structure, but as they interact with the plasma membrane, they form amphipathic structures. (A) The carpet model (Pouny *et al.*, 1992), where the positive part of the peptide interacts with the phospholipids in the membrane, followed by a 'flip' by the hydrophobic part of the peptide towards the hydrophobic core of the membrane, finally leading to the disruption of the membrane, and translocation of the peptide. (B) The pore formation model (barrel-stave) (Gazit *et al.*, 1994). The amphipathic α-helices form bundles, creating a pore in the membrane leading to the peptide translocation. (Picture adapted from Elmquist 2003, with permission.)

of both Tat and the poly-arginine peptides, which do not contain the hydrophobic amino acids necessary for the translocation process.

The 'carpet model' [Fig. 2(A)] was first presented as a model for translocation of some antimicrobial peptides (Pouny *et al.*, 1992; Matsuzaki *et al.*, 1999), but could also be applicable as a model for some CPP uptake due to their demonstrated toxicity at higher concentrations. According to this model, it is a three-step process initiated by the interaction of the peptide with the negatively charged phospholipids, resulting in a change of secondary structure as the basic residues turn towards the membrane surface. The second step involves the rotation of the peptide leading to the interaction between the hydrophobic residues of the peptide with the hydrophobic core of the membrane. In the third step, a small disruption in the lipid packing occurs, which permits the internalization of the peptide. This is a possible mechanism for cellular entry of CPPs; however, it is mostly suggested as a mechanism used by antimicrobial peptides to exert their toxicity.

The third proposed model for cellular translocation is the pore formation (barrel-stave) model [Fig. 2(B)]. This is one of the ways that antimicrobial peptides weaken the bacterial membrane, leading to its depolarization (Gazit *et al.*, 1994). Pore formation is a result of bundles formed by amphipathic α-helical peptides, when the outwardly facing hydrophobic residues interact with the lipid membrane, and the inwardly facing hydrophilic surfaces form the pore.

Since some of the peptide uptake mechanisms seem to be concentration-dependent, it is possible that, depending on the concentration of the CPP, different mechanisms for translocation are used. Although all these three mechanisms show potential to explain membrane translocation of CPPs, none of them provide a satisfactory interpretation of how these peptides are able to translocate cargos 100-fold their own size.

Is a re-evaluation of the mechanism of cellular uptake needed?

The standard protocol for cellular internalization requires fixation of the cells, which is usually carried out in paraformaldehyde (PFA) solution. This is a mild form of cell fixation, as compared to the use of, for example, methanol treatment, which has been shown to cause artefactual uptake of, for example, VP22 (Lundberg and Johansson, 2001). It was recently documented that even mild fixation methods such as PFA treatment can generate artefactual uptake of peptides (Richard *et al.*, 2003). Similar observations were reported in the quantitative flow cytometry (FACS) assays, when the cells were not treated with trypsin. Consequently the membrane-associated peptides were not removed, and the method was not able to discriminate between plasma membrane-associated and internalized peptides. With this novel knowledge, the CPPs Tat and $(Arg)_9$ were shown to enter the cells mostly via endocytosis, and at 4 °C almost no uptake could be observed (Richard *et al.*, 2003). These results confirms that a re-evaluation of the mechanism is needed, at least for these two peptides, and that, when performing peptide internalization experiments, some caution is needed to avoid artefactual uptake.

TOXICITY OF CPPs

As already mentioned, some organisms utilize peptides for antimicrobial self-defence. Depending on the mechanism of toxicity, this might also have an effect on eukaryotic cells. Some antimicrobial peptides, such as buforin, make use of direct binding to DNA and RNA to inhibit protein synthesis (Park *et al.*, 1998), thus leaving the membrane intact, whereas other peptides create pores which disturb the membrane. The toxicity of peptides depends on the amino acid sequence, secondary structure and net charge. Few reports are available about the toxicity of CPPs; however,

CPPs could cause cytoplasmic leakage due to membrane disruption and also interference with the functioning of membrane proteins. Some CPPs, like the model amphipathic peptide, disrupts the plasma membrane during its cell entry through a mechanism similar to the pore forming antimicrobial peptide (Oehlke *et al.*, 1998).

After plasma membrane translocation the peptides might also exhibit cellular toxic effects. Transportan affects GTPase activity (Soomets *et al.*, 2000), and in order to avoid this, a structure–activity study was carried out, and a truncated transportan with minimal GTPase activity was defined. This peptide still retained the ability to translocate across membranes. Unfortunately, intracellular effects of CPPs have not been given much attention, nevertheless, so far no serious side effects of CPPs have been observed in either *in vitro* or *in vivo* applications.

Methods to study toxicity of CPPs

To examine the influence of substances on the plasma membrane, and on cell viability, several methods are available. These include exclusion of colloidal dyes, not taken up by living cells with intact plasma membrane (Melamed *et al.*, 1969), to mitochondrial functionality (Mosmann, 1983) and cytoplasmic leakage assays of cell-introduced probes (Walum and Peterson, 1982). Exclusion of both trypane blue (Scheller *et al.*, 2000) and propidium iodide as well as the mitochondria-activity based MTT assay (Vivès *et al.*, 1997) have been used for evaluation of CPP-induced toxicity. No toxicity for the Tat peptide at concentrations normally used in vector applications was reported; however, depending on peptide concentration (10–100μM) and incubation time, toxicity could be observed. Direct measurement of rapid changes in cell permeability using tritiated 2-deoxyglucose has also been applied to successfully investigate membrane disturbance caused by CPPs (Hällbrink *et al.*, 2001). This study showed that the membrane disturbance was closely related to the hydrophobicity of the peptides.

CPPs AS DELIVERY VECTORS

Peptide-based vectors like CPPs provide a way to deliver a vast range of different biologically active compounds, like proteins for studying cellular processes, as well as antigenic agents. As compared with other delivery vectors, peptide-based delivery is to date the only method that succeeds in delivering a cargo without disturbing the plasma membrane and can be applicable *in vivo*. CPPs possess an appealing set of desirable features for cellular targeting, such as effective delivery *in vivo*, targeting of the nucleus, applicability to all cell types, no apparent size constraint of cargo and seemingly no immunogenic, antigenic or inflammatory properties. Not only have eukaryotic cells been targeted via peptide-enhanced delivery but CPPs are also a potent method for delivering cargo, such as PNA, into bacteria (Eriksson *et al.*, 2002). The only vectors that are comparable to CPPs in *in vivo* delivery are viral vectors, which provide excellent delivery of genetic material, both transiently and stably, although safety and cost issues have made them less desirable.

CPP delivery of proteins

To date there are two methods available for generating chimeric proteins conjugated to a CPP: chemical linking of a synthesized peptide to the protein of interest (Prochiantz, 1996; Pooga *et al.*, 2001), or expression of in-frame recombinant fusions using bacterial expression vectors (Nagahara *et al.*, 1998). An impressive cascade of protein transduction experiments has been described in the literature. Schutze-Redelmeier *et al.* (1996) successfully transduced antigenic peptides into T-cells using penetratin. In an interesting paper Schwarze *et al.* (1999) successfully delivered a biologically active 120 kDa Tat-fusion protein *in vivo* to a variety of tissues of a mouse, including the brain. Another study by Vocero-Akbani *et al.* (1999) showed the potency of the CPP Tat by selectively inducing apoptosis in HIV-infected cells by exploiting the HIV protease. This 'Trojan horse' strategy made use of an engineered Caspase-3 pro-apoptotic Tat-fusion protein, with the endogenous caspase cleavage sites modified and replaced by a 14-residue HIV cleavage site, thus leaving the complex inactivated in non-HIV-infected cells, but promoting apoptosis in HIV-infected ones.

There are also possibilities to deliver non-covalently CPP-linked cargos. As an example biotin modified CPPs were utilized to transport avidin, which binds to biotin with high affinity (Pooga *et al.*, 2001), or simply incubating the CPP with the protein of interest has shown to be sufficient for translocation (Morris *et al.*, 2001).

CPPs in gene therapy

In recent years, the use of oligonucleotides (ONs) and their analogues has attracted a lot of interest. Their potency as sequence-specific ligands of nucleic acids has many appealing features. The most effective vectors for *in vivo* gene delivery to date are viral, although they have some disadvantages such as limited targeting of cells, integration with potential oncogenesis and unwanted immunoresponse.

With the delivery of antisense oligonucleotides against the amyloid precursor protein (APP), CPPs demonstrated their efficacy for delivering non-protein cargos. Antisense effects were observed at as low concentrations as 40 nM, and cellular morphology was altered within 24 h of exposure (Allinquant *et al.*, 1995). Enhanced delivery of genetic material by CPP conjugation has been achieved by several methods, and most recently the delivery of plasmid DNA was shown to be successful by using a branched Tat peptide as a delivery vector (Tung *et al.*, 2002).

Peptide nucleic acid (PNA) has also been used for regulation of gene expression (Nielsen and Egholm, 1999). PNA is a DNA mimic, with an amino acid-derived backbone instead of the sugar-phosphate backbone in DNA. This uncharged backbone stabilizes PNA/DNA and PNA/RNA duplexes, and is protected from degradation by proteases and nucleases. As traditional delivery methods do

not offer efficient delivery *in vivo*, Pooga *et al.* (1998b) successfully utilized a transportan–PNA conjugate, connecting the peptide–PNA via a disulphide bridge. The authors expected that the disulphide bridge would break in a reducing intracellular environment, allowing the liberated PNA to bind to its complementary sequence.

Another study reported the potency of CPP mediated delivery of the Cre recombinase (Jo *et al.*, 2001), a site-specific DNA recombinase from bacteriophage P1, recognizing the *loxP* sites serving as targets of Cre-mediated recombination in the P1 genome. This enzyme can also function as a recombination substrate in mammalian cells, allowing tissue-specific activation or ablation of a gene of choice.

In a recent report the authors combined the viral and CPP systems for enhanced uptake of a recombination-deficient virus, both *in vitro* and *in vivo* (Gratton *et al.*, 2003). This approach improves gene expression and allows the use of lower virus titres, thus decreasing cytotoxicity and immune response *in vivo*.

CONCLUSION

As delivery vectors, cell-penetrating peptides definitely have proven their value. Their ability to effectively deliver hydrophobic macromolecules into practically all types of cells *in vitro*, as well as *in vivo*, without marked levels of cytotoxicity, is impressive. Although the mechanisms of CPP translocation have been studied intensively, this issue has still not been clarified. Recent reports propose that the uptake could involve an endocytotic component, and maybe a re-evaluation of the CPPs mechanisms of cell entry must be considered.

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