

## REVIEW ARTICLE

# Cell-penetrating peptides and antimicrobial peptides: how different are they?

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Some cationic peptides, referred to as CPPs (cell-penetrating peptides), have the ability to translocate across biological membranes in a non-disruptive way and to overcome the impermeable nature of the cell membrane. They have been successfully used for drug delivery into mammalian cells; however, there is no consensus about the mechanism of cellular uptake. Both endocytic and non-endocytic pathways are supported by experimental evidence. The observation that some AMPs (antimicrobial peptides) can enter host cells without damaging their cytoplasmic membrane, as well as kill pathogenic agents, has also attracted attention. The capacity to translocate across the cell membrane has been reported for some

of these AMPs. Like CPPs, AMPs are short and cationic sequences with a high affinity for membranes. Similarities between CPPs and AMPs prompted us to question if these two classes of peptides really belong to unrelated families. In this Review, a critical comparison of the mechanisms that underlie cellular uptake is undertaken. A reflection and a new perspective about CPPs and AMPs are presented.

**Key words:** antimicrobial peptide, cell-penetrating peptide, drug delivery, internalization, translocation mechanism.

## INTRODUCTION

The hydrophobic nature of cellular membranes makes them impermeable for most peptides, proteins and oligonucleotides. Different strategies have been employed to penetrate the membrane barrier and deliver hydrophilic molecules inside the cell for either experimental or therapeutic purposes. So far, microinjection, electroporation, liposomes and viral vectors have been used. Most of these delivery strategies have serious drawbacks, such as low efficiency, poor specificity, poor bioavailability and extensive toxicity [1]. Moreover, they are time-consuming. The endocytic route has been used as an alternative for the import of hydrophilic macromolecules into living cells [2]. However, the proteins engaging in this mechanism stay enclosed within endosomes, and so fail to access the cytoplasm, thus missing their final target.

### Peptides as vectors to introduce macromolecules into cells

An efficient strategy with which to penetrate the membrane barrier was identified by the observation that some intracellular proteins, when added to extracellular medium, were able to pass through the membrane. Tat (HIV-1 transcriptional activator protein) [3] and pAntp (*Drosophila* antennapedia transcription protein) [4] were the first proteins to be identified with this characteristic. The ability to translocate is attributed to basic amino acid sequences in these proteins, and the minimal peptide sequence necessary for the translocation to occur within Tat [5] and pAntp [6] have been elucidated. The observation that these basic peptides allow cellular delivery of conjugated molecules such as peptides [7] or proteins [8] made these molecules attractive, and a new class of vectors, initially known as PTDs (protein transduction domains) [9], but more recently re-baptized as CPPs (cell-penetrating peptides) [10], emerged. This family now includes all the peptides with the ability to translocate across membranes, regardless of whether they are natural, synthetic or chimaeric peptides.

So far, these vectors have been used to translocate a wide range of macromolecules into living cells, including proteins [8,9,11], peptides [7,12], oligonucleotides [13,14], peptide nucleic acids [15] and polysaccharides [16]. Nanoparticles [17] and liposomes [18] have also been internalized by means of CPPs.

### Can AMPs (antimicrobial peptides) also work as vectors?

Most organisms produce gene-encoded AMPs as innate defences to prevent colonization and infection by several microbial pathogens [19–22]. Despite their ubiquity, AMPs can have very distinct sequences and modes of action [23,24]; nonetheless, they usually share several characteristics, such as their short length (a few tens of residues) and their cationicity, typically of charge 4+ or 5+ [25]. Other features of these peptides include their strong interaction with lipidic membranes, a usually broad killing spectrum and their ability to preserve host-cell integrity [23,24].

Clinically these peptides display antimicrobial activity at micromolar concentrations or less, and target bacteria do not seem to readily develop resistance. These properties make AMPs very promising candidates for new generations of drugs to fight antibiotic-resistant strains of pathogens [23,26].

Although most AMPs seem to act mainly at the membrane level [24,25], their translocation into the cytoplasm is not uncommon [27,28]; because of this property, membrane-crossing AMPs have also been used as templates for CPP development [29]. Thus AMPs can have clinical applications both as antibiotics and as precursors of drug transporters.

### HOW DO CPPs TRANSLOCATE ACROSS THE CELL MEMBRANE?

There is no consensus regarding the mechanism of translocation of CPPs; the information available in the literature is controversial. First it was suggested that these peptides translocate by a

Abbreviations used: AMP, antimicrobial peptide; CF, carboxyfluorescein; CPP, cell-penetrating peptide; NLS, nuclear localization signal; pAntp, *Drosophila* antennapedia transcription protein; SV40, simian virus 40; Tat, HIV-1 transcriptional activator protein.

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**Table 1** Source, amino acid sequences and possible internalization mechanism for some examples of peptides that work as CPPs or as AMPs

Name (sequence)	Source [reference] [internalization mechanism(s), reference(s)]
Penetratin (RQIKIWQNRRMKWKK)	pAntp homeodomain (amino acids 43–58) [6] (mainly endocytosis [39], endosomal escape mediated by pH gradient or transmembrane potential [36,53])
Tat (GRKKRRQRRRPQ)	HIV-1 transcriptional activator Tat protein (amino acids 48–60) [5] (mainly endocytosis [40], endosomal escape mediated by pH gradient or transmembrane potential [37])
Pep-1 (Ac-KETWWETWWTEWSQPKKKRKV-cysteamine)	Amphipathic chimaeric peptide with a tryptophan-rich domain and an NLS [57] (physical mechanism mediated by peptide–membrane interaction promoted by pore formation [60] or by transmembrane potential without pores [35])
S4 <sub>13</sub> -PV (ALWKTLLKKVLKAPKKRKV-cysteamine)	Chimaeric peptide with AMP dermaseptin S4 and an NLS [61] (mainly physical mechanism promoted by a transient membrane destabilization [62])
Magainin 2 (GIGKFLHSAKFGKAFVGEIMNS)	AMP from the skin of the South-African clawed frog <i>Xenopus laevis</i> [101] (translocation mediated by toroidal pore formation; peptide molecules translocate stochastically as the pore disintegrates [28])
Buforin 2 (TRSSRAGLQFPVGRVHRLRK)	AMP from the stomach of the Korean common toad <i>Bufo bufo gargarizans</i> [102] (peptide molecules translocate stochastically after the formation and disintegration of a non-permeabilizing pore-like structure [84])
Apidaecins (RP - - - - PRPPHPR (conserved sequence among class members))	AMP from the lymph fluid of several insects [103] (receptor-dependent membrane docking and translocation into target cell [104])

mechanism independent of receptors [30] and independent of the endosomal pathway [5,6]. A physically driven mechanism was suggested, because the cellular uptake at 4 °C and 37 °C was similar [5,6,30,31].

More recent observations led to controversial results, suggesting that the cell localization observed for CPPs is an artefact and results from cell fixation for immunochemistry and cell visualization [32]. The high peptide affinity for membranes may allow CPPs to remain attached to cells during washing. During the cell fixation process, CPPs are released, and the apparent localization inside the cell results therefrom. However, direct observation of translocation in model membrane systems for some CPPs [33–35] supports the existence of physically driven mechanisms governed by spontaneous peptide–membrane interactions. The translocation mechanism issue is thus complex and may differ for different classes of CPPs (Table 1).

### CPPs derived from natural proteins

The CPP derived from pAntp has 16 amino acids and is the sequence necessary and sufficient for translocation to occur [6] (Table 1) and is commonly called ‘penetratin’. The Tat fragment corresponding to residues 48–60 [5] (Table 1), and a shorter fragment (residues 47–57) [18,36,37], have frequently been used in CPP research.

An endosomal pathway for internalization was initially excluded by comparison of the uptake at 4 °C and 37 °C under fixation conditions [5,6,30]. After re-evaluation for the interference of artefacts during fixation, an internalization mediated by endocytosis was concluded for both penetratin [38,39] and Tat peptide [37,40–43]. The basic amino acids are essential for translocation to occur, and membrane binding seems to be the first step prior to endocytic uptake. Heparan sulfate proteoglycans at the cell membrane were proposed to act as receptor for penetratin [42,44–46] and Tat peptide [42,47].

Although it is accepted that these CPPs can enter the cells by endocytosis, there is no consensus in the specific endocytic pathway used for the import of these arginine-rich peptides. A raft-dependent pathway involving macropinocytosis [48] or caveolae

[41,49,50], or a clathrin-dependent endocytosis [47,51,52], were proposed. The dissimilarities among these results can arise from the use of different cell lines, methodologies, labelled peptides, protein-conjugated peptides and different conditions, which can inhibit some pathways while favouring others.

Even in a picture where the endosomal pathway emerges as the physiological uptake of CPPs, the escape from endosomes into the cytoplasm through a physically driven mechanism persists. An escape from endosomes due to acidification was proposed for penetratin and Tat peptide [36,37]. This hypothesis is supported by the results obtained by Gräslund and co-workers [53] with penetratin encapsulated in large unilamellar vesicles. The escape of penetratin occurred only in the presence of a pH gradient. The role of intracellular pH in the internalization of CPPs was also investigated using neutralization agents [38].

A dependence of translocation on a negative transmembrane potential was identified *in vitro* for both penetratin and Tat peptide [34] and *in vivo* for Tat peptide [54]. Terrone et al. [34] suggested that a fraction of the peptide can transverse through the membrane by a transmembrane potential-driven mechanism, whereas the other fraction is internalized by an endosomal pathway. Once inside the endosomes, the transmembrane potential (luminal side positive) drives translocation from the endosomal lumen to the cytoplasm. By contrast, Drin et al. [38] did not find any internalization of penetratin in liposomes, even in the presence of a transmembrane potential. Recently Bárány-Wallje et al. [55], following electrophysiological measurements in planar bilayers, failed to detect translocation, even in the presence of applied voltages.

### Chimaeric peptides

The usefulness of peptides as vehicles to introduce macromolecules into cells led to the development of many chimaeric peptides. Pep-1 (acetyl-KETWWETWWTEWSQPKKKRKV-cysteamine) is a CPP with primary amphipathicity (i.e. amphipathicity resulting from the amino acid sequence itself, not from the folding structure [56]) that comprises a tryptophan-rich so-called ‘hydrophobic’ domain, a hydrophilic domain derived from an NLS (nuclear localization signal) of SV40 (simian virus 40) large T-antigen, and a

spacer between them [57]. A cysteamine group is present at the C-terminus [57] (Table 1).

Pep-1 has been used to introduce large proteins inside cell lines [57–59]. An endosomal pathway was rejected because (1) there was no difference in translocation efficiency at 37 °C and 4 °C [57] and (2) no co-localization of a delivered protein ( $\beta$ -galactosidase from *Escherichia coli*) with different cellular organelles (early endosomes, caveosomes and lysosomes) was detected [59]. By contrast, Weller et al. [58] proposed an endosome-mediated mechanism based on the internalization of Pep-1–protein complexes in the presence or absence of endocytic inhibitors.

Deshayes et al. [60] proposed a transient transmembrane-pore-like structure promoted by  $\alpha$ -helix conformation of the hydrophobic domain when it interacts with membranes. This conclusion was not corroborated by other groups, because no membrane leakage was detected [35,58]. An alternative mechanism, mediated by electrostatic peptide–lipid interactions, was proposed after observation that Pep-1 translocation both *in vitro* [35] or *in vivo* [59] only occurs in the presence of a transmembrane potential.

When Pep-1 was modified at the C-terminus [lack of cysteamine group and grafting of a CF (carboxyfluorescein) group], the membrane affinity and the translocation efficiency was truly impaired, but a small uptake seems to occur by an endocytic mechanism [16].

The chimaeric peptide S4<sub>13</sub>-PV, which results from the combination of a 13-amino-acid sequence derived from the dermaseptin S4 (S4<sub>13</sub> domain) with the NLS from SV40 large T-antigen (see Table 1), was proposed as a potential vehicle to introduce macromolecules into cells [61]. The uptake of this peptide under non-fixation conditions was not decreased in the presence of endocytic inhibitors [62]. An endocytic uptake was only evident at low peptide concentration [63]. The binding of the S4<sub>13</sub>-PV peptide to the cell membrane is promoted by electrostatic interactions with negatively charged components at the cell surface, and a conformation change in the S4<sub>13</sub> domain upon insertion into the bilayers was detected [62]. The translocation of S4<sub>13</sub>-PV across the cell membrane is considered to be a consequence of a transient membrane destabilization produced by peptide–membrane interactions [62]. Endosomal internalization at low peptide concentration suggests that higher peptide concentrations are necessary to induce membrane destabilization.

### Translocation mechanism or mechanisms?

Considering the abovementioned examples, it is clear that the mechanism by which CPPs translocate across cell membrane and deliver their cargoes in the cytosol is far from being totally understood. Although some CPPs are able to translocate by an endocytic pathway, the escape from endosomes demands a physically driven mechanism.

The affinity of each CPP for lipid bilayers may be the key factor for their main mechanism of uptake. Penetratin, for instance, does not show a strong affinity for zwitterionic membranes [46,64,65] and does not induce significant membrane destabilization [66]. Interaction with model membranes only occurs in negatively charged lipid bilayers [46,65]. In studies of the interaction of this peptide with eukaryotic cells, negatively charged proteoglycans presented at the cell surface were regarded as essential for translocation to occur [42,44,45]. Cellular uptake of penetratin occurs via endocytosis, but requires the expression of proteoglycans [42], which demonstrates the importance of electrostatic interactions in increasing the affinity of the peptide for cell membranes [45].

By contrast, Pep-1 has a high affinity for lipidic membranes, even in the absence of negatively charged phospholipids or proteoglycans [67], and it induces significant membrane destabi-

lization [35], which seems to favour internalization. Moreover, the introduction of a CF dye into the hydrophilic domain of Pep-1 and/or deletion of a cysteamine group decreased the peptide's affinity and, consequently, its uptake [16,58], and a slight internalization by endosomal pathway occurs [16]. This suggests that the membrane affinity and the capacity to destabilize it dictate the extent to which a peptide enters the cell by a physical mechanism (a fast process during which the endosomal pathway may be considered stationary) to the detriment of the endosomal pathway.

The hypothesis of the co-existence of endosomal and physically mediated mechanisms was also proposed by Boisseau et al. [68] in a study with maurocalcine, a CPP isolated from the Israeli gold scorpion (*Scorpio maurus palmatus*). A contribution of both mechanisms was identified where the physically driven mechanism results from a transmembrane potential. Moreover, Nakase et al. [69] showed that greater amounts of oligo-arginine were found in the cytoplasm when cells were incubated at 4 °C than at 37 °C. They proposed that, when endosomal pathways are inhibited and an alternative pathway can operate, the peptide is more efficiently translocated to the cytosol. When incubation is at 37 °C, oligo-arginine release in the cytoplasm is difficult, owing to endosome entrapment.

Translocation by a physical mechanism demands not only the existence of a high membrane affinity, but also a promoting force: pH gradients [53] and transmembrane potentials [34] are two possible driving forces. The existence of such driving forces is well understood in the cell context, where in/out media are characterized by the preservation of gradients.

### HOW DO AMPs GET INSIDE CELLS?

The mechanisms by which AMPs gain access to a cell's interior can be classified as pore-dependent and pore-independent, the former being the most usual. In fact, there are relatively few AMPs that do not exert their main function via cell lysis, membrane permeabilization or other forms of bilayer disruption. Few AMPs attack internal targets, and, of those, only a small number can do so without membrane perturbation [70].

### AMPs that induce membrane permeabilization

After the initial peptide–membrane interaction, mechanisms diverge; besides lysis, usually by a mechanism known as the 'carpet' model [71,72], two other models have been proposed for AMP pore formation: the barrel-stave pore and the toroidal pore (for further detailed information, see references [73,74]).

Independently of the pore type, diffusion of free peptide through the pore may not be the principal process of translocation; instead, it has been proposed that it is the peptide molecules that are involved in pore formation that stochastically translocate as the pore disintegrates [28]. Several factors support this statement, the most relevant being the fact that, for AMPs, the local concentration of membrane-bound peptide molecules is usually several orders of magnitude higher than in the aqueous phase (e.g. [75,76]); as such, there will be many more peptide molecules available for pore formation than for pore crossing. In addition, pore diameters are relatively narrow and usually not longer than the length of the peptides (alamethicin barrel stave pores have a diameter of 2–3 nm [77] and those of toroidal mellitin have a diameter of 3–4 nm [74]), preventing or hindering a free diffusion of the peptide; lastly, pore lifetimes are in the microsecond-to-millisecond range (between 40  $\mu$ s for magainin and 200 ms for dermaseptins [28,78]), which is long compared with a single-molecule translocation time, but

probably not long enough to quickly equilibrate inner and outer peptide concentrations.

Given this hypothesis, pore formation can be regarded not only as a membrane perturbation process, but also as an important intermediate step towards cellular invasion by AMPs. This is in agreement with recent findings that indicate that a synergic effect of activity at both membrane and cytoplasm levels may be required to fully explain the mechanisms of some pore-forming AMPs [79–81].

### Non-lytic AMPs

For AMPs that preferentially attack internal cellular targets, similar translocation mechanisms have been reported: for buforin 2, which translocates efficiently, but with little membrane activity [70,82,83], the structure and orientation in the bilayer have been observed to be very similar to those of magainin 2 (Table 1) [83,84]. From these results a model was proposed whereby buforin 2 molecules would form a toroidal pore, just as magainin 2 does, but less stable; this would result in shorter pore lifetimes – with a concomitant decrease in permeabilization – at the same time that the translocation rate would increase because pore disintegration, which is the actual translocation step, would become more frequent [83,84]. This model is supported by results that show that the presence of bilayer components that prevent the formation of toroidal pores (such as dioleoyl phosphatidylethanolamine [28]) inhibit buforin 2 translocation, whereas anionic phospholipids, which decrease the charge repulsions between the cationic peptide molecules, stabilize the pore to a point that significant leakage and flip-flop is observed [84]. Buforin 2 translocation has also been shown to withstand cargo addition, as demonstrated by the attachment of green fluorescent protein [29,85], which makes this peptide a promising candidate for its development into a CPP.

### Other mechanisms of translocation

Apidaecins, which are another class of non-lytic AMPs that are able to kill bacteria with undetectable membrane permeabilization, act by binding to a cytoplasmic target (Table 1) [86]. In this case, translocation has been proposed to occur by specific interaction with a putative membrane permease or transporter in the target bacterial cell; this was suggested by the fact that the all-D enantiomers of the peptides lose the ability to cross the membrane [86]. This characteristic confers a high selectivity on these peptides, which can even be modulated [87], but, on the other hand, the need for a membrane transporter makes apidaecins unappealing as templates for CPP design.

Despite their apparent simplicity, many AMPs have been shown to possess activity-specific regions: through sequence manipulation it has been possible to regulate translocating behaviour, target specificity or antimicrobial efficiency [87–89]. By means of these alterations, it has become possible to broaden the spectrum of CPP template candidates beyond non-cytotoxic translocating AMPs. This has been taken advantage of, for example, in the derivatives of the membrane-active Bac7 peptide [29,88,90], which, unlike their precursor, are not membrane-disruptive, but have retained translocation capabilities.

### CPPs AND AMPs OR JUST MEMBRANE-ACTIVE PEPTIDES?

Membrane translocation is a corollary for membrane permeabilization. Although treated differently by people interested in CPPs and AMPs, the ability to reach the inner leaflet of lipid bilayers is of crucial importance to both. Potentially, all CPPs are AMPs and all AMPs are CPPs. At high enough concentration, pep-

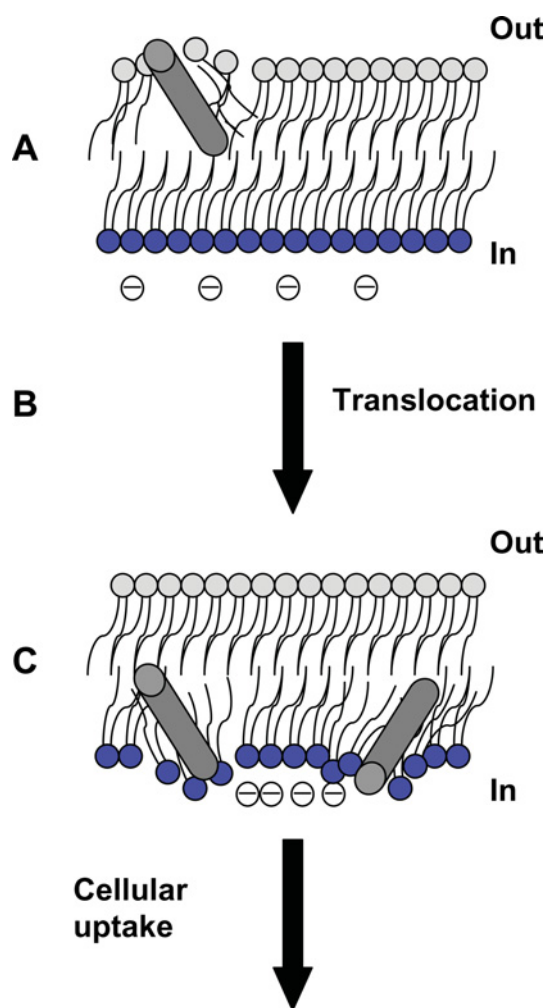
tides reported as CPP perturb membranes and become membrane permeabilizers (see reference [91], in which antimicrobial activity of different CPPs is evaluated), whereas AMPs may reach cytoplasmic targets before membrane permeabilization. This is not surprising, because both CPPs and AMPs are very similar molecules: short cationic peptides. It should be stressed that both classes cannot be differentiated on account of their structure because there is a wide diversity of conformations within each class of peptides [25,92]. The attention devoted to both CPPs and AMPs is very application-oriented, which dictates why these very similar classes of molecules are considered in such a separate fashion. CPPs are mainly studied by people focusing on gene therapy and drug delivery. AMPs are mainly regarded as tools to fight bacterial infections. However, from the molecular mechanistic point of view, the separation of these peptides into two groups is really rather academic.

Cellular membranes of target cells where the activity of these two peptides are evaluated are quite different. CPPs are generally evaluated against mammalian cells, whereas the target of AMPs is the bacterial cell. The major differences rely on anionic-lipidic and cholesterol contents. The bacterial membrane has a higher percentage of negatively charged lipids and does not contain sterols [24]. Different effects reported with CPPs and AMPs can arise from the differences in membrane composition, factors which modulate peptide affinity and membrane destabilization.

Considering the overlap between the mode of action of CPPs and AMPs, it does not seem reasonable to obstinately seek an exclusive answer to the question whether CPPs enter cells through endocytic or physical processes. As indicated above in the present Review, depending on circumstances, the same peptide may potentially use both [16,63,68]. Moreover, endocytic entrapment has to be followed by physical membrane translocation if the peptide is to reach the cytoplasm. The physical pathway can be mechanistically described by the interaction equilibrium between the peptides in the medium and the outer leaflet of membranes, perturbation of bilayers, translocation among leaflets and a second equilibrium of the peptides between the inner leaflet of the membrane and the reducing conditions of the cell interior [67,93–95] (Figure 1). A more effective or faster formation of a membrane-disturbing structure, mediated by the AMP magainin, was identified when a transmembrane potential was present [96].

Certain chimaera peptides, such as Pep-1, may even be considered a 'blend' between AMPs and CPPs. Although reported as a CPP, Pep-1 is a strongly amphipathic cationic peptide, rich in basic amino acids and tryptophan, having a proline residue in its sequence. These are classical characteristics attributed to AMPs. The ability to cysteine-bridge monomers, which greatly improves translocation efficiency, further increases the similarities to AMPs. Not surprisingly, Pep-1 uses mainly physical routes to translocate membranes [35,57,59]. However, this route is not always operative [16], and endocytic pathways are alternatives. The results obtained with Pep-1 confirm the co-existence of endocytic and physically mediated pathways. Such co-existence was previously proposed by other authors [97] using other CPPs, but this proposal was largely overlooked. The kinetic factor is important, as progress through physically driven pathways is rapid compared with that through endocytic pathways: when both physical and endocytic pathways are operative, the physical pathway is dominant, owing to faster kinetics [67,93].

These peptides are able to destabilize the membrane (fusion and vesicle aggregation were detected) without evidence for pore formation or flip-flop [35,66]. A 'membrane-thinning' effect was proposed for the AMP magainin 2 [98], in which the peptide aggregates on the surface of the membrane and the decreased local surface tension allows the peptide to intercalate the membrane.



**Figure 1** CPP translocation by a physically driven process can be regarded as a composite of three sequential steps

(A) The peptide (dark-grey cylinder) inserts in the bilayer outer interface (light-grey head-groups with fatty acid tails) and causes local membrane perturbation. (B) Owing to a membrane gradient (e.g. transmembrane potential, pH gradient) or concentration effects, the peptide overcomes the hydrophobic core of the bilayer by an unknown mechanism. (C) The peptide is released from the inner leaflet of the membrane (blue head-groups with fatty acid tails) to the cytoplasm. In a model artificial system (e.g. a vesicle) the system would tend to an equilibrium that can be accounted for by three different partition constants, one for each of the elementary steps (A, B and C).

Flexible sealing between peptide side groups and lipid head-groups minimize leakage during the peptide passage through the membrane [29].

A pore-formation mechanism was proposed for MPG (a 27-residue amphipathic peptide) and Pep-1 [60,99], which is also a common mechanism used by antimicrobial peptides. In the case of a transmembrane pore, a large pore would be necessary to allow the passage of attached macromolecules, a situation that compromises cell viability and all the significance of these peptides as vehicles. In some cases pore formation can explain the translocation of the peptides *per se*; however, these pores are not large enough to explain the translocation of proteins [28].

The history of CPP research can be summarized from two different periods, with a sudden change of paradigm in 2001 [32], later confirmed in 2003 [100]. First, the physical paradigm dominated. CPPs were considered to cross bilayers by a physical process. Since 2001–2003 there has been a tendency to think the

opposite. Reality may not be so black-and-white, and this rather simplistic view of physically driven versus endocytic mechanisms seems inadequate. The CPP research community should go back to basics and redefine CPPs on the basis of their cargo translocation ability rather than their stand-alone peptide properties.

Most of the CPP research focuses on the peptides' membrane-translocation ability in the absence of cargoes. It is thus crucial to develop new methodologies to detect and quantify translocation of peptide-mediated cargo translocation in vesicles and cells.

As to the peptides themselves, and their interaction with lipid bilayers, it may be that the frontiers between fusogenic peptides, AMPs and CPPs become so undefined that, in the near future, only the unified broad-scope title of 'membrane-active peptides' will make sense.

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