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Review

# Cell-penetrating peptides: tools for intracellular delivery of therapeutics

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**Abstract.** The main problem of therapeutic efficiency lies in the crossing of cellular membranes. Therefore, significant effort is being made to develop agents which can cross these barriers and deliver therapeutic agents into cellular compartments. In recent years, a large amount of data on the use of peptides as delivery agents has accumulated. Several groups have published the first positive results using peptides for the delivery of therapeutic agents in relevant animal models. These peptides, called cell-penetrating peptides (CPPs), are short peptides (fewer than 30 residues) with a net positive charge and acting in a receptor- and energy-independent manner. Here, we give an extensive review of peptide-mediated delivery systems and discuss their applications, with particular focus on the mechanisms leading to cellular internalization.

**Key words.** Cell-penetrating peptides; mechanisms of membrane translocation; conformations; pore-like structure; peptide-mediated delivery.

## Introduction

Cellular internalization of large hydrophilic therapeutic agents such as proteins or nucleic acids is still a challenging task because of the presence of the plasma membrane, which constitutes an impermeable barrier for such molecules. In order to circumvent this problem, several methods of carrier-mediated delivery systems have been developed. Among them, much attention has recently been given to the use of peptide-based delivery systems. Compared with other systems, such as those based on cationic lipids [1–4] or polyethyleneimine (PEI) [5–7], for example, the use of peptides with cell-penetrating properties has several advantages. These are mainly due to the various possible modifications arising from the peptide sequence, which yield carriers addressing different cellular subdomains and/or able to transport various types of cargoes. However, the pathway of cellular uptake may be equally sequence-dependent. Some peptides are used for delivery of nucleic acids and promote their delivery through the endosomal pathway, whilst others can cross membranes independent of the endosomal pathway.

In this review, we describe the most popular cell-penetrating peptides and their use as transfection agents and, where possible, try to correlate their transfection characteristics with their physicochemical properties. Among them, we will mainly focus on peptides derived from protein transduction domains or their kin for classification reasons (transportan [8], penetratin [9], Tat [10] and VP22 [11]), amphipathic peptides (MAP [12], KALA [13], ppTG20 [14], proline-rich peptides [15], MPG-derived peptides [16] and Pep-1 [17]) and three peptides which cannot be fit into the classes cited above (loligomers [18], arginine-rich peptides [19] and calcitonin-derived peptides [20]). The sequences of the leader compounds of each family are reported in table 1.

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Table 1. Sequences of the CPPs discussed in this review and classified into the three families, as described in the text.

| Names | Sequences | Res. | Ref. |
|---|---|---|---|
| *Peptides deriving from protein transduction domains and assimilated* | | | |
| Penetratin | RQIKIWFQNRRMKWKK | 16 | [9] |
| Tat(48–60) | GRKKRRQRRRPPQ | 13 | [10] |
| Transportan | GWTLNSAGYLLGKINLKALAALAKKIL | 27 | [8] |
| VP22 | DAATATRGRSAASRPTERPRAPARSASRPRRPVD | 34 | [11] |
| *Amphipathic peptides (secondary and primary)* | | | |
| MAP | KLALKLALKALKAALKLA | 18 | [12] |
| KALA | WEAKLAKALAKALAKHLAKALAKALKACEA | 30 | [13] |
| ppTG20 | GLFRALLRLLRSLWRLLLRA | 20 | [14] |
| Trimer | $(VRLPPP)_3$ | 18 | [15] |
| P1 | MGLGLHLLVLAAALQGAWSQPKKKRKV | 27 | [80] |
| MPG | GALFLGFLGAAGSTMGAWSQPKKKRKV | 27 | [16] |
| Pep-1 | KETWWETWWTEWSQPKKKRKV | 21 | [17] |
| *Other cell-penetrating peptides* | | | |
| Arg9 | RRRRRRRRR | 9 | [19] |
| Loligomer | Branched Polylysine + NLS | 24 | [18] |
| hCT(9–32) | LGTYTQDFNKFHTFPQTAIGVGAP | 24 | [20] |

## Peptides derived from protein transduction domains

Many CPPs were designed from sequences of membrane-interacting proteins, such as fusion proteins, signal peptides, transmembrane domains and antimicrobial peptides. Within these sequences, short sequences called protein transduction domains or PTDs proved to efficiently cross biological membranes without the need of a carrier or of a receptor and to deliver peptides or proteins into intracellular compartments [21, 22]. Many investigations suggested that the use of PTD-based peptides could be of major importance for therapies against viral diseases or cancers. Among these, the third helix of the homeodomain of *antennapedia* called penetratin [9, 23], the Tat peptide derived from the transactivating protein Tat of human immunodeficiency virus-type 1 (HIV-1) [10, 21, 24], transportan [8] and VP22 [11] were used to improve the cellular uptake of peptides, proteins and oligonucleotides.

### Penetratin

Most applications of penetratin are related to the transfer of proteins such as those involved in the cell cycle progression [25] and induction of apoptosis [26]. This carrier peptide is efficient for the transfer of small proteins (fewer than 100 residues), but its efficiency is poorer for larger proteins [27]. Penetratin can also be used for transfection of nucleic acids, such as antisense oligonucleotides [28, 29], peptide nucleic acids (PNAs) [30] and double-stranded DNAs, with, however, much poorer transfection efficiency for the latter [27].

The cellular uptake mechanism of penetratin is still matter of debate, and two models have been proposed so far. The first one is based on the formation of inverted micelles [27, 31, 32], the second on the existence of peptide-lipid interactions inducing a local electroporation-like membrane permeation [33]. However, a direct membrane translocation process has been questioned, and a mechanism involving mainly endocytosis has been proposed [34]. Finally, a good compromise suggests that cellular internalization could be mediated by both endocytosis and direct translocation [35].

In spite of numerous investigations [9, 36–45], from the conformational point of view the situation is also rather unclear. Although this peptide shows a high propensity to adopt an $\alpha$-helical conformation as revealed by spectroscopic investigations carried out in trifluoroeihanol (TFE) and SDS-containing media, an antiparallel $\beta$-sheet structure was identified for penetratin engaged in a lipid-containing air-water interface [46]. Moreover, in the presence of phospholipid vesicles, penetratin adopts a $\beta$-sheet structure with, however, a small amount of helical contribution at low peptide concentrations, suggesting that the conformation of penetratin could be concentration-dependent [39, 42].

### Tat peptide

The transcription-transactivating (Tat) protein of HIV-1 is a protein of 101 residues which consists of three functional domains: an acidic N-terminal region required for transactivation activity, a cysteine-rich DNA binding domain and a basic domain comparable to a nuclear localization sequence. One main characteristic of the Tat protein lies in its ability to cross the plasma membrane of neighbouring cells [47]. Proteins fused with either fragment 37–72 or 1–72 of Tat were successfully delivered into mice 20 min after intravenous injection, and the protein of interest was detected in several tissues [10]. Later, it was shown that the minimal peptide-promoting membrane translocation was

the 49–57 basic domain [19, 48]. Presently, the Tat peptide has been used for transfer of a broad variety of macromolecules, such as fusion proteins and nucleic acids.

Oligonucleotides have been most often internalized in the form of covalent Tat/oligonucleotide conjugates [49]. Tat can also form complexes which are rapidly internalized and which are stabilized by electrostatic interactions occurring between the positive charges of the peptide and the negative charges of the phosphate groups in nucleic acids [50]. Furthermore, DNA transfection can be improved by the use of Tat di- or trimers [51]. Tat can also improve the efficiency of other vector systems. As an example, liposomes are more efficient in transferring plasmids when Tat molecules are associated at their surface [52, 53].

As for the mechanism involved in the cellular internalization process, it is still controversial [54], and it appears now that it could occur through a macropinocytosis process dependent on lipidic microdomains, more specifically lipid rafts [55, 56] or through clathrin-dependent endocytosis [57].

### Transportan

Transportan is a synthetic peptide the sequence of which is built from the N-terminal fragment of the neuropeptide galanin linked through a lysine residue to mastoparan [8]. Similarly to other peptides derived from PTDs, cellular internalization of transportan can be achieved at 4 °C and in the presence of specific inhibitors of some endocytotic pathways. Transportan and its analogues can cross epithelial layers more efficiently than penetratin [58].

This carrier peptide has been used for the transfer of proteins in vitro [59], for PNAs, both in vitro [60, 61] and in vivo [30], small interfering RNA (siRNA) [62] and is also able to transfer double-stranded oligonucleotides by hybridization to a complementary PNA which is covalently linked to the carrier [63]. The induced activity is independent of the presence of a receptor and also appears to be independent of energy and temperature, but the internalization pathway is still a matter of debate [59]. Recent results suggest that internalization could occur according to two processes. The first corresponds to the uptake of complexes at the membrane surface followed by translocation by induced invagination of the membrane [64]. The second process corresponds to interaction at the membrane surface followed by direct penetration of small-sized complexes into the cellular cytoplasm. Finally, most structural investigations show that transportan adopts a helical structure in the presence of phospholipids or SDS [42, 65, 66].

### VP22-derived peptide

VP22 protein is generated by herpes simplex virus-type 1 (VHS-1) virus [67]. This protein is expressed in infected cells and then penetrates into neighbouring cells and enters their nuclei [11, 68]. The 40 residues of the C-terminal region are responsible for messenger RNA (mRNA) transfer activity. When fused to other peptides or proteins, this peptide remains capable of membrane translocation. Owing to this property, VP22 peptide has been used for the transfer of functional proteins in vitro [69] and in vivo [70]. The use of VP22 as a transfecting agent has allowed testing of some apoptosis-inducing proteins [71] and improvement of in vivo anti-tumoral response to an antiviral gene [72].

However, the use of this carrier suffers for some weaknesses, which are mainly related to the final nuclear localization of the cargo, thereby reducing the number of potential applications. Finally, it should be emphasized that all observations on cellular localizations were carried out on fixed cells and cannot therefore exclude artifactual observations, as already reported for Tat [73].

### Amphipathic peptides

An amphipathic molecule can be defined, in short, as consisting of two domains: a hydrophilic (polar) domain and a hydrophobic (non-polar) domain. For peptides, the amphipathic character arises from either the primary structure or the secondary structure. Primary amphipathic peptides are defined as the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues. Secondary amphipathic peptides are generated by the conformational state, which allows positioning of the hydrophobic and hydrophilic residues on opposite sides of the molecule (see fig. 1).

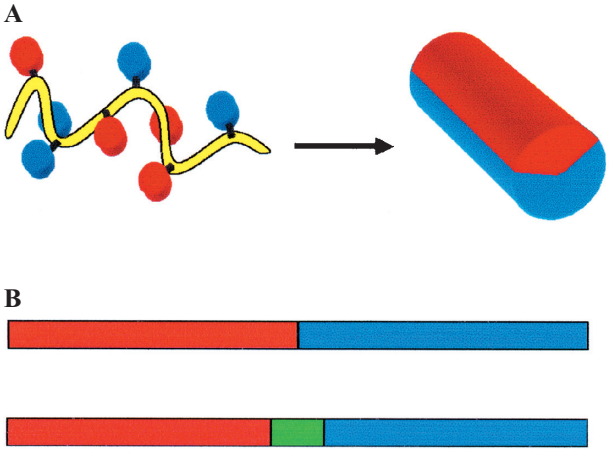


Figure 1. Schematic representation of amphipathic peptides. (*A*) The amphipathic character appears after folding into a helical conformation. (*B*) The appearance of the amphipathic character does not require folding.

**Secondary amphipathic peptides**

Most of the secondary amphipathic peptides used as drug delivery agents adopt an $\alpha$-helical structure. Since the $\alpha$-helix contains 3.6 residues per turn, the residues, hydrophilic for example, must be in positions i, i+3/i+4, i+7 and so on.

A large number of vector peptides exhibit this residue distribution. This is particularly the case of the MAP (model amphipathic peptide) family. Comparison of the internalization efficacy of a series of peptides designed on the basis of MAP suggests the existence of a tight relationship between amphipathic character and cellular internalization [74]. While a true MAP promotes internalization of a cargo, a non-amphipathic analogue cannot. From the comparative study of a series of 18-residue-long amphipathic peptides differing in their primary sequence, charge or hydrophobic/hydrophilic balance, it appears that the essential criterion for translocation resides in the amphipathic character of the peptide [75].

Among the other secondary amphipathic peptides, the 30-residue-long peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA) can specifically be used for destabilizing the membranes of endosomes in a pH-dependent process [76]. This destabilizing effect is also observed on liposomes by examining GALA-induced liberation of calcein. However, although GALA has a membrane destabilizing effect, the negative charges of the glutamyl residues preclude binding of the peptide to nucleic acids and therefore its use as a carrier peptide. In order to overcome this problem, several peptides, such as KALA, have been designed. This latter peptide was obtained by replacing some alanines by lysines, with a simultaneous reduction in the number of glutamic acids. These modifications, which do not modify the amphipathic character, allow binding and cellular delivery of DNA through an endocytosis-mediated pathway [13].

Two other secondary amphipathic cell-penetrating peptides have been designed on the basis of the sequence of peptide JTS1 (GLFEALLELLESLWELLLEA) [77]. Substitution of all negatively charged residues by lysine or arginine has yielded to peptides ppTG1 and ppTG20, respectively [14], which have both membrane-destabilizing and DNA binding properties. Conformational studies reveal that both have a strong tendency to adopt a helical conformation, both in solution and in a membrane-mimicking environment and that in vivo they have, transfection efficiencies similar to that of KALA with, however, a slight improvement for the Arg derivative compared with ppTG1. It should be mentioned that Lys-to-His modifications lead to a loss of transfection activity.

Finally, a last important family of secondary amphipathic peptides was recently proposed. This family is based on the polyproline II conformation. The polyproline II helix contains three residues per turn and can be transformed into a proline-rich amphipathic helix by replacing prolines in positions i+1, i+7, i+13… by charged residues (designated by X), while residues in positions i/i+2, i+6/i+8 i+12/i+ 14 … are replaced by hydrophobic ones (designated by Z) [78]. The peptides of general sequence $(ZXZPPP)_n$ are at the basis of the peptides $(VXLPPP)_n$, where X = Arg, Lys or His and n= 1, 2 or 3 [79]. Their translocation into HeLa cells was investigated thanks to fluorescently labeled forms (carboxyfluorescein –(CF)– linked at the C-terminus [15]). Observations reveal that the most efficient peptide is obtained for n = 3 and X = Arg. Although this peptide is less efficient than Tat and pAntp, owing to its lack of toxicity further studies using this peptide as a carrier of plasmids are currently in progress.

**Primary amphipathic peptides**

As already mentioned, primary amphipathic peptides are the result of the sequential assembly of hydrophobic and hydrophilic domains. The first one is required for membrane anchoring and for complex formation with hydrophobic cargoes. The hydrophilic domain is required to address a subcellular compartment, to improve the solubility of the vector and for complex formation with hydrophilic negatively charged molecules [80].

There are three major families of primary amphipathic peptides: those derived from a signal peptide (SP) [81, 82], from a fusion peptide (FP) as found in the MPG family [16, 82], and the tryptophan-rich sequences forming the Pep family [17]. These three families have a common hydrophilic domain, the nuclear localization sequence (NLS) of SV40 large T antigen: PKKKRKV. All these peptides bear a WSQ sequence which acts as a linker between the hydrophilic and hydrophobic domains, thereby maintaining their integrity. The selected SP sequence corresponds to that of the light chain of the immunoglobulin of *Caiman crocodylus*, while the FP of MPG was chosen from HIV-1 fusion protein gp41. For Pep peptides, the hydrophobic sequence was selected from the dimerization motif at the interface of HIV-1 reverse transcriptase. All of these peptides are acetylated and bear a cysteamide group at their N- and C-termini, respectively. The peptides of the SP family enter cells very rapidly at 4 °C as well as at room temperature. Since endocytosis inhibitors have no effect on cell entry, the endocytosis pathway has been ruled out to explain the mechanism of internalization. These peptides have been used for the intracellular delivery of oligonucleotides [82] or porphyrin [83] derivatives, which are covalently linked to the carrier though the cysteamide moiety. However, they have proved to be toxic when used at concentrations higher than 10 μM due to pore formation, which generates membrane depolarization [84].

Attempts to overcome this property were made by designing the MPG family based on the association of a fusion sequence to the above-mentioned NLS and linker. Several

variants of the parental peptide have been tested. The first one (MPG-W) is the result of a $W^7$-to-F substitution and results in efficient nuclear localization in fibroblasts [80, 82, 85]. This behaviour prompted us to synthesize other variants in order to control the addressed subcellular compartment for nucleic acid cargoes. This was achieved by a K-to-S substitution in position 23, corresponding to the second lysine of the NLS sequence, generating the vector peptide MPG-$\Delta^{NLS}$. This substitution was made to reduce nuclear addressing by decreasing the number of positive charges, thereby also reducing the stability of the complex formed with the negatively charged nucleic acids. MPG-$\Delta^{NLS}$ was shown to efficiently transfer single- and double-stranded oligonucleotides, RNAs [16, 86], siRNAs [87, 88], double-stranded phosphorothioate oligonucleotides [89] and more sophisticated oligonucleotides, such as $N^{3'} \rightarrow P^{5'}$ thio-phosphoramidates, without formation of any covalent linkage [90, 91].

Another type of primary amphipathic peptide, Pep, was recently developed. It differs from MPG in the nature of the hydrophobic sequence and was designed with the aim of delivering peptides, proteins or PNAs [17, 92]. Pep-1, the leader peptide of the Pep family, forms complexes with proteic cargoes which are rapidly delivered into a great variety of cell lines and applied in cellulo and in vivo [93, 94]. Pep-1 is now commercially available under the name of Chariot.

## Other peptides

Most CPPs were designed on the basis of protein transduction domains or on the basis of their amphipathic character. Nevertheless, some CPPs were developed using other criteria. Some of them are based on the role of positive charges in the translocation process. These are cationic polypeptides, such as polylysine, polyhistidine and polyarginine, or dendrimeric polycationic molecules. Others arise from small proteins that can be internalized by cells such as calcitonin.

### Polyarginine-based peptides

The investigations carried out with Tat and penetratin revealed that the role of positive charges is crucial for translocation. A comparative study of several cationic polypeptides showed a higher efficiency for polyarginines compared with polyhistidines or polylysines [95]. Amongst polyarginine peptides, $Arg_7$ and $Arg_9$ have been the most widely used, in vitro for the transfer of peptides [96] and in vivo for the transfer of proteins [97]. $Arg_7$ and $Arg_9$ have been used in vivo for the transfer of cyclosporine [98] and human catalase [99], respectively. Although most assays were carried out using covalent carrier-cargo conjugates, polyarginines have also been used for transfer of genes through formation of complexes involving the gene and several polyarginine molecules [100].

Similarly to other carrier peptides, such as Tat or VP22, the internalization process of polyarginines remains controversial due to questionable fixation methods. To address this issue, several experimental conditions were explored, and among them, the role of the heparan sulphates was pointed out [101]. A four-step process was proposed for the $Arg_9$ peptide, described as binding to the heparan sulphates at the cell surface, then internalized via a heparan sulphate-specific endocytosis procedure; after heparinase-mediated degradation of the heparan sulphates and release of the polyarginines, which promotes leakage of vesicular membranes, the peptides are released into the cytoplasm.

### Calcitonin-derived peptides

Human calcitonin (hCT) is a hormone secreted by the C cells of the thyroid and is involved in calcic homeostasis and used for the treatment of diseases such as hypercalcemia or osteoporosis [102]. This hormone is able to

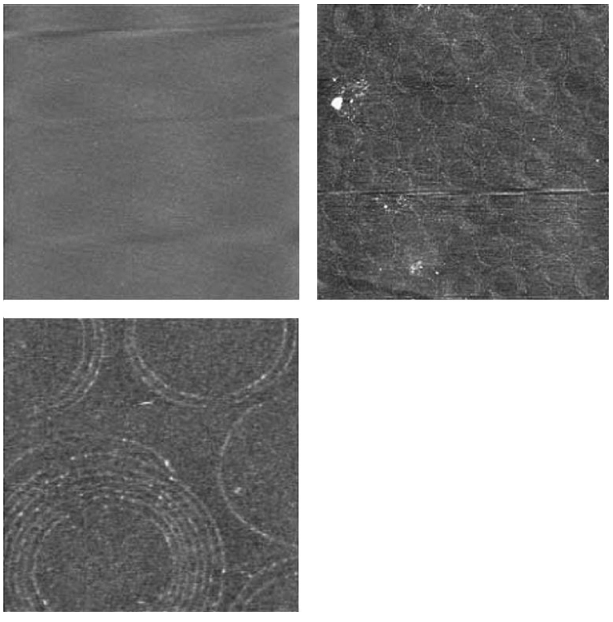

Figure 2. Atomic force microscopy imaging of transferred mixed monolayers composed of dioleoylphosphatidylglycerol and hCT (9–32) at various lipid/peptide ratios. The monolayer was obtained by spreading the desired mixture dissolved in a chloroform/methanol (3/1 v/v) mixture at the water surface. After evaporation of the solvent, the films were transferred at a constant surface pressure (20 mN/m) onto freshly cleaved mica. Examination was made on the hydrophobic side. Upper images: Lipid/peptide ratios are 0.1 (left) and 0.5 (right). Scan size 5 μm × 5 μm. Lower image: Zoom of the image at the 0.5 ratio. Scan size 833 nm × 833 nm. (Taken from [108]). These images show that the peptide does not modify the lipid monolayer (dark background) nor can it adopt a well-defined organization according to the lipid/peptide ratio (clear spirals).

cross the nasal epithelium, which allows its use in the form of a nasal spray which has proved to be as efficient as intravenous injections [103]. Structure-activity relationship studies have indicated that the C-terminal fragment can cross the nasal epithelium, while the N-terminal domain is required for receptor recognition but not for membrane penetration. Hence the 9–32 fragment was investigated with respect to its ability to behave as a carrier peptide [20].

The 9–32 fragment of hCT, called hCT(9–32) has been used for transfer of green flurescent protein (GFP) through cellular membranes via a covalent carrier-cargo linkage [104] and also for the delivery of daunorubicine into several cell lines [105]. More recently, another derivative of hCT has been developed in which an NLS has been branched on a lysine side chain. This latter compound enables the transfer of a plasmid through formation of a complex [106].

Investigations on the mechanism of hCT(9–32) cellular internalization have pointed out the temperature, time and concentration dependence, thereby suggesting an endosome-mediated pathway [107]. This was confirmed by the fact that the well-known endosomial labelling fluorescein-isothiocyanate (FITC)-dextran co-localizes with the peptide. Physicochemical investigations have also confirmed that peptide concentration is a key factor in determining the internalization process. Indeed, low peptide concentrations do not disturb membrane organization [108] (fig. 2), while high concentrations – i.e. concentrations similar to those used for transfection experiments – strongly modify this organization (fig. 3) [109].

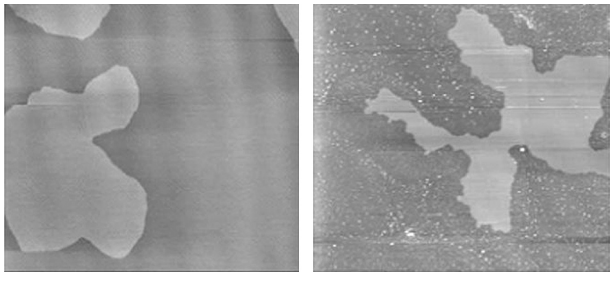

Figure 3. Visualization by atomic force microscopy of the formation of aggregates of hCT(9–32) in the dioleoylphosphatidylcholine-enriched fluid phase of mixed dioleoylphosphatidylcholine/dipalmytoylphosphatidylcholine bilayers. After formation of the bilayer by deposition of unilamellar vesicles on a freshly cleaved mica surface, the medium was incubated in a solution of the peptide at the desired concentration. Owing to the bilayer nature, the observations are now made on the hydrophilic side. Left: Without peptide; the dark domains correspond to the fluid phase, while the protruding gel phase is clearer. Right: with the peptide after 150 min. incubation with 50 μM. The fluid phase is still in dark and is covered by dots made of aggregates of hCT(9–32), while the gel phase is also slightly modified with formation of rough contours. Scan size: 10 μm × 10 μm. (Taken from [109]).

**Loligomers**

Loligomers can be compared with dendrimers due to their ramifications, which contain mainly positively charged residues. They contain a C-terminal region with three residues which are linked to positively charged sequences through an oligolysine sequence. These positively charged sequences contain the NLS of SV40 large T antigen associated with a pentalysine motif generating a cytoplasmic translocation signal. Owing to the presence of the NLS, loligomers accumulate in cell nuclei in an energy-dependent manner [18]. They have been used to transfer cytotoxic compounds [110] and also to transfer genes [111]. However, the use of loligomers is restricted due to their toxicity.

## Mechanisms of translocation

Identification of the mode of action of CPPs is crucial for the design of future generations of CPPs. The present status concerning this aspect is still matter of debate if not controversial. Endocytosis or not endocytosis, that is the question! Based on the currently available literature, the appropriate and well-known answer that comes immediately to mind is: it all depends. [112]. The following section will highlight points which need further clarification and mechanisms which appear well established.

Investigation of the mechanism of internalization requires identification of several physicochemical properties of the carrier peptides. First, it is crucial to elucidate the type of interaction that the peptide can elicit in the presence of membrane components (phospholipids, receptors, heparan sulphates and so on). It is also necessary to identify the structural criteria, mainly the peptide primary and secondary structures, which can influence the internalization process. Finally, three main entry mechanisms can be examined: direct penetration into the membrane, translocation through formation of a transient structure and endocytosis-mediated entry.

With respect to the interaction of CPPs with the plasma membrane, most data stress the role of positive charges, which allow direct electrostatic interactions with phospholipid headgroups. However, some differences can be noticed depending on the nature of the CPP. As an example, penetratin differs from transportan in that the former interacts preferentially with negatively charged membranes, whereas interactions of the latter do not depend on charges [42]. Furthermore, comparison of internalization properties of all-L with those of all-D peptides [19, 32] indicates that this process is not receptor-mediated, as confirmed by the use of giant unilamellar vesicles [45]. Other investigations point out the importance of the heparan sulphates present at the cell surface. Indeed, internalization of Tat, penetratin and polyarginines is inhibited upon degradation of the heparan sulphates, by

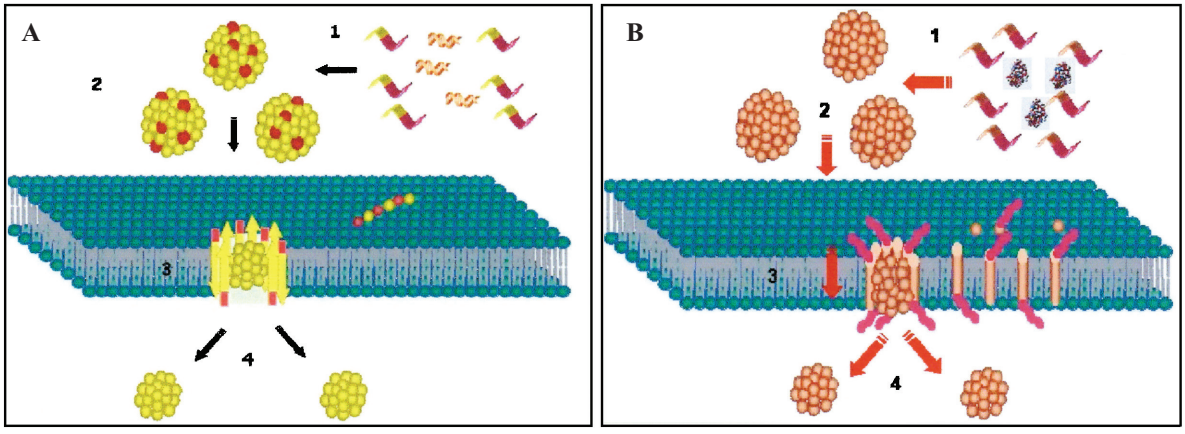


Figure 4. (*A*) Proposed model for MPG-mediated membrane translocation of nucleic acids. The four steps correspond to formation of the complex; membrane uptake of the complex; translocation through the bilayer; and release into the cytoplasm. The colour convention is red, NLS; yellow, hydrophobic sequence. (Taken from reference [121]). (*B*) Model of Pep-1-mediated transfer of proteins through lipid bilayers. The steps are similar to those of *A*. (Taken from [122]).

addition of heparin or sulphated polysaccharides or heparan sulphate-deficient cell lines [101, 113, 114]. However, other authors claim that Tat and penetratin can enter cells independent of the presence of heparan sulphates [45, 115].

Characterization of structural criteria associated with efficient CPPs remains to be improved, as only a few studies have been devoted to this issue. Up to now, several mechanisms have been proposed [116]. The first one describes direct penetration through the plasma membrane and has been proposed for Tat. The unfolded fusion protein interacts first with the membrane through electrostatic interactions and then crosses the membrane directly. Once inside the cell, the fusion protein is refolded thanks to the chaperone system. However, this mechanism has been recently questioned, and both an internalization mechanism involving macropinocytosis [55] and a clathrin-dependent endocytosis mechanism have been proposed [57].

A second mechanism accounting for translocation of penetratin has been proposed based on the formation of inverted micelles [32]. In this model, a penetratin dimer interacts with the negatively charged phospholipids, thereby inducing formation of an inverted micelle inside the lipid bilayer. The inverted micelle structure allows the peptide to remain in a hydrophilic environment. Such a mechanism is, however, still matter of debate, since non-symmetric distribution of penetratin between the inner and outer membrane leaflets generating an electric field has been shown. Upon increasing the amount of peptide on the outer leaflet, the electric field reaches a critical value which can generate an electroporation-like phenomenon [33].

A third mechanism accounting for cellular internalization which has to be considered is endocytosis. Most investigations are based on identification of the cellular localization of CPPs or localization of the cargoes by means of fluorescence or by the use of inhibitors of endocytosis. Unfortunately, artifacts arising during preparation of samples (fixation for example) generate questionable information with respect to endocytosis [54, 73, 117]. Finally, other investigations show that cellular entry of penetratin requires energy and enters by endocytosis, that polyarginines interact with heparan sulphates, followed by endocytosis, and that Tat is internalized through a macropinocytosis-mediated mechanism. Although these latter investigations clearly indicate that endocytosis is involved in the internalization of CPPs, it appears that different mechanisms could occur simultaneously. This is confirmed by the behaviour reported for penetratin and transportan for which both membrane translocation and endocytosis, occurring simultaneously, have been suggested [35, 118].

A final mechanism was recently suggested for internalization mediated by peptides belonging to the family of primary amphipathic peptides, namely MPG and Pep-1. On the basis of physicochemical investigations, including circular dichroism, and Fourier transform infrared and nuclear magnetic resonance spectrometries [119–122] associated with electrophysiological measurements and investigations dealing with the use of systems mimicking model membranes such as monolayers at the air-water interface and transferred monolayers, two very similar models have been proposed. Both are based on formation of transient porelike structures. The main difference between the model proposed for MPG and that proposed for Pep-1 is found in the structure giving rise to the pore structure. For MPG, it is formed by a $\beta$-barrel structure (fig. 4A) [121], while that of Pep-1 depends on association of helices (fig. 4B) [122]. For both peptides strong hydrophobic phospholipid-peptide interactions have been detected, and in both models the folded parts of the

carrier molecule correspond to the hydrophobic domain, while the rest of the molecule (linker + NLS) remains unstructured.

## Conclusions and perspectives

Although some cell-penetrating peptides are beginning to be applied in the medical field, some weaknesses are still encountered, in particular due to the lack of specificity toward targets. Thus, that some modification of their sequences or the introduction of non-natural amino acids bearing side chains that can improve their specificity is required. However, it is known that even minor sequence modification, can strongly modify the ability of peptides to act as drug carriers. It is therefore essential to identify precisely the criteria which can define an efficient cell-penetrating peptide with a high degree of drug transfer. One possibility, which remains to be more thoroughly explored, lies in elucidation of the mechanism(s) leading to membrane translocation. This requires understanding the interactions of these carrier peptides with their membrane components in association with the structural consequences of these interactions. These criteria must to be satisfied by the CPPs which will be designed in the future and which will be at the origin of the next generation of carrier peptides.

*Acknowledgments.* We apologize to our colleagues whose contributions are not cited due to reference limitation.

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