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Review

Cell-penetrating peptides: 20 years later, where do we stand?

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ABSTRACT

Twenty years ago, the discovery of peptides able to cross cellular membranes launched a novel field in molecular delivery based on these non-invasive vectors, most commonly called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs). These peptides were shown to efficiently transport various biologically active molecules inside living cells, and thus are considered promising devices for medical and biotechnological developments. Moreover, CPPs emerged as potential tools to study the prime mechanisms of cellular entry across the plasma membrane. This review is dedicated to CPP fundamentals, with an emphasis on the molecular requirements and mechanism of their entry into eukaryotic cells.

1. Cell-penetrating peptides: what are they?

The identification of proteins that can enter cells was first reported in the late eighties, contradicting the acknowledged understanding that the plasma membrane is impermeable to hydrophilic molecules. Thus, it has been demonstrated that the Trans-Activator of Transcription (Tat) protein of the Human Immunodeficiency Virus was able to efficiently enter tissue-cultured cells and promote the viral gene expression [1,2]. Moreover, Antennapedia homeodomain, a transcription factor of Drosophilia melanogaster, was also shown to enter nerve cells and regulate neural morphogenesis [3]. The interesting spontaneous entry of both proteins led to extensive structure/function studies to find the shortest amino acid sequence necessary for the uptake. This resulted in the identification of the first CPPs: Tat peptide, corresponding to the basic domain of HIV-1 Tat protein [4,5] and penetratin, corresponding to the third helix of the Antennapedia homeodomain [6]. Ever since, various peptides showing the same penetrating capacities have been discovered or rationally designed.

1.1. Definition and classification of CPPs

The field of CPPs evolved rapidly, ever since the first sequences were described. This makes it hard to have a general definition covering the characteristics of the different CPPs discovered. So far, one can say that CPPs are short peptides (generally not exceeding 30 residues) that have the capacity to ubiquitously cross cellular membranes with very limited toxicity, via energy-dependent and/or independent mechanisms, without the necessity of a chiral recognition by specific receptors. Most common CPPs are positively charged peptides, though the presence of few anionic or hydrophobic CPPs was also demonstrated. A primary or secondary amphipathic character is also implicated but not strictly required for the internalization.

According to their origin, we can distinguish three main classes of CPPs: peptides derived from proteins, chimeric peptides that are formed by the fusion of two natural sequences, and synthetic CPPs which are rationally designed sequences usually based on structure–activity studies (Table 1). Other attempts to classify CPPs, in spite of their diversity, were based on the physico-chemical characteristics of the sequences (e.g., their amphipathicity [7], or their hydrophobicity [8]). A recent review summarizes the different classifications and the physico-chemical properties of the so-far described CPPs [9].

1.2. Applications

CPPs can transport inside living cells a variety of covalently or non-covalently linked cargoes, as has been reviewed for

Abbreviations: CPP, cell-penetrating peptide; MALDI, matrix-assisted laser desorption; TOF, time of flight; MS, mass spectrometry; ON, oligonucleotide; EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; CS, chondroitin sulfate; HS, heparan sulfate; GAG, glycosaminoglycan; EIPA, ethylisopropylamiloride

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nanoparticles [18], peptides [18,19], proteins [20,21], antisense oligonucleotides [20,22], small interfering RNA [23], double stranded DNA [18] and liposomes [18].

The transport of the smallest cargo to large 120 kDa proteins had been successfully carried out both in vitro and in vivo. For instance, activable CPPs (ACPPs) were recently employed in vivo to target cancer cells over-expressing metalloproteinase-2 [24], while treatment of various inflammatory diseases by inhibition of NF-κB was also effective in vivo by coupling the inhibitors to different CPPs [25]. Tumor-targeting was also achieved in vivo for the *(D)*R_8–doxorubicin conjugate [26]. It is difficult to keep track of the various applications because the field is emerging rapidly. Recently, a novel class of intrinsically bioactive CPPs, baptized bioportide, made an appearance with the description of a CPP sequence derived from cytochrome c that mimicked the apoptotic role of the entire protein once it entered inside cells [27,28]. Another in vitro study on mouse neuronal hypothalamic cells revealed that the N-terminal sequence derived from the prion protein could penetrate cells and disabled the formation of prions [29].

2. Entry into cells: how do they do it?

Studies in the field of CPPs evolve around three research areas: (i) using CPPs as vectors to transport various macromolecules in vitro and in vivo for targeted cellular therapies, (ii) defining the structural basis of the internalization capacities in order to engineer new CPPs with optimum activity, (iii) elucidating the mechanisms of cell entry that remain subject of controversy in the literature. These different areas are tightly connected because in order to better use CPPs as vectors, one should understand how these peptides are crossing cellular membranes: what are the structural requirements, how do they interact with the various cellular components to drive their way inside the cells, and what are their final localizations.

2.1. Methods to study the mechanism of CPP uptake: in search for the perfect technique?

Various biological and biophysical methods are used to study the internalization mechanism, to localize and to quantify CPPs and their cargoes inside cell. Yet, every method has its pitfall and in order to have access to the large picture one should use a combination of different approaches.

2.1.1. In cellulo approaches

Studies in the cellular context mainly aim to follow the CPP and/or the cargo uptake, or to reveal the molecular mechanisms of the internalization. The methods used are generally indirect, based on fluorescence (and to a lesser extent on radiolabelling) or on the detection of the biological activity of the cargo [30]. Another method that enables direct quantification of the intact CPPs inside the cells or bound to the cellular membranes had been developed, based on matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) [31,32]. Other biophysical approaches are also used in living cells, such as electron microscopy to study membrane structures induced by CPPs and the peptides distribution [33,34] and in cell Raman spectroscopy to reveal the peptide secondary structure in cellular compartments [35].

2.1.1.1. Fluorescence-based protocols. These are the most common methods used. The peptides are covalently coupled to a fluorophore and measuring the fluorescence (fluorimetry) will enable indirect quantification of the peptides, while confocal microscopy allows localizing the probes inside living cells. Though convenient, many drawbacks of such methods exist. For instance, the indirect detection through the fluorescent label does not allow defining if the peptide is still linked to the fluorescent probe once inside cells; thus, the molecular integrity of the internalized entities cannot be demonstrated. Moreover, quenching of the fluorescence due to the accumulation in subcellular compartments and transitory binding partners may also lead to inaccurate results [36]. In addition, CPP functionalization by fluorophores changes the physico-chemistry of the peptides, thus influences negatively or positively their internalization efficiency and increases their toxicity (*cf.* Box 1). Indeed fluorophores are not only hydrophobic moieties but some of them also contain negative charges that change the overall net charge of CPPs. For example, carboxyfluorescein can introduce two negative charges on peptides since fluorescein contains a carboxylic acid and is generally added at the N-terminal primary amine of peptide sequences.

Box 1 CPP labeling effect. Beside other experimental factors that affect the uptake mechanism (cell type, incubation time, temperature . . .), the label attached covalently or non-covalently to the CPPs will definitely influence the interaction and the subsequent mechanism of internalization of the studied peptides. Fluorophores, for example, are generally highly hydrophobic and are shown to greatly modify the interaction with the lipid bilayer [34,41], to increase the uptake as well as to affect the cellular distribution of the CPPs [37,42,43]. Moreover, it was demonstrated that fluorophores change the flexibility and the conformation of the peptides [44]. Biotin on the other hand is generally less hydrophobic than the commonly used fluorophores, and was shown not to affect the interaction with model membranes [45]. Nevertheless, the presence of specific transporters for biotin was described in some cell lines like intestinal and hepatic cells, and was used to improve the delivery of macromolecules [46,47].

Thus, the physico-chemical characteristics of the probe should always be taken into consideration when assessing the mechanisms of internalization. However, the fact that our interest in CPPs relies on their vectorization capacities, adding a probe will give us information about conjugated CPP-cargo behavior. Yet, this does not exclude the importance of deciphering the molecular basis of the peptides/cells interactions because A to Z analysis of every CPP-cargo is not the solution for a rapid development of the field of applications.

An indirect protocol has been developed to study the intracellular localization of CPPs, which does not require their labeling with fluorophores [37]. This approach is based on CPP staining once inside cells, by using for example biotin–streptavidin interaction. Biotinylated peptides are thus stained by streptavidin-coupled fluorophores. But this involves cell fixation, which is believed to induce artifactual results due to a non-efficient fixation [38], allowing vesicle entrapped and membrane-bound peptides and proteins to be redistributed in new cellular compartments (mainly cytosolic) after the permeabilization step [38–40].

Finally, an important point should also be kept in mind when using fluorescence-based protocols, which is the necessity to eliminate the membrane-bound CPPs before any analysis. Methods to reduce signals from surface bound CPPs, such as quenching of surface-bound fluorophores, heparin washings and trypsin treatments, are generally employed. For instance, in the protocol described above based on staining of internalized biotinylated CPP, the membrane-bound peptide is quenched by unlabelled avidin [37].

2.1.1.2. Functional assays in cells. In order to get a direct read-out of the peptide entry into cells, methods to detect the biological activ-

Table 1
Origin and sequences of some of the studied CPPs.

Peptide	Origin	Sequence	Reference
Protein-derived			
Penetratin	Antennapedia (43–58)	RQIKIWFQNRRMKWKK	[6]
Tat peptide	Tat(48–60)	GRKKRRQRRRPPQ	[5]
pVEC	Cadherin(615–632)	LLIILRRRIRKQAHAHSK	[10]
Chimeric			
Transportan	Galanine/Mastoparan	GWTLNSAGYLLGKINLKALAALAKKIL	[11]
MPG	HIV-gp41/SV40 T-antigen	GALFLGFLGAAGSTMGAWSQPKKKRKV	[12]
Pep-1	HIV-reverse transcriptase/SV40 T-antigen	KETWWETWWTEWSQPKKKRKV	[13]
Synthetic			
Polyarginines	Based on Tat peptide	$(R)_n$; $6 < n < 12$	[14,15]
MAP	de novo	KLALKLALKALKAALKLA	[16]
R_6W_3	Based on penetratin	RRWWRRWRR	[17]

ity of conjugated molecules or cargoes have been widely developed [48,49]. These approaches are indeed very appropriate for the purpose of therapeutic and biotechnological applications. Nevertheless, measuring the biological activity of the cargo introduces many parameters such as the affinity of the cargo for its intracellular target, the efficiency of cargo modification by the target and potential reversibility of the enzymatic reaction, which hamper the rationalization of the results and complicate the analysis of the internalization mechanism.

A widely used example is the splicing redirection assay [50,51], where CPPs are used to internalize antisense ONs whose activity results in the upregulation of the luciferase gene expression. The splicing reporter system workers consist on a recombinant plasmid (pLuc/705) carrying the luciferase gene interrupted by a mutated human β-globin intron 2 (IVS2-705). The mutation in the intron causes aberrant splicing of luciferase pre-mRNA, preventing translation of luciferase. However, if the aberrant splice site is masked by antisense ONs the pre-mRNA of luciferase will be properly processed, yielding to the expression of the protein whose activity can be evaluated by luminescence. Stably transfected HeLa pLuc 705 cells have been used to evaluate the CPP-based delivery of various ONs, as well as to gain insight on the mechanisms of internalization [52–54].

Another system is based on the Cre-mediated recombination of an enhanced green fluorescent protein (EGFP)-reporter gene giving rise to expression of EGFP. The recombination can only take place upon exogenous Cre-protein delivery to the nucleus [55,56].

2.1.1.3. Quantification of internalization in cells. An approach relying on MALDI-TOF MS was developed in our laboratory in order to quantify the amount of internalized peptides inside living cells [31,32]. Moreover, we can have access to the amount of both internalized and plasma membrane-bound peptides. MALDI-TOF MS is a very sensitive method, with limits of detection in the femtomole range. To get an absolute quantification in biological samples, we should surpass sample heterogeneity that may lead to signal suppression. Moreover, an internal standard is required, because the ionization and the detection in MALDI are strongly dependent on the nature of the molecule studied. For that, the CPPs are synthesized with a spacer of four glycines on the N-terminus ([^{1}H]CPP), bearing a biotin tag for purification purposes. The spacer will allow us obtaining the internal standard peptide, where the four glycines are bideuterated ([^{2}H]CPP). This helps overcoming the risk of molecular discrimination between the analyte ([^{1}H]CPP) and the internal standard ([^{2}H]CPP) during purification, sample preparation for MS analysis and the desorption–ionization steps.

With this method, the quantity or concentration of peptide internalized together with its molecular integrity (intact or degraded) can be determined within the same experiment. In addition, by using proteolytic digestion, we can discriminate between the amount of membrane-bound and internalized peptides since 10–100 fold differences between those two amounts are generally observed [57–60]. The disadvantage of this method is that it does not give any information about the intracellular localization of the peptide. However it proved to be valuable to decipher the mechanisms of internalization (endocytosis versus direct translocation) and compare the relative internalization efficiencies of CPPs.

2.1.2. In vitro approaches the usefulness of antimicrobial peptides

For in vitro approaches, the CPP field have particularly benefited from the field of antimicrobial peptides (AMPs). These two peptide families should indeed be considered as related membrane-active peptides [61]. Most of CPPs and AMPs are positively charged, and the majority of AMPs are also amphipathic α-helices. The relative contribution of the positively charged and hydrophobic domains is very important for the membrane activity of AMPs [62]. Upon interaction of AMPs with membranes, the positive charges cluster at the lipid–peptide interface establishing strong electrostatic interactions with the negatively charged phospholipid membrane of pathogens. The non-polar face of the peptides will then insert into the membrane through hydrophobic interactions, and cause increased permeability of the membrane through different perturbation mechanisms [63].

As for AMPs, various biophysical approaches are employed to analyze in vitro the molecular interactions between CPPs and cellular components, such as carbohydrates and lipids, in order to surpass the complexity of the biological systems. Methods with model membranes or lipid bilayers have been widely used to determine the interaction, insertion and direct translocation of CPPs through the inert plasma membrane [64]. Peptide secondary structure following interaction with lipids or polysaccharides was also studied mainly by circular dichroism and infrared spectroscopy [60,65,66].

Most in vitro studies of membrane translocation by CPPs rely on fluorescence techniques [67] with the same pitfalls as described for studies with cells. However, other techniques that do not require labeling of the CPP sequences brought a lot of information regarding membrane perturbation or supramolecular reorganization events that are evoked by these peptides: ^{31}P NMR, differential scanning calorimetry, calcein or fluorescein-entrapped liposome leakage and small angle X-ray scattering [7,59,60,68,69]. For all these assays, membrane models are used.

The curvature of the bilayers is important for the membrane behavior of CPPs. Several studies using large or giant unilamellar vesicles (LUV and GUV) have been reported to analyze the translocation properties of CPPs. LUVs are vesicles classically obtained by extrusion of multilamellar vesicles. With the size of 100 nm, LUVs are considered as vesicles with little or no defects and widely used for translocation studies of fluorescent-labeled peptides or calcein leakage assays. LUVs with smaller size (50 nm) can be assimilated

as models for small, highly curved intracellular vesicles [70]. GUVs are vesicles larger than 1–10 μm diameter that can be obtained by spontaneous formation or by electroformation, and are assimilated as models for cell plasma membrane. More recently, giant vesicles derived from the plasma membrane of cells were used by Pooga and co-workers to study the internalization of six classical CPPs. The composition of these vesicles mimics the one of the cell membranes yet lacks the machinery necessary for endocytosis [71].

Penetratin internalized into GUVs and not into LUV except in the presence of a transbilayer potential [72–74]. More evidence was added for transmembrane translocation dependence on transbilayer potential of penetratin, with a light-generated proton pumping system in LUVs [75]. The same methodology, with bacteriorhodopsin (BR) asymmetrically introduced into large unilamellar vesicles (LUVs), was used to induce a pH gradient across the lipid bilayer that was shown to facilitate membrane translocation for intermediately hydrophobic CPPs (pVEC, M918), and much less for hydrophilic CPPs (Tat, R_9) [76]. Membranes of GUVs are more flexible than typical LUVs, and topical, positive and negative curvatures should be more easily formed in GUVs, as reported for penetratin with fluid membrane domains [77].

Penetratin, Tat and R_9 do not evoke liposome leakage [72,78] but a primary amphipathic CPP such as TP10 can [79]. These data show that different translocation mechanisms should explain the differences in membrane behavior of CPPs.

2.2. Structural requirements for CPPs internalization

Though not specifically required for the cellular uptake, the electrostatic interaction of basic CPPs with the negatively charged proteoglycans and phospholipids on the cell surface is generally acknowledged to be the first step for the cellular entry [80–84]. Moreover, membrane binding and insertion is also an important feature of CPPs, especially amphipathic ones, which can lead to endocytic pathways or direct translocation [66,85,86]. Interaction with the different components of the plasma membrane, be it electrostatic or hydrophobic, is strongly controlled by the positive charge number and density, the hydrogen bonds, the size and the secondary structure of the peptides [87–89].

2.2.1. Positive charge and "Magic Arginine"

Since the cell wall constitute an array of negative charges, CPPs generally exploit their basic residues in order to drive their way inside the cell. Thus, the logical first interaction between the positively charged CPPs and the plasma membrane is of electrostatic nature, where the peptides will bind to the negatively charged polysaccharides and lipids before finding their way to the internalization active membrane sites. In fact, the role of the positive charge in enhancing the uptake of macromolecules was demonstrated a long time ago, when Ryser and Hancock found that the uptake of albumin by tumor cells was boosted if the protein is mixed with high molecular weight poly-lysines [90]. This crucial role of positively charged residues was then proved for different CPPs sequences. For instance, truncation or alanine substitution of any of the charged residues within the basic region of Tat peptide markedly reduced the rate of uptake [91], and a decrease in the uptake of penetratin was also observed after alanine scanning of the basic residues [92].

A closer look led to the finding that arginine residues were more effective in terms of internalization than lysines, and that replacing lysine residues with arginine increased the rates of the uptake [14,91]. Thus, the uptake efficiency is attributed to the guanidinium headgroup of the arginine side chain rather than to the positive charge alone [93,94]. Moreover, it was demonstrated that the number of arginine residues is also crucial, with polyarginines of 7–15 residues presenting an optimal uptake [14,15].

In fact, the guanidinium group forms bidentate hydrogen bonds with negatively charged phosphate, sulfate and carboxylate groups on the cell surface, in contrast to the situation with ammonium cations in lysines that can donate only one hydrogen bond. This will result in counteranion scavenging that will help attenuating the polarity of the guanidinium group by producing a polar ion pair complex capable of diffusing into the membrane [93]. In oligo/polymers, this counteranion scavenging will also help in attenuating charge repulsion between nearby guanidinium entities, while in the case of nearby ammonium groups the charge repulsion is attenuated by reduction of pKa values. This will result in thermodynamically more stable complexes between oligo/polyarginines compared to monoarginines [95]. Moreover, Sakai et al. further demonstrated that the counteranion-mediated phase transfer of oligoarginine from water into chloroform was more efficient at a cation/anion ratio of 2 [96], and that phosphate and sulfate counteranions were more efficient than carboxylate in mediating phase transfer. Various other theoretical studies shed the light on the importance of guanidinium–phosphate interactions and the physico-chemical state of the arginine-side chain [97,98].

2.2.2. Hydrophobicity and "Tryptophan Power"

Hydrophobic residues, when present in the sequences of CPPs, play a major role in the interaction with the plasma membrane bilayer and thus are thought to enhance the peptide translocation across this membrane [99–101]. For instance, any single residue mutation of the N-terminal hydrophobic sequence of pVEC peptide (LLILL) led to a decrease of the cellular uptake of this CPP [100]. On the other hand, vesicle leakage was induced when adding a tryptophan residue or a fluorescein isothiocyanate (FITC) dye to the unlabeled sequences of R_6 and Tat peptide, showing that changes in hydrophobicity strongly affected the translocation mechanism [41]. Nevertheless, the hydrophobic character should be taken with caution because deeper insertion in the lipid membrane might lead to a less efficient internalization, probably because the peptides will be stuck in the plasma membrane [102,103]. For instance, it has been shown that the mutated (W48F,W56F)penetratin was more deeply inserted than the wild type penetratin in the hydrophobic core of negatively charged bicelles [104]. Indeed, a minimal flexibility of the positively charged entities is required in order to induce internalization.

Among hydrophobic residues, aromatic functional groups play a role beyond hydrophobicity. Non-covalent interactions of aromatic π-electron density have been extensively studied and demonstrated to be crucial for biological functions [105]. Aromatic residues, especially tyrosine and tryptophan, are predominant at the membrane surface of membrane proteins and were demonstrated to have favorable free energies of insertion into the bilayer interface [106,107]. In addition, it was demonstrated that tryptophan residues are involved in membrane destabilization processes [108,109]. Tryptophan was found a crucial residue for the uptake of CPPs, and its role was mainly assessed for the interaction with the lipid plasma membrane bilayer [17,85]. For instance, alanine-scanning study confirmed that except for Trp residues, no particular hydrophobic residue is required for penetratin internalization [92], while addition of a tryptophan to the C-terminal end of R_7 peptide enhanced the uptake of this latter [110].

Recent studies have uncovered an extended role of Trp in the mechanism of internalization of CPPs. Rydberg et al. have indeed shown that increasing the number of Trp residues in oligoarginine sequences enhances the uptake efficiency of the peptides, albeit the affinity of peptides for phospholipids is not affected [111]. In this study, the uptake pattern differed between peptides with different tryptophan contents and compositions, showing both endo-

cytotic and non-endocytotic uptake mechanisms. The same group also described that penetratin and PenArg (that contain two Trp residues) binding to sulfated sugars is stabilized by hydrophobic interactions and result in clustering of heparin [112]. We also demonstrated a direct role of the number of Trp in the interaction with sulfated sugars and in the internalization efficacy of basic CPP sequences [60]. Thermodynamic data indicated that Trp residues within basic peptide sequences increase the affinity of these latter for chondroitin (CS) and heparan sulfates (HS). These peptides also adopt a β-strand structure in complex with CS and HS and lead to the formation of large and stable aggregates. Basic peptides without Trp are structured as α-helices in complex with CS and HS and do not induce the formation of observable aggregates [60]. With regard to the molecular mechanisms of these interactions between CPP containing Trp residues and glycosaminoglycans (GAGs), the first binding step should involve ion-pair formation between all basic peptides and GAGs. Arg and Trp residues could contact the sugar units either by electrostatic and bidentate hydrogen bond interactions with the sulfates or by hydrophobic interactions to the sugar rings, respectively. Trp residues may also bind to sulfate groups of GAGs, possibly through π–anion interactions [113–116]. In addition, it is well-known that Trp and Arg side chains from two different peptide β-strands can also interact through π–cation non-covalent bonds and evoke self-assembly of peptides, which should lead to the formation of β-sheet aggregates in complex with GAGs. Finally, π–cation interactions between Trp and Arg residues can modify the pKa of the guanidinium side chain and increase the abundance of the protonated form of arginyl residues, and thus the interaction of the peptide with negatively charged GAGs [60].

2.2.3. Peptide secondary structure

The secondary structure of CPPs was mainly studied when interacting with model membranes, in order to explain the membrane perturbation and subsequent internalization. Mostly unstructured in aqueous solution [66,85,86,102,117,118], CPPs adopts various structures following the interaction with lipids. This variability, even for the same sequence, is certainly a result of the different experimental conditions employed such as: peptide/lipid concentrations and ratios, buffer conditions (pH, ionic strength, etc.), temperature, and others. In contact with model membranes, Tat and short polyarginines remain unstructured, while penetratin had been shown to adopt an α-helical structure [65,104,119–121] and a β-strand or a β-turn conformation [122,123]. Although one should remind that dynamics of peptide structure in cells might be faster than time-resolved detection techniques, a recent in cell Raman study showed that penetratin is mainly random coil and β-strand in the cytoplasm, and β-sheet in the nucleus [35].

It is still not clear if a correlation between the peptide secondary structure and its ability to translocate exists. For instance, it was shown that an α-helical structure is not necessary for the internalization of penetratin [120,124]. What should be kept in mind, however, is the importance of the structural flexibility, as observed by the group of Divita who suggested that the structural polymorphism and malleability of CPPs could be important for the membrane interaction and internalization route [88], and that depending on the structure adopted the peptide will favor one route of entry on another [86]. "Chameleon-like" properties at biomembrane surfaces were also described for penetratin by Magzoub et al., where the authors suggested that the peptide, generally random coiled in water, tends to fold into an α-helix or to adopt a β-fold depending on the conditions [65]. Structural polymorphism correlates with the fact that the peptides have to interact with different lipid environments, and hence to adapt their conformation, as suggested by Brasseur and co-workers [125].

2.3. Mechanisms of internalization of CPPs: one route for entry?

The first reported studies for penetratin, Tat peptide and R_9 indicated that these peptides enter the cells by a passive, temperature- and receptor-independent process [5,6,15,124], which is not sensitive to endocytosis inhibitors [5,126]. It was thus suggested that the peptides are entering via direct translocation through physical perturbation of the plasma membrane. But all these studies were based on confocal microscopy in fixed cells, and it was later demonstrated that fixation prior to confocal laser scanning microscopy of cells incubated with fluorescence-labeled CPP could lead to artifactual results [38]. Since 2003 [38,127], an emphasis has been given to the role of endocytosis in the uptake of CPPs, but with different emerging techniques, it appeared that both endocytosis and direct translocation across the plasma membrane can occur at the same time [128]. In addition, recent studies suggest that a receptor-mediated entry is not ruled out for some CPP-conjugates. For instance, Ezzat et al. demonstrated that PepFect14 peptide forming non-covalent nano-complexes with ONs enter HeLa cells through class-A scavenger receptors (SCARAs) [129].

An important feature when summarizing the different mechanisms described is the parameters that affect the uptake: the CPP alone or coupled to a small or high molecular weight cargo, the nature of the link between the peptide and the cargo, the cell line used, the extracellular peptide concentration, the incubation time,...[57,130,131]. It is thus difficult to make a general rule out of the specific experimental conditions published.

All the mechanisms used by CPPs to enter are somehow connected and occur simultaneously, and the down-regulation of one pathway might lead to an up-regulation of the other. In the following section, we have tried to summarize the various findings regarding the cellular entry, with an emphasis on some of the most studied peptides: penetratin, Tat and oligoarginine.

2.3.1. Endocytosis

Endocytosis of CPPs consists of two steps: endocytic entry followed by endosomal escape. This latter is crucial in order to avoid degradation of the cargo in lysosomes, and to enable the cargo to reach its extra-endosomal target and exert its biological activity. The favorable conditions for endosomal escape (e.g. the physicochemical properties of the CPPs) are not yet known, giving an additional reason for studying the various CPP/molecule interactions.

Endocytosis is a natural process occurring in all cells. It can be triggered by electrostatic interactions with cell surface proteoglycans or by direct interaction with the plasma membrane. When bound to GAGs, CPPs might enter following the recycling cycle of GAGs that are constantly internalized [132,133], or more effectively CPPs can trigger endocytosis via clustering of GAGs, activation of intracellular signals and actin remodeling [57,82,89,134]. All known types of pinocytic routes were described for the entry of the different CPPs and mainly CPPs–cargo complexes.*Macropinocytosis:* Macropinocytosis was demonstrated to be implicated in the internalization of polyarginines [135], and to a much less extent of penetratin. These observations relied on the inhibition of macropinosome formation by ethylisopropylamiloride (EIPA), and of F-actin polymerization by cytochalasin D. Moreover, the authors described an induced rearrangement of the actin cytoskeleton after treatment of cells with R_8, but no colocalization of R_8 and R_{16} oligomers, suggesting different mechanisms implicated in the entry. Later, Duchardt et al. demonstrated that macropinocytosis for R_9 prevails at concentration less than 10 μM [130]. As for Tat peptide, inhibiting macropinocytosis also led to a decrease in the uptake, suggesting macropinocytosis as a route of entry [56,136].

A recent study [41] proposed that Tat can stimulate its own uptake by macropinocytosis: they suggested that following internal-

ization of the peptide via a pore-formation mechanism, Tat will interact with the actin cytoskeleton which will trigger macropinocytosis. Other studies, however, reported that after binding and clustering of proteoglycans, the peptides will induce activation of a small GTPase, Rac1, which will result in actin remodeling and induce macropinocytosis [137,138]. Finally, the group of Dowdy confirmed macropinocytosis as the route for Tat uptake, with the necessity of the presence of plasma membrane active proteins but not surface GAGs or sialic acids for the uptake [55].

Clathrin-mediated endocytosis: Clathrin-mediated endocytosis was shown to be implicated for the uptake of penetratin, Tat peptide and other CPPs after inhibition by hyperosmolar medium [139]. Moreover, while various studies demonstrated the implication of clathrin dependent endocytosis for Tat peptide [140–142], other suggested that the uptake of Tat was not affected by dynamin1, an inhibitor of clathrin-mediated endocytosis [136], and that the uptake in clathrin knock-down cells was not affected [143].

Caveolae-mediated uptake: Caveolae-mediated uptake was also demonstrated for some CPPs, such as Tat peptide that colocalized with caveolae marker caveolin-1 [144], even though various other studies gave contradictory results. For instance, treatment of HeLa and Chinese hamster ovary (CHO) cells with nystatin and filipin III, inhibitors of caveolae-dependent endocytosis, did not affect the internalization of Tat peptide [140], and caveolin knock-out was shown not to affect the uptake of Tat peptide in baby hamster kidney cells [143]. Finally, raft-dependent endocytosis (caveolae-dependent or not) was shown to also play a role in the internalization of penetratin, Tat peptide and others [145].

Noteworthy, the distinction between the various endocytic routes in most of the cited studies relied on the use of chemical inhibitors (Table 2) and the detection of the colocalization of the peptides with protein makers. Such methods can have negative side effects, including poor specificity, high dependence on the cell line used and even sometimes decrease in cell viability [146,147]. Thus, the interpretations of the results must be made with caution.

Direct translocation across the plasma membrane: Direct translocation involves destabilization of the plasma membrane, in an energy- and temperature-independent manner. Evoked at first as the mechanism of internalization of CPPs, then refuted as an artifact of fixation, direct translocation was later confirmed using fluorescence in living cells, quantification of the uptake at 4 °C and in the absence of endocytosis (e.g. ATP depletion) and using various biophysical approached in model systems [57,71,148–150]. Noteworthy, while endocytosis in inhibited at 4 °C, direct translocation is also decreased for membrane dynamics and fluidity are affected at such low temperature [151,152]. Thus, assessing direct translocation at low temperatures in living cells leads to an under-estimation of this latter.

Table 2
Endocytic chemical inhibitors frequently used.

Inhibitor	Mode of action
Clathrin-mediated endocytosis	
Hyperosmolar conditions	Dispersion of plasma membrane
Potassium depletion	Clathrin lattices
Cytosolic acidification (NH_4Cl; Chloroquine; Bafilomycin A)	Inhibits the budding of clathrin-coated pits
Chlorpromazine	Loss of clathrin and AP2 adaptor complex from the cell surface
Macropinocytosis	
Amilorides	Inhibitors of Na^+/H^+ exchange
Cytochalasin D; Latrunculins	Disassembly of the actin cytoskeleton
Lipid raft-dependent endocytosis (caveolae-dependent or not)	
Methyl-β-cyclodextrin	Cholesterol extraction
Nystatin; Filipin	Cholesterol sequestration
Statins	Inhibition of cholesterol synthesis
Clathrin- and caveolae-mediated endocytosis	
Dynasore	Inhibition of dynamin GTPase activity

Various hypotheses were reported to explain direct translocation of CPPs across the lipid bilayer (Fig. 1).

Inverted micelle formation: Inverted micelle formation was first proposed to explain the direct translocation of penetratin [124] (Fig. 1A). In this model, the basic residues will interact with the negatively charged phospholipids in the plasma membrane, and the subsequent interaction of hydrophobic residues with the membrane core induces the destabilization of the bilayer forming a negative curvature (i.e. invagination of the membrane) [66]. The concomitant reorganization of the neighboring lipids [153] leads to the formation of the inverted micelle that encapsulates the peptide(s) in its interior. Membrane disruption releases the peptide in the intracellular side. Substitution of tryptophan residues by phenylalanine inhibited the formation of inverted micelles, as revealed by ^{31}P-NMR experiments [117], showing an important role of this residue in the interaction with the bilayer, not only in terms of hydrophobicity. Molecular dynamic simulation further demonstrated the inverted micelle hypothesis, with an emphasis on the role of hydrogen bonding between the guanidinium headgroups and phosphate groups [154]. Various other studies describe membrane deformation (tubulation, multivesicular structures) upon the interaction of CPPs with model and cellular membranes [34,155].

Adaptive translocation: The capacity of guanidinium headgroups to form bidentate hydrogen bonds will give oligoarginines either a hydrophilic or hydrophobic character depending on the associated counteranion. Based on this capacity, the interaction between guanidinium-rich peptides and the phosphate lipid headgroups will mask the peptide charge, attenuating its polarity and enabling its adaptive diffusion into and across the membrane [156] (Fig. 1C). This diffusion is driven by the membrane potential of the plasma membrane [93], thus the peptide should carry a positive net charge. This explains the fact that a minimum number of arginine residues is necessary to observe internalization.

Pore-formationmodel: Molecular dynamic simulations and electrophysiology experiments [157,158] evoked the formation of transient toroidal pores that will enable the passive diffusion of Tat and arginine-rich peptides across the plasma membrane(Fig. 1B). Briefly, the interaction between the positive side chains and the phosphate groups leads to the accumulation of the peptides in the outer leaflet, positioned between the phosphate and the carbon chains of the lipids. The accumulation of the peptides causes a thinning of the bilayer, and the attraction between the side chains of arginine and lysine and the headgroups of the distal layer leads to the formation of a transient pore through which other peptides diffuse carrying with them the attached phospholipids. This evokes that the length of the side chain of arginine residues also contributes to the more efficient insertion and translocation compared to the lysine side chains [91]. Studies with other CPPs, based in addition on fluorescence and calorimetric methods, also suggested the formation of transient pores as a mechanism of direct translocation [79,159–161]. Palm-Apergi et al. proposed that the disturbance of the plasma membrane induced by the formation of the pores will trigger the membrane repair response (MRR) induced by the calcium efflux into the cells. Internal vesicles will fuse together and serve as a patch to reseal the membrane [33].

Electroporation-like permeabilization: Electroporation-like permeabilization was described by Binder and Lindblom for the direct passage of penetratin across negatively charged membranes. They suggest that above a threshold peptide concentration, the asymmetric charge distribution between the outer and the inner compartments will cause a transmembrane electrical tension. The membrane is thus permeabilized in an electroporation-like manner which creates transient defects that enable the peptides to

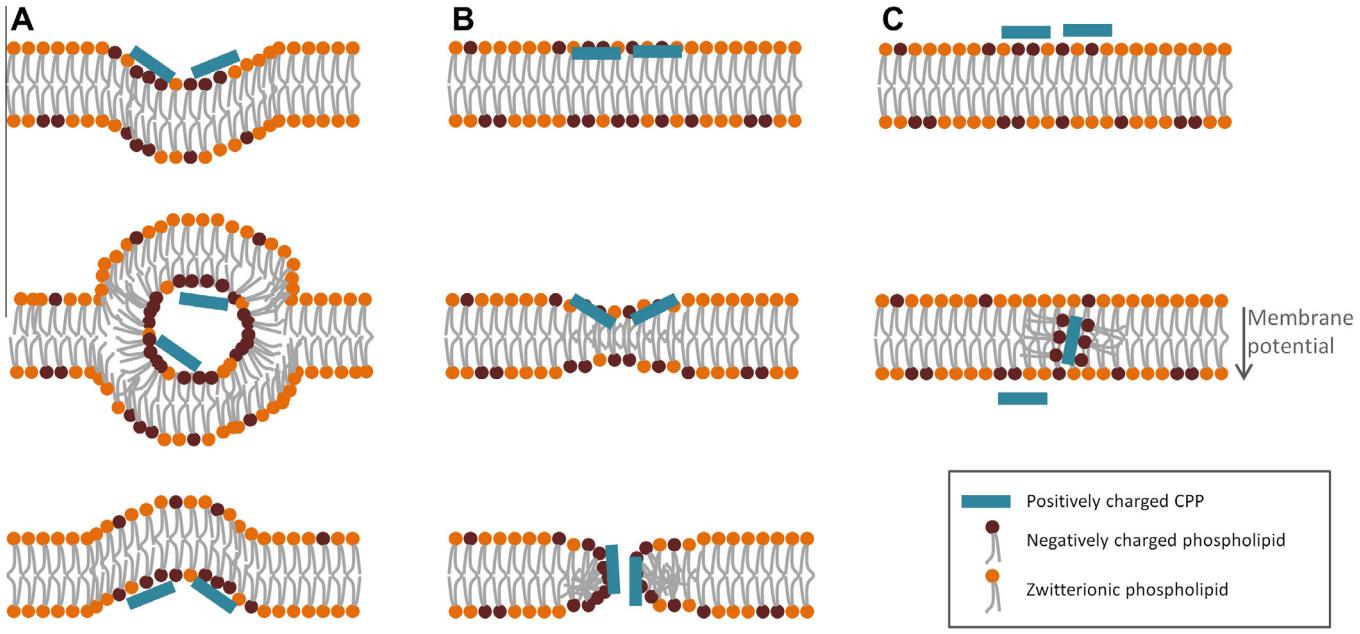

Fig. 1. Examples of the proposed mechanisms for direct translocation. (A) Inverted micelle formation. (B) Pore-formation. (C) Adaptive translocation.

distribute to both leaflets [149]. Indeed penetratin was shown to remain at the interface between the phospholipid bilayer and the aqueous medium without a deep insertion into model membranes [162]. This mechanism was later disputed by Su et al. [123] who demonstrated by solid-state NMR that penetratin was distributed in both leaflets even at low peptide concentration.

Entry at microdomains boundaries: Regions between different membrane domains can have packing defects that are more susceptible for peptide entrance or can act as nucleation sites for pore formation. These domains can be formed by the preferential interaction of the peptide with anionic lipids for example, which will lead to a reorganization of the plasma membrane [153]. Another possibility was evoked by the group of Brock, relying on the action of acid sphingomyelinase (ASMase) that will translocate to the extracellular leaflet of the plasma membrane and hydrolyses SM, thus creating ceramide-enriched domains. CPPs are believed to enter at the interface between these domains and the bulk of the plasma membrane [163].

3. Perspectives

Although the exact mechanisms of CPP internalization is still indecisive, several studies pointed to the importance of the interaction with, and the clustering of, GAGs or negatively charged phospholipids in the internalization of CPPs [57,59,60,80,134,164–166].

On one side, with the use of cell lines devoid of certain types of GAGs, and the concomitant observation of decreased internalization efficacy, the implication of GAGs in internalization of CPPs is no longer questioned in the field [26,55,57,164]. Exploiting GAGs as a portal to the cytoplasm of cells deserve however more studies on the intracellular traffic and fate of CPPs or CPP-cargoes.

When CPPs enter through endocytosis, endosomal escape, thus membrane translocation, occurs within cells and releases CPPs into the cytosol [167–172]. Endosomal escape would involve phospholipid clustering as a first transient membrane destabilization step, as antimicrobial peptides do to exert their toxic action [166]. Direct translocation of CPPs at the plasma membrane would occur primarily through negative curvature formation [48] or at membrane domain boundaries [163]. Albeit several recent studies brought strong data for the existence of direct membrane translocation [34,41,57,60,71,173–175], a better physical description of these mechanisms of entry in cells is still an open challenge for the next years.

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