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Translocation Mechanism(s) of Cell-Penetrating Peptides: Biophysical Studies Using Artificial Membrane Bilayers

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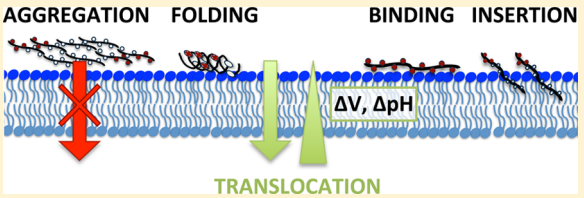
ABSTRACT: The ability of cell-penetrating peptides (CPPs) to cross cell membranes has found numerous applications in the delivery of bioactive compounds to the cytosol of living cells. Their internalization mechanisms have been questioned many times, and after 20 years of intense debate, it is now widely accepted that both energy-dependent and energy-independent mechanisms account for their penetration properties. However, the energy-independent mechanisms, named "direct translocation", occurring without the requirement of the cell internalization machinery, remain to be fully rationalized at the molecular level. Using artificial membrane bilayers, recent progress has been made toward the comprehension of the direct translocation event. This review summarizes our current understanding of the translocation process, starting from the adsorption of the CPP on the membrane to the membrane crossing itself. We describe the different key steps occurring before direct translocation, because each of them can promote and/or hamper translocation of the CPP through the membrane. We then dissect the modification to the membranes induced by the presence of the CPPs. Finally, we focus on the latest studies describing the direct translocation mechanisms. These results provide an important framework within which to design new CPPs and to rationalize an eventual selectivity of CPPs in their penetration ability.



Cell-penetrating peptides (CPPs) are small cationic sequences, defined by the capacity to cross cell membranes and deliver cargoes into cells. The first CPP sequences to be developed were derived from natural proteins. For example, Penetratin is a fragment of the homeodomain of Antennapedia, a transcription factor from *Drosophila*.[1] This sequence is responsible for the internalization of the whole protein, which plays the role of the cargo and has a critical role in cell−cell communication during neuronal morphogenesis. Since their discovery at the beginning of the 1990s, the use of CPPs has become a popular strategy for achieving intracellular access.[2−4] Nevertheless, the exact mechanism(s) by which CPPs are able to cross biological membranes remains to be definitively proven.[5]

In living cells, there is evidence of both energy-independent penetration ("direct translocation") and energy-dependent mechanisms (endocytosis and macropinocytosis).[6,7] Whereas direct translocation at the plasma membrane prevents sequestration of the cargo in endosomes, energy-dependent mechanisms may lead to premature degradation of the biologically active cargo moiety bound to the CPP during the endosomal maturation. But if the cargo reaches its cytosolic or nuclear target after endocytosis, this means that it has efficiently escaped from endosomes. Both direct translocation and endosomal escape require that the CPP and its cargo cross membrane bilayers.[8] As a consequence, to improve the efficiency of the cargo delivery, it is the propensity of CPP to cross membranes that needs to be dramatically enhanced. It is currently challenging to rationally design more effective translocating sequences, in particular because the mechanism(s) explaining how these cationic peptides translocate through biological membranes remains to be accurately elucidated. Translocation has been investigated by using both living cells and artificial membrane models such as lipid vesicles. Whereas the former model is *a priori* ideal for the study of CPP internalization, it is only with the latter that snapshots of the translocation mechanism at the molecular scale have been obtained. Herein, we will review the different results obtained with the most well-known CPPs (Penetratin, Tat, Arg9, R6/W3, Pep-1 and TP10) regarding their translocation mechanism(s) using vesicles as model membranes (Table 1). We have limited this review to these peptides because only these have been fully characterized.

The first step of the internalization process of a cationic CPP may be viewed as a concentration step from the extravesicular medium to the membrane surface. These interactions may induce folding and/or an oligomerization of the CPP mediated by the presence of phospholipids (PLs). In response to the interaction with the CPP, the lipid bilayer may be profoundly disturbed. Lipids are reorganized and eventually recruited by

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Table 1. Six CPPs Described Herein[a]

CPP	sequence	length	net charge	molecular weight	amphipathicity	secondary structure in the presence of phospholipids	ref
Penetratin	RQIKIWFQNRRMKWKK	16	+7	2247	secondary	α-helix, β-sheet	1
Tat	GRKKRRQRRRPPQ	13	+8	1719	nonamphipathic	random coil	9
Arg9	RRRRRRRRR	9	+9	1423	nonamphipathic	random coil	10
R6/W3	RRWWRRWRR	9	+6	1514	secondary	α-helix	11
Pep-1	KETWWETWWTEWSQPKKKRKV	21	+5	2846	primary	α-helix	12
TP10	AGYLLGKINLKALAALAKKIL	21	+4	2183	primary	α-helix	13

[a]The sequence and some of the physicochemical properties of Penetratin, Tat, Arg9, R6/W3, Pep-1, and TP10 are reported.

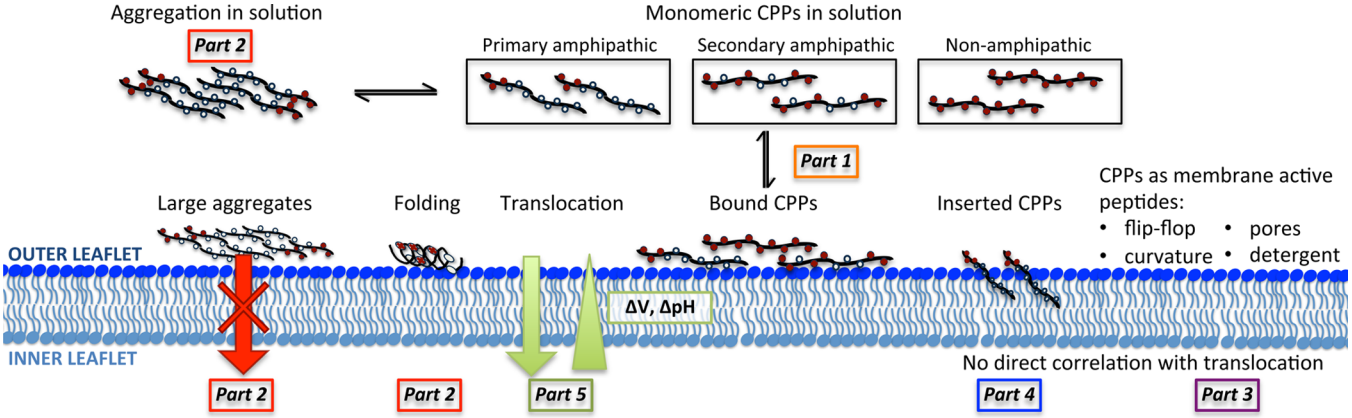

Figure 1. CPP–membrane interaction can be split into several different phenomena. All of them can promote and/or hamper the translocation of the CPP through the membrane. These phenomena will be described in the first four parts of this review (sections 1–4) to understand the physical state of CPPs in interaction with membranes at thermal equilibrium. The translocation event is a dynamic phenomenon that occurs transiently and will be the subject of the last section (section 5).

CPPs. Membrane symmetry can be additionally perturbed. Following adsorption and peptide–lipid interaction at the water–membrane interface, CPPs can also insert into the membrane. We will successively review these steps in detail (Figure 1).

Are these peptides able to shuffle through membrane bilayers, and how does translocation proceed? These remain the central questions. To probe the translocation event, various experimental procedures have been developed. Because of the broad variety of experimental setups, PLs, and CPPs investigated, the literature is very rich but difficult to interpret and may at times even seem to be contradictory. As a consequence, the penetration of CPPs into vesicles itself remains under debate. We will present the most recent experimental data obtained for the direct translocation of peptides and discuss the various mechanisms that have been proposed to explain the translocation of CPPs through the membrane.

1. THE AFFINITY OF CPPS FOR MEMBRANES CONCENTRATES THEM ON THEIR SURFACE

The concentration of CPPs on membrane surface involves various partners, including PLs. The interaction between PLs and CPPs may originate from various contributions: electrostatic interactions, hydrophobic effects, hydrogen bonding, and folding. In this first part, we analyze the strength and relative weight of these different contributions.

The affinity of CPPs for membranes can be rationalized by classifying them into three groups depending on their amphipathicity. This widely used classification not only is convenient but also provides real insight into the mechanisms of binding of CPPs to lipid bilayers. In this classification, Pep-1 and TP10 are considered as primary amphipathic CPPs. Their primary sequences contain well-defined cationic and hydrophobic domains. Penetratin and R6/W3 have both cationic and hydrophobic residues, but the repartition of these residues is not ordered in their primary sequence. However, in an α-helical conformation, they display one hydrophobic side and one cationic side. They are thus considered as secondary amphipathic CPPs. Tat and Arg9 are polycationic peptides and hence are nonamphipathic CPPs.

1.1. Affinity of Primary Amphipathic CPPs for Model Membranes. Primary amphipathic CPPs avidly bind membranes, even in the absence of any anionic PLs (partition constant of $\sim 10^4$ M^{-1}). Their affinity for membranes has been quantitatively determined by titration of an aqueous solution of the peptide with large unilamellar vesicles (LUVs) followed by fluorescence (either by using a tryptophan residue present in the sequence or by adding an extra fluorophore to the native sequence).[14] Surface plasmon resonance experiments have also been performed.[15] These results strongly suggest that the interaction between primary amphipathic CPPs and membranes is essentially governed by hydrophobic interactions, even if the affinity for membranes slightly increases in the presence of anionic PLs.[14,16,17]

The affinity of Pep-1 seems to be independent of the physical state of the membrane. However, fluorescence self-quenching of tryptophan occurs in the liquid-ordered phase (L_o). This phenomenon may be related to an uneven repartition of the peptide on the LUV surface. Pep-1 might indeed be concentrated on the defects in the packing of the PLs.[14] Similarly, observation by fluorescence microscopy of the

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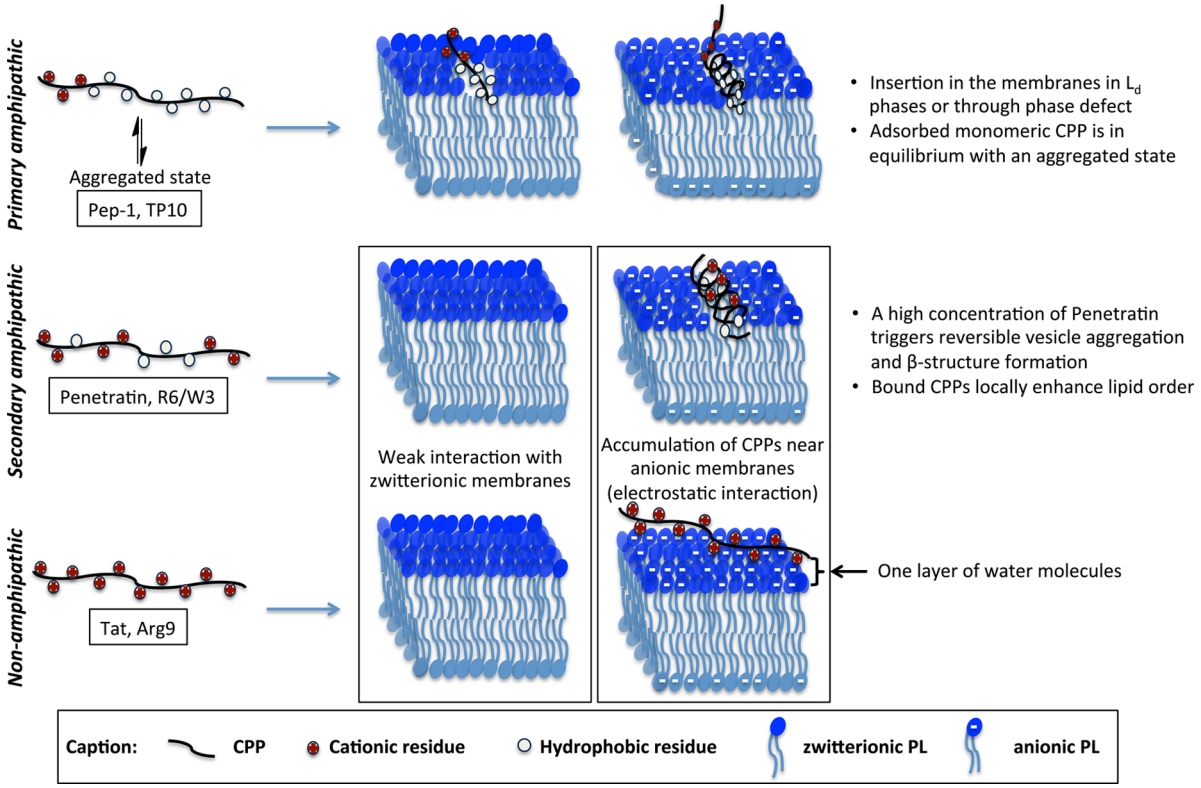

Figure 2. How CPPs bind and insert into the lipid bilayer. Affinity, structure, and insertion into lipid bilayers of primary amphipathic, secondary amphipathic, and nonamphipathic CPPs. CPPs are random coils in solution. Primary and secondary amphipathic CPPs can adopt a defined secondary structure undergoing a structural change triggered by the membrane. Primary amphipathic CPPs can insert into both membranes with low and high percentages of anionic headgroups. In contrast, secondary amphipathic and nonamphipathic CPPs insert in only membranes that have a high concentration of anionic lipids.

interaction between Pep-1 and supported membranes has shown that it displays a higher affinity for curved membranes.[18]

The TP10 peptide exhibits a clear preference for liquid-disordered (L_d) regions of giant unilamellar vesicles (GUVs).[19] The preference of TP10 for L_d domains, where PLs are less tightly packed, is in agreement with its penetration properties.

This particularly high affinity of primary amphipathic CPPs for phase defects or disordered regions of membrane bilayers might be related to an interaction both with the hydrocarbon core and with the headgroups of the PLs (Figure 2).

1.2. Affinity of Secondary Amphipathic CPPs for Model Membranes. Secondary amphipathic CPPs are less prone to interact with membranes composed of only zwitterionic PLs. Nevertheless, they interact with negative membranes as efficiently as primary amphipathic CPPs do.

The affinity of Penetratin has been extensively studied over the past 15 years and is now well-documented. Binding isotherms have been determined by titration using NBD-labeled Penetratin[20] (Penetratin labeled with the 7-nitrobenz-2-oxa-1,3-diazole fluorophore) or unlabeled Penetratin using the fluorescence properties of tryptophan residues in the sequence.[21−25] Whereas the apparent constant is $\sim 10^6$ M^{-1} for LUVs containing ≥20% anionic PLs, it drops rapidly for less anionic LUVs. Similar binding affinity has been measured for R6/W3.[26] The apparent strength of this interaction is essentially due to the accumulation of CPPs next to the membrane because of electrostatic interaction. Indeed, Penetratin accumulates immediately above the membrane surface at a local concentration of ∼500 μM, whereas the concentration of the free peptide is in the micromolar range. The effective partition constant, which evaluates the other contribution to membrane affinity, is smaller and corresponds to the partition constant of other membrane-active peptides containing hydrophobic residues (80 M^{-1}).[27] Interestingly, whereas the apparent partition constant depends on the membrane charge density, the partition constant does not.[21] This shows that Penetratin does not interact specifically with any of the tested PLs polar heads but rather that Penetratin accumulates on anionic membranes because of nonspecific electrostatic interactions.

The affinity of Penetratin for anionic membranes was also quantified using isothermal titration calorimetry (ITC). Binder and Lindblom found apparent binding constants similar to those obtained by fluorescence-based assays.[28] Moreover, they showed that the affinity of Penetratin for membrane bilayers is essentially driven by enthalpy. As a consequence, the binding of Penetratin to membrane surfaces is not favored by the classical hydrophobic effect (i.e., desolvatation of the peptide upon the membrane, driven by entropy) but by the nonclassical hydrophobic effect (hydration of the PLs polar heads and the packing density of the phospholipids) and lipid-induced peptide conformational change. PL ordering induced by Penetratin has been experimentally observed.[29] This packing is expected to be exothermic and can explain part of the heat released during ITC experiments.[28] Membrane binding can also induce an exothermic conformational transition of CPPs (random coil to α or β structures) that contributes to the binding energy (Figure 2).[30]

1.3. Affinity of Nonamphipathic CPPs for Model Membranes. Nonamphipathic CPPs do not contain fluoro-

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genic residues. Thus, binding studies using optical techniques require chemical derivatization.[31,32] Membrane staining of neutral GUVs by fluorescent Tat is non-negligible and increases with the content in anionic PLs but decreases in the presence of salts, demonstrating both that electrostatic interactions between Tat and membranes are fundamental but also that other interactions exists (as for secondary amphipathic CPPs). Two-dimensional diffusion coefficients for this peptide have been determined using single-molecule tracking and reveal that Tat diffuses faster than PLs on membranes. Its diffusion is also independent of the phase state of the bilayer. These observations indicate that this peptide floats on the membrane bilayer (Figure 2).[32] The equilibrium position above the membrane surface for the Tat peptide corresponds to the minimum of the electrostatic potential arising from both the Coulombic attraction and the Born repulsion. Tat is located ~0.3 nm above the surface of the membrane, thus leaving a small gap filled with one layer of water molecules. Quantitative analysis of membrane binding has also been performed by titration using a tryptophan-labeled Tat analogue, similar to Pep-1 or Penetratin.[22,33]

Ziegler and Seelig also performed ITC experiments. They obtained results compatible with the fluorescent study and also observed that the electrostatic contribution is large and accounts for 80% of the binding energy between peptides and membrane.[34] A slight hydrophobic contribution accounts for 20% of the interaction. They showed that the affinity of peptide for membranes is an enthalpy-driven phenomenon below 37 °C but that the binding entropy increases with temperature. As a consequence, nonelectrostatic interactions are due to hydrophobic effects (desolvation of the peptide).

The affinity of Arg9 for anionic membranes has also been evaluated and shown to be high.[26,35] The electrostatic contribution for the binding of this nonamphipathic CPP to membranes is high but interestingly accounts for only 33% of the binding energy. This is surprising given the polycationic nature of Arg9. Nevertheless, the partition constant also contains a hydrogen bonding effect, which is enhanced with arginine in comparison to that with lysine and can explain the measured differences between both peptides.

In summary, the affinity of CPPs for membranes can originate from several contributions: Coulomb interaction, hydrogen bonding, the hydrophobic effect, and conformational transitions. The different behaviors between CPPs can be explained by the relative weights of these contributions in the binding free energy. Primary amphipathic CPPs are prone to adsorb on membrane bilayers, regardless of whether they are anionic, because of the large hydrophobic contribution to membrane binding. They can penetrate the bilayer, particularly if it is in the disordered phase or presents packing defects. For secondary amphipathic CPPs, the electrostatic contribution increases and is even the largest contribution for anionic membranes. They are thus expected to interact preferentially with anionic regions of eukaryotic cell membranes. However, PL-induced CPP conformational change and CPP-induced PL ordering increase the binding energy of secondary amphipathic CPPs for membranes. In contrast to the two previous CPP subgroups, nonamphipathic CPPs (Arg9 and Tat) interact only with anionic membrane bilayers. Nevertheless, even for this group, the other contributions to the affinity are noticeable.

2. EVOLUTION OF THE FOLDING OF CPPS NEAR MEMBRANES

Unstructured in buffered aqueous solutions, CPPs can evolve near membranes and adopt well-defined secondary structures. The folding of CPPs occurring in the presence of membranes has been related to their penetration properties,[36] but the opposite conclusion has also been drawn.[37] In the following section, we will describe the main structural transitions that occur when some of the most well-known CPPs are exposed to a new environment. We will also discuss the eventual formation of supramolecular aggregates of CPPs.

2.1. Structural Characterization of CPPs in Aqueous Buffer: CPPs Are Essentially Unstructured. In aqueous solutions, CPPs are essentially unstructured.[26,38−44] Tat and Arg9 are fully disordered, whereas Penetratin, R6/W3, TP10, and Pep-1 might have a low level of secondary structure.[40,41] At millimolar concentrations, only Pep-1 presents a helical conformation in pure water.[39]

2.2. Structural Characterization of CPPs in the Presence of Phospholipids: α and β Structures Have Been Observed. Eiríksdóttir et al. recently proposed a new classification of the different CPPs (starting from their structural characteristics) in three groups that could explain their respective internalization pathways.[38]

2.2.1. "Helix" Subgroup. Pep-1, TP10, and R6/W3 are members of the helix subgroup.[38] These peptides are random coil in the presence of zwitterionic phospholipids but can fold as a helix in the presence of negatively charged PLs (20−100% content in PG) (Figure 2).[26,38,39,43,45]

Remarkably, Pep-1 can form stable cell-permeable complexes via hydrophobic interaction between its tryptophan-rich domain and the cargo moiety.[39,46] This interaction does not induce a folding of Pep-1, which remains essentially unstructured. Nevertheless, upon binding to the membrane of the Pep-1−cargo complex, this CPP still experiences a conformational transition to a helical structure.[39]

2.2.2. "β-Sheets" Subgroup. In the presence of PLs, the secondary structure of Penetratin depends dramatically on the experimental conditions. Eiríksdóttir et al. consider Penetratin as a member of the β-sheets subgroup because this CPP is susceptible to switching from a random coil in solution to a β-sheet under certain conditions. Nevertheless, in the presence of PLs, Penetratin may also remain unfolded or even form α-helices.

Penetratin is essentially random coil in the presence of zwitterionic PLs.[38,40−42,47] As reported by Magzoub et al., the degree of helicity increases with the presence of anionic PG headgroups.[47] Anionic PG headgroups (20%) seem to be sufficient for Penetratin to trigger a helical conformation (Figure 2). The secondary structure of Penetratin seems then to be insensitive to the zwitterionic/anionic PL ratio for membranes having 20−50% PG (with an approximately 1/100 CPP/PL ratio).[21,47] This latter observation is in agreement with the fact that partition constants of Penetratin are the same for zwitterionic and anionic PLs. However, for a higher PG content, the α-helicity decreases in favor of β-sheet structures.[38,47,48]

Penetratin is exquisitely sensitive to membrane charge density and to the CPP/PL ratio. At a high CPP/PL ratio (between 1/30 and 1/1), Penetratin forms antiparallel β-sheets. Surprisingly, the α-helix to β-structure switch observed as the ratio of CPP/PL increases has also been associated with vesicle

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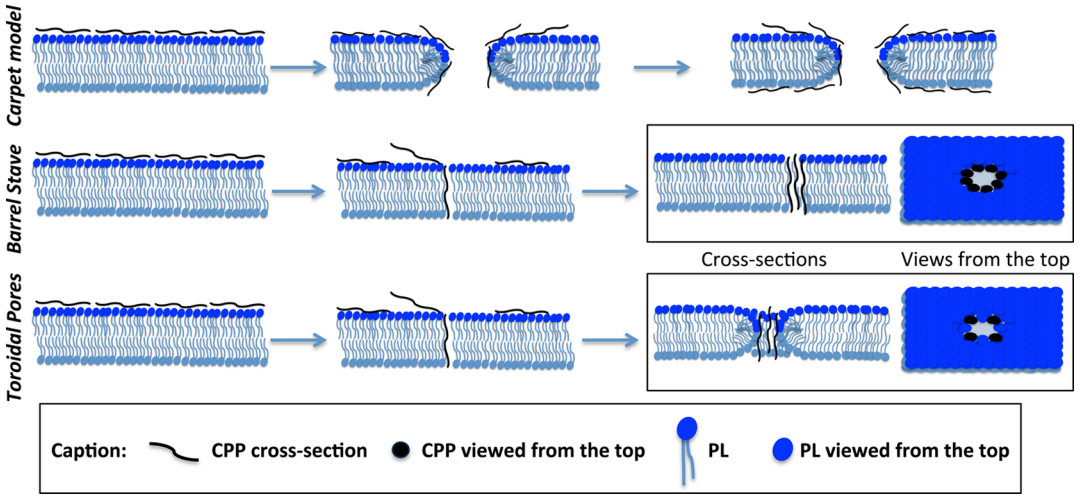

Figure 3. Different mechanisms postulating how CPPs may perturb membranes. In the carpet model, CPPs bind and accumulate on the bilayer surface. Having exceeded a threshold concentration, they insert into the hydrophobic core. This induces a micellization and disrupts the bilayer, as a detergent would do. In the barrel-stave model, CPPs aggregate and insert into the membrane bilayer forming a well-defined pore. In the toroidal pore model, CPPs bind to the surface, aggregate, and induce the lipid monolayers to bend (tilt) continuously, thus adopting a saddle-splay curvature.

aggregation, as assessed by turbidity measurements. Interestingly, this aggregation is transitory, because the turbidity fades. The system relaxes to dispersed vesicles of the same diameter as the initial vesicles with concurrent formation of α-helices.[40,41,49] The only possible interpretation for this phenomenon is that after a short phase of accumulation of Penetratin on the membrane, penetration of Penetratin into the vesicles induces disaggregation of the vesicles.[49]

2.2.3. "Disordered" Subgroup. Contrary to Pep-1 and Penetratin, Tat and Arg9 remain unfolded and are members of the disordered subgroup.[38] Both peptides adopt no specific secondary structure, regardless of their environment (Figure 2).

It should be pointed out that only scarce data have been obtained that correlate the folding with the penetration of the CPPs. We have shown that spontaneous translocation of CPPs occurs both in DOPC/DOPG LUVs (DOPG higher than 80% in moles) and in DOPC/DOPG/cholesterol LUVs (with 30% DOPG in moles). This percentage of anionic PLs is sufficient to generate stable helical secondary structures. As a consequence, it can be postulated that direct translocation of Pep-1, TP10, R6/W3, and Penetratin occurs when they are in a helical conformation, whereas Arg9 and Tat translocate as random coils.

2.3. Formation of Supramolecular Structures: Aggregation of CPPs. *2.3.1. Aggregation of CPPs in Solution.* The aggregation of CPPs in solution might influence their binding properties and furthermore change their internalization pathway(s). Pep-1, being a primary amphipathic peptide, is expected to form micelles. The critical micelle concentration (CMC) has been determined by measuring the surface tension at the air−water interface and is low (0.5 μM).[38,39] Tryptophan quantum yield measurements also show a concentration dependence, which can be explained by the formation of aggregates. The apparent critical concentration is ∼3.4 μM, probably corresponding to the formation of larger aggregates.[14] Similarly, TP10 is clearly amphipathic and has a very low CMC (38 nM), whereas Arg9, Tat, and Penetratin have no interfacial properties and do not aggregate in solution.[38]

2.3.2. Aggregation of CPPs on Membranes. Most importantly, aggregation of CPPs can occur on the membrane. This phenomenon can be triggered by a high concentration of the CPPs on negative PLs but also may be induced by conformational changes.

The aggregation of Penetratin on cell membranes has been postulated as being an important feature for cell penetration. More recently, the oligomerization of Penetratin has been reported on various model membranes. Electron spin resonance (ESR) has been performed with Penetratin tagged with a nitroxide paramagnetic spin-label.[47,50] The intensity of ESR spectra showed a high dependency on the CPP/PL ratio, and this dependency has been attributed to magnetic coupling phenomena, suggesting great proximity between the spin-labels. This dependency has only been reported for negatively charged vesicles and has been observed in the same CPP/PL ratio range as the α-helix to β-structure transition.

Aggregation of Pep-1 has been directly observed by microscopy at the grazing angle geometry using fluorescently labeled Pep-1.[18] The diffusion of clusters of Pep-1 molecules on the membrane surface of GUVs has been followed, and their diffusion coefficients have been determined, revealing that radii of Pep-1 aggregates span more than 30−100 PLs. Similar evaluation of the diffusion coefficient for the Tat peptide on GUVs has been performed by Ciobanasu et al.[31,32] They reported the absence of aggregates of this CPP. Nevertheless, the aggregation of Tat peptide in the presence of a large proportion of highly anionic PLs has not been investigated.

To investigate the relationship between the aggregation of CPPs and the direct translocation phenomenon, we performed a kinetic analysis of the penetration of CPPs inside anionic LUVs.[51] We showed that both Arg9 and R6/W3 translocate through membranes as dimers. Transient aggregation or at least dimerization may occur on this membrane, probably because of the shielding of positive charges of these CPPs by the anionic heads of the PLs. Such a phenomenon can occur *in vivo* because of the heterogeneity of cell membranes, which can exhibit negatively charged domains. As a consequence, the penetration of CPPs by direct translocation might not be regarded as an interaction between a single CPP and a membrane bilayer.[51,52]

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3. CPPS AS MEMBRANE-ACTIVE PEPTIDES

Membrane-active peptides such as cell-penetrating, antimicrobial, and cytolytic peptides are all known to perturb cell membranes. This is inherent to their biological role. In this section, different aspects of the bilayer modifications induced by CPPs will be discussed. Lipid packing modification, membrane permeabilization, or alteration of the membrane asymmetry will be the topic of section 3.1. The connection between membrane curvature and the efficiency of membrane translocation will be dissected in section 3.2.

3.1. CPPs Induce Lipid Reorganization. The large majority of CPPs are cationic; their spontaneous translocation across the hydrophobic membrane core is not a passive diffusion and requires lipid rearrangement, but even when the peptide binds, the membrane experiences a series of structural changes.[53−55]

3.1.1. CPPs Perturb the Lipid Packing, Membrane Organization, and Membrane Fluidity. A widely used model that describes the lipid packing defects induced by the presence of CPPs is the carpet model. In this model, the peptides accumulate on the bilayer surface. Above a critical threshold local surface density, CPPs disrupt the membrane bilayer in a detergent-like manner (Figure 3).[56]

However, some other modifications induced by the presence of the CPPs are more subtle. Joanne et al. found that both CPPs and antimicrobial peptides (AMPs) induce specific lipid segregation of binary lipid mixtures, unsaturated lipids being preferentially recruited, before lipids with higher phase transition temperatures.[57] According to Lamaziere et al., the membrane tubulation induced by Penetratin is inhibited in L_o but favored in the L_d membranes. Both results suggest a certain role of lipid mobility, for achieving the required rearrangements for peptide internalization.[58,59] Penetratin is able to induce dynamical phase separation of PL bilayers, in concomitance with Penetratin-rich clusters and transient domain formation.[60] The internalization of Penetratin into cells and the extent of PL reorganization in model membranes induced by the CPP were found to be correlated.[61] Walrant et al. suggested that the increased lipid bilayer fluidity induced by R6/W3 facilitates peptide translocation and endocytosis.[62] Moreover, Arg9 increases the bilayer fluidity and affects the fatty acid chain packing rendering the lipid bilayer more prone to reorganization.[26]

3.1.2. CPPs and AMPs (pore formation and phospholipid flip-flop). To cite the author's statement in an interesting review, "Potentially, all CPPs are AMPs and all AMPs are CPPs".[63] In the case of AMPs, these perturbations are important enough to generate permanent defects in the lipid bilayer leading to membrane permeabilization (Figure 3).[64−68] Similarly, at high CPP/PL ratios, membrane perturbation has been observed. For example, Herce et al. showed that Arg9 is able to destabilize the bilayer creating transient pores.[69] Herce and Garcia investigated the mechanism of membrane translocation for the Tat peptide by molecular dynamics simulations. According to their model, Tat peptides act cooperatively: once they exceed a certain threshold local surface density (when the surface is locally "crowded"), they sequester the phosphate groups of the phospholipids located on the outer leaflet of the membrane bilayer and attract the phosphate groups located on the inner leaflet. As a consequence, the bilayer becomes thinner. Arginine and lysine side chains on the Tat peptide are inserted into the lipid bilayer and nucleate the formation of a transient transmembrane pore (half-life of <1 μs). Once the pore is formed, the Tat peptides translocate across the membrane by diffusing along the walls of the pore.[70] Several experiments demonstrated how "crowding" of Tat peptides on the membrane surface might occur before translocation.[34,71]

AMPs might have the propensity to induce PL flip-flop. For example, the AMP magainin 2 rapidly scrambles the phospholipids between both leaflets. This phenomenon has been related to the formation of toroidal pores through the bilayer. According to this model of pores, PLs can freely diffuse along the walls of the pore and give rise to an apparent PL flip-flop (Figure 3).[72] We recently demonstrated that CPPs can also catalyze PL flip-flop, but via a very different mechanism. Indeed, the addition of CPPs to a suspension of vesicles induces only a weak PL flip-flop. Moreover, the stoichiometry between the amount of translocated CPPs and flipped PLs is directly proportional to the charge of the considered CPP: one anionic PL flips per cationic residue present in the sequence of the CPP. CPPs thus mediate flip-flop of PLs via the formation of neutral hydrophobic CPP−PL complexes, and reciprocally, the translocation of CPPs through biological membranes is thus mediated by PLs. This translocation model corresponds to the formation of inverted micelles and will be further discussed in section 5.3.[51]

These examples highlight the fact that biological membranes are far more complex than an inert hydrophobic barrier. In the translocation process, the reorganization of the membrane is fundamental and the two counterparts, CPPs and PLs, are always intimately associated.

3.2. Coupling Membrane Curvature to Function. A complex balance of repulsive and attractive forces between lipid molecules drives the self-assembly of asymmetric lipid bilayers.[73,74] The connection among pore formation, invaginations, protrusions, and Gaussian curvature has been explored in the literature. Irrespective of its molecular origin, a spontaneous curvature generates a spontaneous tension. The shape of lipid molecules determines the spontaneous curvature.[75] Some lipids induce negative membrane curvature (PE), while PC, PG, and PS possess a cylindrical shape and self-assemble to give flat membranes. It is possible to find an explicit link between the local chemical composition and the bending properties of a membrane (L_d phases favor saddle shapes, and L_o phases preferentially segregate into the lower-curvature areas).[76,77] The membrane spontaneous curvature has been studied in connection with the occurrence of elastic instabilities, tubulations, and budding processes.[78]

In general, accumulation of CPP in LUVs is less efficient than in GUVs. To explain the lack of internalization into LUVs, Persson et al. proposed a correlation among membrane curvature, membrane tension, and the ability of Penetratin to translocate across model lipid membranes.[79]

Negative Gaussian and saddle-splay curvature deformations are thought to be the major events leading to translocation of both CPPs and AMPs.[80] The ability of the Tat peptide to translocate across cell membranes can be related to the induction of negative Gaussian curvature, which is topologically required for pore formation.[81] More generally, peptides favoring negative curvatures were shown to be more efficient for membrane translocation.[82] Penetratin, Arg9, and R6/W3 were found to induce morphological membrane perturbations in plasma membrane spheres.[83] The formation of invaginations in membranes and the consequent GUV deformation can result in tubular pearling.[84] The membrane fluidity is critical for the

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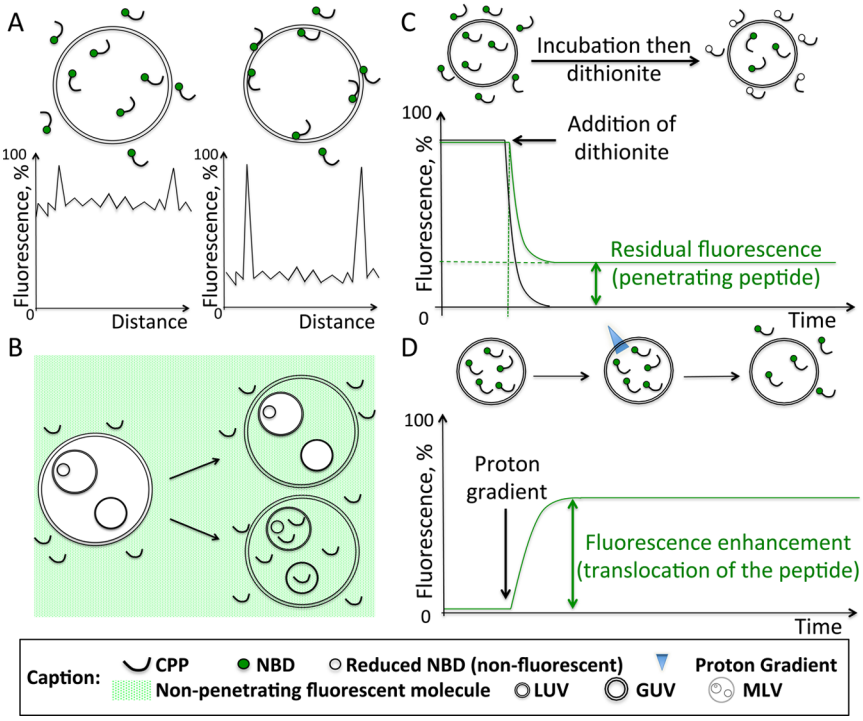

Figure 4. Insight into some protocols that assess the direct translocation of CPPs through membrane bilayers. (A) It is possible to visualize GUVs by confocal microscopy. Fluorescently labeled CPPs can be either homogeneously distributed (left) or mainly adsorbed on the lipid bilayer (right). In the second case, direct translocation is difficult to assess. (B) MLVs in the presence of a small dye such as a tracer can be used to evaluate CPP translocation without using fluorescently labeled CPPs. If CPP membrane permeabilization of the bilayer is associated with its translocation, the dye will be able to penetrate within the different compartments of an MLV (bottom scheme). If the CPP-induced membrane permeabilization does not induce CPP translocation, the small vesicles present in the lumen of the largest one will not contain any dye (top scheme). (C) Schematic representation of the experimental setup used to measure the accumulation of NBD-labeled peptides inside LUVs. Sodium dithionite is added to quench the fluorescence of the noninternalized NBD peptide. The ratio between the residual and the initial fluorescence gives the amount of internalized NBD-CPP. (D) Evaluation of the release of labeled CPP molecules entrapped in LUVs. If the creation of a proton gradient provokes the escape of a fluorescently labeled CPP from the lumen of LUVs, an increase in the measured fluorescence will be observed.

occurrence of these peptide-induced membrane deformations, as shown by the fact that Penetratin is able to induce tubulation ("physical endocytosis") in L_o membranes of GUVs but not in raftlike domains of membranes.[58,59] The curvature and elasticity of the lipid bilayer are thus exceptional features characterizing the behavior of membranes toward the interaction with membrane-active peptides.[85]

With regard to the membranes, what are the features that make them more permeable to peptides? Unequivocal assessments are difficult to establish, given the complexity of membranes and CPP−PL interactions. We could refer to either the ability of peptides to form inverted micelles with PLs, the ability to induce deformations, or their insertion into the bilayer. We will address this question in the next section.

4. INSERTION OF CPPS INTO THE LIPID BILAYER

CPPs are random coils in solution and tend to assume a more defined secondary structure when interacting with model membranes. According to Deshayes and co-workers, CPPs folded as an α-helix are able to insert deeper in membranes.[38] The orientation of a peptide in a membrane depends not only on its secondary structure but also on the lipid composition. The different modes of insertion of primary, secondary, and nonamphipathic CPPs will be described in the following sections.

4.1. Insertion of Primary Amphipathic CPPs into Model Membranes. Troeira Henriques et al. investigated the translocation properties of amphipathic Pep-1, focusing their attention not only on its ability to translocate and destabilize membranes but also on its conformation and orientation within the lipid bilayer. Insertion of Pep-1 into the hydrophobic domain of the membrane is thought to promote membrane destabilization. By calculating the α-helix mean tilt angle, they found that in DPPC membranes, Pep-1 is intercalated with a defined orientation close to the membrane normal, while in more fluid membranes, such as POPC, Pep-1 shows no preferred orientation.[43]

As with Pep-1, TP10 forms amphipathic α-helices when bound to membranes. Yandek et al. proposed a thermodynamic cycle to rationalize the transfer of TP10 from water to a bilayer core that includes the following steps: helix formation at the membrane surface followed by internalization into the bilayer core region of the α-helical peptide with a tilt angle of 90°.[17] Both Pep-1 and TP10, which fold as α-helices when bound to membranes, are able to penetrate the bilayer and permeabilize membranes.[86]

4.2. Insertion of Secondary Amphipathic CPPs into Model Membranes: Penetratin. In the presence of DMPC, the two tryptophan residues of Penetratin display the same wavelength and quenching constant as in aqueous buffer, indicating that they are not associated with vesicles, but in the presence of DMPG or DOPG vesicles, the tryptophan emission peak is shifted to 340 nm, indicating that the tryptophans are partially buried in vesicles.[40] Penetratin also strongly interacts

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with negatively charged small unilamellar vesicles (SUVs), and Trp48 is more deeply inserted into the membrane.[87]

Similarly, Penetratin interacts with POPG/POPC (30/70) and pure POPG vesicles. The lack of distinction between the two tryptophan residues can be rationalized as an indication that Penetratin folded as an α-helix lies flat parallel to the bilayer, close to the surface.[48] The insertion of the tryptophan residues has been evaluated to be around 10−13 Å from the bilayer center.[22]

The relationship between the Penetratin secondary structure triggered by the interaction with charged vesicles and its insertion within the lipid bilayer is ambiguous and strongly depends on the model system (i.e., SUVs vs LUVs).[40,87] The exact nature of a triggered CPPs folded state is not a prerequisite for lipid binding and cell internalization, and the "choice" between the two types of structure is driven by the interaction that leads to the more stable conformation.

4.3. Insertion of Nonamphipathic CPPs into Model Membranes. The transfer of the arginine residues of membrane-active arginine-rich peptides, such as Tat and Arg9, into DOPC lipid bilayers is "non-additive". The limiting step of this transfer is the formation of the first water defect allowing the penetration of the first arginine. Once this step is overcome, the energetic cost for transferring additional arginine residues is minimal, if not favored. Nevertheless, the translocation process might strongly depend on the exact composition of the membrane.[88] Vorobyov et al., by calculating the free energy for arginine binding and lipid bilayer crossing, observed similar behavior in bilayers composed of zwitterionic lipids or anionic/zwitterionic lipid mixtures.[89] Furthermore, the favorable enthalpy for the energetics of lipid binding of polyarginine increases with an increase in polymer chain length.[44]

In summary, the interactions and orientations of peptides in membranes depend on the sequence of the CPPs, the nature of the interacting lipid bilayer, and the model system. The relationship between membrane insertion and the translocation properties should be taken with care.[90] Almeida and Pokorny dissected the translocation machinery of these membrane-active peptides from a thermodynamic point of view, as proposed by White and Wimley.[91] They concluded that the mechanism of action of amphipatic α-helical peptides is driven by the thermodynamics of the insertion into membranes from the surface-bound state.[45] Later, by studying the behavior of three AMPs, they found that the key step for peptide activity is not peptide insertion but more likely binding of the peptide to the membrane.[92]

5. TRANSLOCATION EVENT: HOW TO REACH THE OTHER SIDE OF THE LIPID BILAYER?

Artificial membrane bilayers are attractive models of natural membranes for the analysis of direct translocation mechanism(s) because all energy-dependent processes are abolished. The use of artificial membranes in studying the translocation process will be discussed in this section, as well as the use of transbilayer electrochemical gradients to mimic the gradients occurring through cell or endosome membranes.

5.1. Qualitative and Quantitative Protocols for the Assessment of CPP Translocation through Artificial Membrane Bilayers. *5.1.1. Direct Visualization of the Translocation of CPPs.* The internalization of CPPs inside GUVs can be directly observed by confocal microscopy using fluorescently labeled peptides (Figure 4).[22,31,44,79,93−96] This strategy is very convenient but leads to several artifacts.[93] To avoid false-negative results, several authors have reported on the use of multilamellar vesicles (MLVs) instead of GUVs.[95,97,98] The translocation was assessed by the observation of smaller fluorescent vesicles in the lumen of the outer leaflet (Figure 4B). To avoid CPP labeling, Wheaten et al. investigated the translocation of native CPPs through MLVs in the presence of a small dye as a tracer.[94] If the investigated CPP perturbs the membrane while crossing it, the tracer dye will penetrate successively in the different small vesicles (Figure 4B). Such a protocol has the major drawback of being insensitive if CPPs translocate silently. Huang et al. reported an interesting translocation assay based on the use of two giant inverted micelles, which can re-create a membrane bilayer when put into contact.[99] This strategy is attractive because single translocation events can be monitored and also because membrane asymmetry can be precisely controlled. Finally, if such protocols providing an easy picture of the translocation process are attractive, they are both time-consuming and, quantitatively speaking, poorly informative. Therefore, many authors prefer LUVs as model membranes.

5.1.2. Assessment of the Direct Translocation of CPPs through LUV Membrane Bilayers Using Fluorescence-Based Protocols. Quantitative fluorescence assays are based on the high sensitivity of the quantum yield to the direct environment of the probe. The principles of some of these experiments are summarized in Figure 4 and have been reviewed by Henriques et al.[66]

5.1.3. Assessment of the Direct Translocation of CPPs Using Isothermal Titration Calorimetry. Binder and Lindblom reported the observation of a direct translocation of CPPs using ITC.[28,100] For anionic LUVs, containing <50% anionic PLs, the released heat remains constant until the peptide saturates the membrane and then drops to zero. This released heat corresponds to the binding of the CPP to the membranes. For higher anionic contents, the released heat is more important and changes over the course of the titration experiment. The shape of the curves can be interpreted only if one considers that direct translocation occurs.

In summary, fluorescence spectroscopy has been extensively used to probe the internalization of CPP into vesicles. Some other strategies have been developed, such as ITC and quantitative mass spectrometry assays.[101] Nevertheless, the data obtained concerning direct translocation through bilayers seem contradictory.

5.2. Direct Translocation Can Spontaneously Occur through Membrane Bilayers. Direct translocation of CPPs was first questioned in 2000 by Thorén et al. and in 2001 by Drin et al.[25,96] Whereas Thorén et al. assessed the direct translocation of Penetratin through GUVs, Drin et al. concluded that Penetratin is not able to cross LUVs. Since then, divergent results have been described. Direct translocation of the six CPPs described herein has been observed through GUV membranes using confocal microscopy and fluorescently tagged CPPs (Figure 4A).[22,44,79,94,96,101] A rapid equilibration of concentration between the outside medium and the vesicle lumen has been described for Tat, Penetratin, and TP10.[22,31,94] A threshold of anionic PL content has been reported for the translocation of Tat, whereas TP10 translocates even through neutral membrane bilayers.[31,94] Penetratin requires only a low content of anionic PLs. Nevertheless, such studies have to be assessed with caution because of the high sensitivity of GUV membranes.[102] Moreover, Bárány-Wallje et

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al. reported no entry for the same CPPs under analogous experimental conditions.[93]

Spontaneous direct translocation has also been investigated with SUVs and LUVs (Figure 4C). Even for these model membranes, divergent results have been described. Several studies have shown that CPPs cannot penetrate spontaneously inside such curved vesicles.[22,79,93,103] However, spontaneous translocation of CPPs has been reported through membranes made of binary PL mixtures having a high content of anionic headgroups (>50 mol %).[51,100,101] Binder and Lindblom reported the existence of a high threshold of requirement for membrane negative charge density for Penetratin for the observation of the direct translocation phenomenon.[100] We have not always observed such a requirement, which seemed dependent upon the membrane composition. The anionic content can be lowered for ternary PL mixtures. Nevertheless, no obvious general rule could be derived to rationalize the various internalization kinetics and yields.[51] Interestingly, we observed that Arg9, Tat, and R6/W3 can cotransport nonpenetrating peptides inside LUVs when covalently bound to them.

5.3. Mechanism(s) of Direct Translocation. To date, various models have been proposed to rationalize CPP translocation, which is frequently considered as a perturbation of the bilayer (Figure 3). Until recently, no direct validation of these models has been obtained, except for the formation of inverted micelles, for which the data are in agreement with their formation.[104] However, the CPP/PL stoichiometry could not be determined. Similarly, the formation of pores has been occasionally observed but never fully characterized.

The key difference between these models is the behavior of the PLs upon peptide translocation. For example, PL mixing between the two leaflets of the membranes is expected if transmembrane pores are formed,[72] whereas no mixing should occur if the membrane-thinning model is valid. For the inverted micelle model, the anionic PLs should neutralize the charges of the CPPs and a precise stoichiometry between the translocated CPPs and the flipped PLs should be observed.[53] An assay was set up to test these models. An unlabeled CPP was incubated with LUVs selectively labeled on their outer leaflet by NBD-tagged anionic PLs. Addition of dithionite induces the fluorescence quenching of NBD. In the absence of CPPs, the fluorescence on the outer leaflet was completely quenched upon addition of dithionite, showing that NBD-PLs remained only on the outer leaflet. The addition of CPPs perturbs the membrane asymmetry. We showed that if n CPP molecules enter per LUV and if p is the global positive charge of the CPP, $n \times p$ PLs flipped from the outer leaflet to the inner leaflet of LUVs. This showed that CPPs enter as a neutral complex $[(CPP^{p+})(phospholipid^{-})_p]$, in perfect agreement with the inverted micelle model (Figure 5).[51]

We expected to observe that upon internalization, almost 50% of the incubated CPP would reach the inner leaflet. However, we never observed such a steady state. Depending on the membrane composition and the CPP, we barely obtained a 20% internalization yield, and not the expected 50% of CPP on each leaflet. To interpret this surprising result, we proposed that CPPs enter as small aggregates inside vesicles. The requirement of an oligomerization rate-limiting step prior to internalization would explain why CPPs enter slowly inside LUVs after a rather fast first step.

Alternatively, this surprising lack of equilibration of the CPPs on both leaflets can be explained by the arguments developed

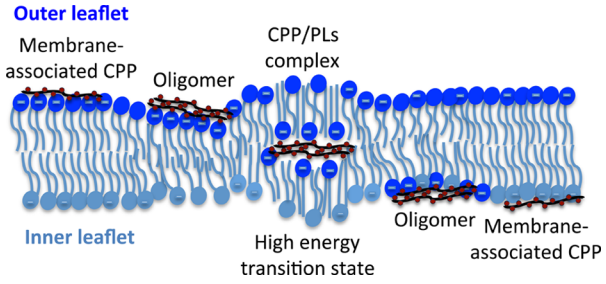

Figure 5. Direct translocation of CPPs can be mediated by the formation of inverted micelles. After CPP binds to the membrane, CPP oligomerization can induce the formation of a CPP−anionic PL inverted micelle-type complex. The neutral $[(CPP^{p+})-(phospholipid^{-})_p]$ complex can translocate through the hydrophobic core of the membrane. As a consequence, anionic lipids are flipped from the outer to the inner leaflet of the membrane bilayer.

by Binder and Lindblom to interpret the apparent existence of a threshold for CPP translocation.[100] If we explained the sensitivity of the internalization rate to concentration by a statistical effect, Binder and Lindblom gave an explanation based on the evolution of the activation energy of the translocation. The energy barrier to cross the membrane is high because it requires the formation of a highly curved inverted micelle structure. The height of this activation barrier indubitably depends on the lateral stress on the membrane bilayer induced by the asymmetric presence of the CPP. Binder and Lindblom demonstrated in a very simple and elegant way that the asymmetric distribution induced the presence of an electrical field through the membrane. This field induces the so-called Maxwell stress and generates a lateral pressure, which tends to bend the membrane. Binding of a CPP on a negatively charged surface also modifies the repulsion between the anionic headgroups of the PL. This creates an area imbalance between both leaflets and also facilitates the creation of inverted micelles. Both effects are in agreement with the dependency of the energy barrier with the surface density of adsorbed CPP. This explains why CPPs do not equilibrate between both leaflets in the absence of another electric field.

5.4. Direct Translocation through Membrane Bilayers Is Modulated by Transbilayer Electrochemical Gradients. The translocation yields increase systematically in the presence of transmembrane electrochemical gradients. Only Bárány-Wallje et al. reported the absence of translocation in the presence of a transmembrane potential.[93] This is probably due to the use of the highly rigid DPhPC/DPhPS mixture.

A transmembrane potential can be created by adding valinomycin to LUVs containing potassium in their lumen placed in a sodium-buffered solution.[105,106] Terrone et al. studied the translocation of NBD-labeled Penetratin into LUVs with a large diversity of membrane compositions in the presence or absence of a transbilayer potential.[106] With a negative potential inside the vesicles, the penetration is enhanced and leads to an increased level of accumulation of CPP in the LUV lumen. Very interestingly, the authors noticed that the internalization yield increases with the propensity of PL to form inverted micelles. Similarly, Troeira Henriques et al. investigated translocation of Pep-1 in the presence of a transbilayer potential.[107,108] They showed that a negative transbilayer potential induced the translocation process through the bilayer, whereas no translocation occurred in the absence of such a potential or in the presence of a positive one.

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Interestingly, Troeira Henriques et al. and Huang et al. reported the transport of β-galactosidase or horseradish peroxidase through artificial membrane bilayers when each was incubated in the presence of a large excess of Pep-1 before the translocation assay.[99,109] Direct translocation of the enzyme has been shown to depend on the presence of a transbilayer potential. The translocation of large CPP−protein complexes is unlikely to occur through the formation of inverted micelles. Formation of stable membrane pores is also excluded, but the translocation can occur through the formation of transient pores as shown by conductance measurements.[99]

To shed light on the endosomal escape mechanism, transmembrane pH gradients were created using LUVs as a model system. The endosomal internalization pathway is indeed characterized by a progressive acidification of the endosomes. Such pH gradients have been generated using nigericin, an ionophore, or bacteriorhodopsin, a light-driven protein proton pump.[8,110−112] In the absence of transbilayer pH gradients, no escape of Penetratin from the endosome lumen has been observed (LUVs with a low anionic PL content). Upon acidification, ∼30% of the Penetratin previously concentrated inside LUVs escaped in ∼100 min, whereas the extent of escape of Tat and Arg9 does not significantly increase under the same conditions.[8] Interestingly, the driving force for the escape of Penetratin from LUVs is not the transbilayer potential generated by the transmembrane proton gradient, as has been shown using valinomycin, but the proton gradient itself (Figure 4D).[110] Considering that Penetratin does not have any residue sensitive to pH changes, in this pH range, the authors proposed that the pH gradient might have an influence on the lipid bilayer. Nevertheless, it is not clear how the pH gradient drives the escape of Penetratin from LUVs.

Because of the use of various experimental protocols, much evidence in favor of the existence of a direct translocation through artificial membranes has been obtained (Figure 4). This phenomenon is not observed on rigid membranes or on membranes having a low binding affinity for peptides. The vector effect of CPPs has even been described for noncovalent complexes between CPPs and proteins or nonpenetrating moieties covalently bound to CPPs. Direct translocation into anionic vesicles occurs and is modulated by lipid composition and various extrinsic parameters such as membrane potential. All of these factors influence the kinetics of translocation (activation energy and concentration of the peptide close to the membrane). Two key parameters can be modulated to increase the internalization yields: the CPP dimerization and the mutual adaption between CPP and PLs to form inverted micelles. These highly curved structures are indeed energetically difficult to form. It is still not clear how the PL composition finely modulates CPP translocation, and this remains a crucial question, one that needs to be tackled to design new CPPs and improve the efficiency of the existing ones.

6. CONCLUSION

Many studies have been undertaken using membrane models to probe the different parameters influencing the interaction between CPPs and the cell surface. Herein, we have presented the interaction between CPPs and PLs, but similar studies have been conducted with surface glycosaminoglycans, which also participate in the concentration of CPPs on cell surfaces.[102] Via the use of membrane models, we now have a good understanding of the parameters that drive CPP−cell surface interactions.

Another contribution of model membranes is the possibility to directly probe the perturbations induced by CPPs. This can help rationalize the eventual cytotoxicity of some CPPs and to possibly prevent it. Indeed, CPPs are neutral vectors for drug delivery, without any biological activity other than their penetration property (considered as undesired side effects). Nevertheless, CPPs as well as other membrane-active peptides slightly perturb the membrane organization. The accumulation of CPPs on the membrane and the eventual insertion of the CPPs have been fully characterized using membrane models.

A very important application of model membranes in the field of CPPs comes from the possibility of efficiently screening thousands of peptides for their penetrating properties. This will be very advantageous for two reasons. First, this screen will yield new penetrating sequences with eventual new properties. Second, by extension of the number of CPP sequences, this might help determine penetration rules. Such a strategy was adopted by Marks et al.[95] They found new CPPs by screening peptides designed starting from the Arg9 sequence. Interestingly, the new CPPs they found have a low percentage of arginine residues. This is probably related to the weakly anionic nature of the artificial membrane bilayer they used (10% PG only). New polycationic CPPs would indubitably have been selected using membranes containing a higher content of anionic PLs. It is currently not straightforward to choose an adequate model membrane to design a suitable CPP for both *in vitro* and then *in vivo* applications. Nevertheless, the CPPs found by Marks et al. have very interesting properties because they can translocate rapidly through multiple weakly anionic membrane bilayers, which cannot be usually crossed by Arg9 or Tat CPPs.

In summary, much has been done to understand the physicochemical properties of CPPs at the thermal equilibrium position and to find new penetrating sequences, but only scarce data have been obtained for the direct translocation of CPPs through membrane bilayers. This is intrinsically a dynamic phenomenon, which is challenging to probe and requires the design of new experimental protocols. We recently validated one translocation mechanism: CPPs can cross membrane bilayers as neutral and hydrophobic $[(CPP^{p+})\text{-}(phospholipid^{-})_p]$ complexes (Figure 5). This mechanism corresponds to the inverted micelle model (or adaptive translocation model). However, this does not mean that the other mechanism(s) might not be valid under other experimental conditions, especially with different lipid compositions or in the presence of membrane proteins. As a consequence, one important research route is the elucidation of the translocation mechanism. In particular, the influence of cargoes on the translocation is crucial. Indeed, the physicochemical properties of the cargo (hydrophobicity, charge, and size) might dramatically affect the properties of CPPs. CPPs can induce the translocation of nonpenetrating cationic peptides covalently bound to them. Very interestingly, we demonstrated that the translocation of these CPP−cargo conjugates proceeds via the same inverted micelle mechanism.[51] The influence of anionic cargoes has never been probed but is likely to decrease the internalization efficiency by shielding the positive charges of the CPP, and despite its high level of physiological importance, the translocation of large cargoes (>50 amino acids; the homeodomain of Antennapedia contains 60 residues) conjugated to CPPs has never been

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evaluated. Another research route is the in-depth understanding of the CPP features that permit them to translocate through membrane bilayers. It is indeed currently impossible to predict if a peptide will translocate. Moreover, it is also very challenging to rationalize the very different penetration properties if subtle changes in the membrane composition are introduced. The complex interplay between a given CPP and a type of PL allowing its direct translocation is what is called the "mutual adaption". The rules of mutual adaption still remain to be understood to propose the first rational design of new penetrating vectors.

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ABBREVIATIONS

AMP, antimicrobial peptide; CMC, critical micelle concentration; CPP, cell-penetrating peptide; DMPC, dimyristoylphosphocholine; DOPC, dioleoylphosphocholine; DOPG, dioleoylphosphoglycerol; DPhPC, diphytanoylphosphocholine; DPhPG, diphytanoylphosphoglycerol; ESR, electron spin resonance; GUV, giant unilamellar vesicle; ITC, isothermal titration calorimetry; L_d, liquid-disordered lipid phase; L_o, liquid-ordered lipid phase; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PC, phosphatidylcholine phospholipid headgroup; PE, phosphatidylethanolamine phospholipid headgroup; PG, phosphatidylglycerol phospholipid headgroup; PL, phospholipid; POPC, palmitoyloleoylphosphocholine; POPG, palmitoyloleoylphosphoglycerol; PS, phosphatidylserine phospholipid headgroup; SM, sphingomyelin; SUV, small unilamellar vesicle.

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