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Interactions of amphipathic carrier peptides with membrane components in relation with their ability to deliver therapeutics

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Abstract: To identify rules for the design of efficient CPPs that can deliver therapeutic agents such as nucleic acids (DNAs, siRNAs) or proteins and PNAs into subcellular compartments, we compared the properties of several primary and secondary amphipathic CPPs. Studies performed with lipid monolayers at the air–water interface have enabled identification of the nature of the lipid–peptide interactions and characterization of the influence of phospholipids on the ability of these peptides to penetrate into lipidic media. Penetration and compression experiments reveal that both peptides interact strongly with phospholipids, and observations on Langmuir–Blodgett transfers indicate that they can modify the lipid organization. Conformational investigations indicate that the lipid–peptide interactions govern the conformational state(s) of the peptides. On the basis of the ability of both peptides to promote ion permeation through both natural and artificial membranes, models illustrating the translocation processes have been proposed. One is based on the formation of a β-barrel pore-like structure while another is based on the association of helices. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cell-penetrating peptides; conformation; membrane uptake; peptide-lipid interactions; amphipathic peptides

Cellular internalization of large hydrophilic therapeutic agents such as proteins or nucleic acids is still a challenging task because of the presence of the plasma membrane, which constitutes an impermeable barrier for such molecules. Circumventing this difficulty requires the assistance of agents that facilitate the crossing of this barrier. Among them, peptides are very powerful tools, and two main strategies are involved: (i) covalent linkage between cargo and vector, thereby forming a conjugate and (ii) formation of a complex between the two partners.

To elucidate the activity of multifunctional CPPs, which can act as carriers of biologically active materials and identify their potential targets, it is crucial to localize the peptides in biological material. Furthermore, the presence of proteases in biological media leads to the rapid degradation of all L-peptides, and the fate of the various resulting peptide fragments may provide important information for the design of resistant materials. One of the easiest to handle and most efficient methods to examine these different aspects of peptide fate consists of the use of fluorescent labels that are covalently linked to the peptide. This approach may also provide information on the possible targets of compounds that could be carried by the peptide. Up to now, most of the investigations have dealt with side-chain labeled peptides, which makes them restricted to functional amino acids. However, as functional side-chains are often involved in the biological role of peptides, it appeared that a peptide labeling that deals with *C*- or *N*-terminals or both, which participate less often in the biological activity, could provide an appropriate tool for the localization of these materials. Such a labeling, at least that of the *C*-terminal, can be easily achieved through the use of mercaptoamide peptides obtained after removal of disulfide linked peptide-resin, which allows further chemical reactions dealing with the – SH group. This type of strategy allows the building up of the peptide by the conventional Fmoc method [1,2]. Subsequently the labeling can be made selectively after complete solid-phase peptide synthesis. Hence, the peptide can be labeled or not at its *N*-terminal when on the resin, while the *C*-terminal labeling occurs after cleavage from the resin [3].

Although, some carrier peptides have begun to be used in the medical field, some weaknesses are still encountered, in particular owing to the lack of specificity toward targets. This implies that some modifications of their sequences or the introduction of non-natural amino acids bearing side-chains that can improve their specificity is required. However, it is now known that modifications in their sequences, although minor, can strongly modify their ability to act as a drug carrier. It is therefore necessary to identify precisely

Abbreviations: CPP, Cell Penetrating Peptide; AEDI, aminoethyldithio 2-isobutyric acid; TBTU, *O*-(1H-benzotriazol-1-yl)-N,N,N′N′-tetramethyl-uronium tetrafluoroborate; TCEP, Tris(2-carboxyethyl)phosphine; NLS, Nuclear Localization Sequence; DPPC, Dipalmitoylphosphatidylcholine; DOPC, Dioleoylphosphatidylcholine; DPPG, Dipalmitoylphosphatidyl-glycerol; DOPG, Dioleoylphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; CD, Circular Dichroism; FTIR, Fourier Transform InfraRed; cpi, critical pressure of insertion; CMC, Critical Micellar Concentration; SDS, Sodium Dodecyl Sulfate.

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the criteria that can define an efficient CPP with a high degree of drug transfer ability. One possibility that remains to be more thoroughly explored lies in the elucidation of the mechanism(s) leading to membrane translocation. This requires the understanding of the interactions of these carrier peptides with membrane components as well as the structural consequences of these interactions. These criteria have to be satisfied by the CPPs that will be designed in the future and that will be at the origin of the next generation of carrier peptides.

SYNTHESES

The peptides (see subsequent text for the sequences) were synthesized using AEDI-Expansin resin with the Fmoc/tBu system as described previously. Fmoc amino acids were activated by the addition of equimolar amounts of TBTU and HOBT diluted to 0.3 ~ with 5.5% NEM in DMF. Coupling was obtained by running this solution through the reaction column for a standard 45 min. Double couplings (45 min) were performed at crucial steps where, according to our experience, a single coupling leads to a strong lowering of the yield and thus to a strong increase in difficulty at the purification step. As an example, for MPG these double couplings concern residues Thr14, Ala17, Lys24, Arg25 and Val27. Fmoc deprotections were achieved by percolating 20% piperidine in DMF. After deprotection of the terminal amino groups, the side-chain protected peptidyl-resins were washed with DCM, diethylether (4×) and dried under vacuum.

MPG or Pβ: AcGALFLGFLGAAGSTMGAWSQPKKKRKV-Cya
Pα: AcGALFLAFLAAALSLMGLWSQPKKKRKV-Cya
Pep-1: AcKETWWETWWTEWSQPKKKRKVCya
With Ac = CH_3–CO–and Cya = NH–CH_2–CH_2–SH

All protected peptidyl-resins were deprotected for 4 h with TFA/ethanedithiol/thioanisole/phenol/H20 (94 : 4 : 2 : 2 : 2). The reactor content was then filtered out and washed with DCM (4×), DMF (4 × 1, ethanol (3 × 1, H20 (3×) and finally with sodium acetate buffer 0.05 M, pH 4.5.

Every deprotected peptidyl-resin reacted with TCEP HC1 (4 equiv. per mole of peptide on the polymer) in degassed 0.05 M sodium acetate buffer (pH 4.5)/DMF (1/1) for 16 h. The reactor content was then filtered out and washed with H_2O (3×) and ethanol (2×). The resulting crude peptide solutions were evaporated and freeze dried. The yields of crude peptides were over 99%.

After side-chain deprotection, the peptides were purified by semi-preparative HPLC in isocratic conditions and the purity of all collected fractions was checked by analytical HPLC.

THE PEPTIDES OF THE MPG FAMILY

Peptides of the MPG family are based on the association of a fusion sequence (GALFLGFLGAAGSTMGA) with the NLS (PKKKRKV) and linker (WSQ). Several variants of the parental peptide have been tested. The first one is the result of a W^7 to F substitution and results in efficient nuclear localization in fibroblasts [4]. This behavior prompted us to synthesize other variants to control the addressed subcellular compartment for nucleic acid cargoes. This was achieved by a K to S substitution at position 23 corresponding to the second lysine of the NLS sequence, generating the peptide vector MPG–Δ^{NLS}. This substitution was made to reduce nuclear addressing by decreasing the number of positive charges, thereby also reducing the stability of the complex formed with the negatively charged nucleic acids. MPG–Δ^{NLS} was shown to efficiently transfer single- and double-stranded oligonucleotides, RNAs [5,6], siRNAs [7,8], double stranded phosphorothioate oligonucleotides [9] and more sophisticated oligonucleotides such as $N^{3'} \rightarrow P^{5'}$ thio-phosphoramidates without formation of any covalent linkage [10,11]. Furthermore, to improve the understanding of the influence of the conformational state, another analogue called Pα has been designed, and we expect it will adopt a helical conformation, at least in its hydrophobic domain. It must be noted that the presence of the cysteamide moiety is required otherwise a severe lowering of the transfection power is observed. This latter point also holds for peptides of the Pep family.

Studies at the Air–Water Interface: Adsorption at Lipid-containing Interfaces

The ability of peptides of the MPG family (MPG and Pα) to introduce itself into phospholipid monolayers spread at the air–water interface was monitored by measuring variations in surface tension at different initial pressures, and by injecting a given peptide concentration in the subphase. This first requires determination of the saturating surface pressures induced by the peptides at a lipid-free air–water interface.

Various penetration experiments were carried out using four different phospholipids, the headgroups (zwitterionic or negatively charged) and the physical state (liquid expanded or liquid condensed) of which were varied. The various plots of pressure variation *versus* the initial surface pressure are reported in Figure 1.

This figure reveals first that for peptide Pα, when the lipid is in a liquid condensed state, the nature of the headgroups has no influence on penetration, and the cpi obtained by extrapolation at initial pressure (Πi) = 0 of the variation of surface pressure ($\Delta\Pi$) induced by the peptide is high (37 mN/m) (Figure 1(A)). In the case of peptide MPG, although still high,

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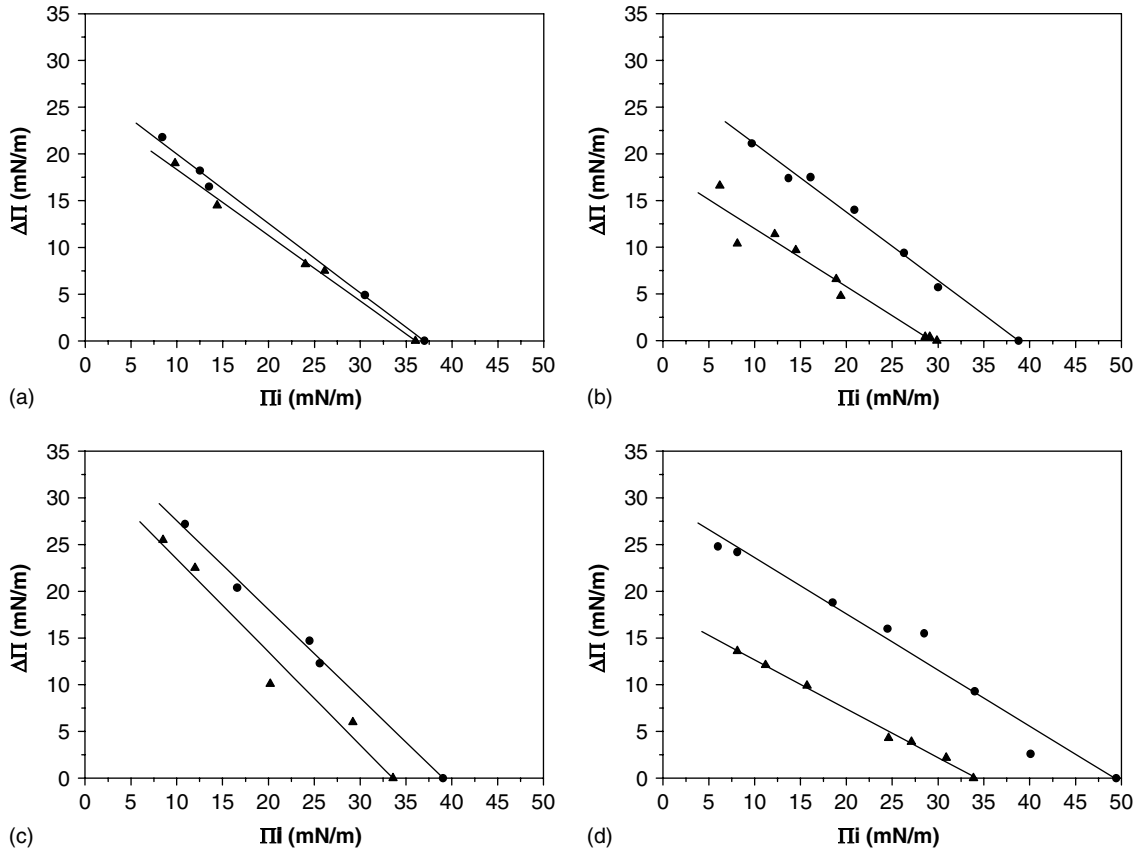

Figure 1 Surface pressure variations as a function of the initial pressure of the phospholipid monolayer. Peptide concentration in the subphase was 0.25 μM. (A) Pα in the presence of ▲ DPPC and ● DPPG. (B) MPG in the presence of (▲) DPPC and (●) DPPG. (C) Pα in the presence of (▲) DOPC and (●) DOPG. (D) MPG in the presence of (▲) DOPC and (●) DOPG. Reprinted from *Biochemistry*, **43**, Deshayes S, Plénat T, Aldrian-Herrada G, Divita G, Le Grimellec C, Heitz F, primary amphipathic cell-penetrating peptides: structural requirements and interactions with model membranes, 7698–7706, Copyright (2004), with permission from ACS.

thecpi increases from 30 to 38 mN/m for DPPC and DPPG, respectively, indicating a better uptake by negatively charged phospholipids (Figure 1(B)). For lipids in the liquid-expanded state, insertion depends on both the initial conformational state of the peptide and the nature of the headgroups. In all cases, the cpi are high (see Figure 1(C) and (D)). Furthermore, the difference between the cpi occurring between negatively charged and neutral phospholipids is weak for the helical peptide (about 10 mN/m) compared to the other (about 17 mN/m) (compare Figure 1(C) and (D)). Further examination of Figure 2 also provides information on the interactions occurring between the two peptides and the phospholipids considered here. These are based on the pressure value measured for an air–water interface at low lipid content (i.e. at low initial pressure). The finding of surface pressures greater than those obtained for the pure peptides at saturation is indicative of strong peptide–lipid interactions [13]. This is the case of MPG, particularly in the presence of negatively charged phospholipids and of Pα with DOPG.

Compression Isotherms

Figure 2 shows the miscibility diagrams issued from the compression isotherms obtained for peptides MPG and Pα when mixed with various phospholipids, DOPC, DOPG, DPPC and DPPG. Isotherms of the pure lipids are in agreement with those already reported [14,15]. Isotherms were analyzed by examining the variation of the mean molecular area (those related to contributions of both the peptide and the lipid) as a function of the peptide/lipid ratio at a given and constant surface pressure. These variations are reported in Figure 3 and correspond to a pressure of 20 mN/m, which is below that of the collapse. Note that the same trends are observed at a pressure of 10 mN/m. Close examination of these insets indicates that, for all situations examined here, small but significant and reproducible deviations from linearity are observed and that several situations are encountered depending on the peptide–lipid combination. Interestingly, for lipids in the liquid-expanded state, namely DOPC and DOPG, positive and negative deviations from linearity occur depending on the peptide conformation. The nonlinear variation of the mean

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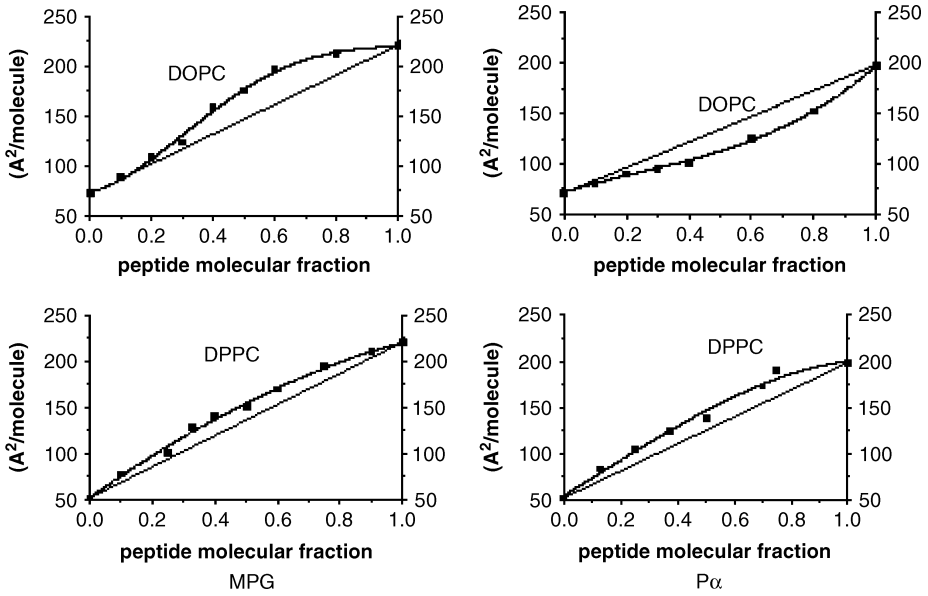

Figure 2 Variations of the mean molecular areas for MPG and Pα as a function of peptide/lipid ratios. The data are issued from the compression isotherms. Reprinted from *Biochemistry*, **43**, Deshayes S, Plénat T, Aldrian-Herrada G, Divita G, Le Grimellec C, Heitz F, primary amphipathic cell-penetrating peptides: structural requirements and interactions with model membranes, 7698–7706, Copyright (2004), with permission from ACS.

molecular area provides a good argument, suggesting that the peptides and lipids are miscible and interact [16–18]. This further indicates that MPG peptide interacts with DOPC and DOPG with contraction of the mean molecular area, while peptide Pα interacts with these phospholipids with an expansion of the mean molecular area. When investigating with phospholipids in the liquid condensed state (DPPC and DPPG), all peptide–lipid combinations reveal an expansion of the mean molecular area, indicating that the peptides interact with and are miscible with DPPC and DPPG [12].

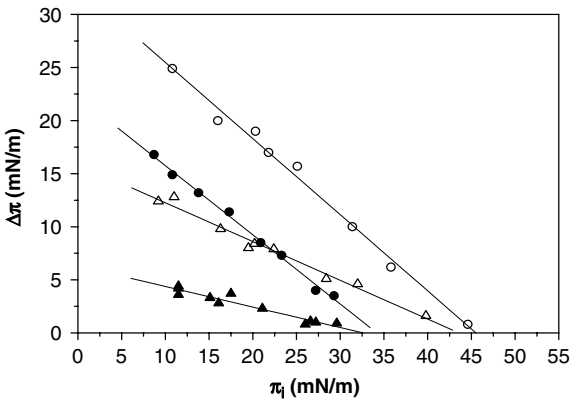

Figure 3 Variation of the surface pressure as a function of the initial surface pressure of the phospholipid monolayer. (Δ) DOPC, (O) DOPG, (●) DPPG, and (▲) DPPC. Extrapolation at zero initial pressure gives the critical pressure of insertion. Reprinted from *Biochemistry*, **43**, Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F, insight into the mechanism of internalization of the cell penetrating carrier peptide Pep-1 through conformational analysis, 1449–1457, Copyright (2004), with permission from ACS.

Mass Spectrometry Analysis

In positive ion mass spectrometry experiments, the dominant complexes detected under a variety of conditions were (1 : 1) MPG/lipid complexes. PGs generally showed stronger binding affinities for MPG than PCs. Zwitterionic PC head groups are overall neutral, whereas PGs are negatively charged. The fact that DLPG and DMPG bind more tightly to MPG than DLPC and DMPC, respectively, shows the significance of the electrostatic interaction in stabilizing peptide–lipid complexes.

Like MPG, Pα exhibited affinity for both PC and PG, although, as was the case for MPG, PC binds less tightly to Pα than PG. It is again clear that electrostatic interactions contribute more heavily to stabilizing the interaction binding between fusion peptides and PG as compared to PC.

Detailed binding specificities between selected phospholipids and model fusion peptides have been examined. DMPG, which carries a negative charge, exhibits a stronger binding to MPG and Pα than DMPC, whose nature is zwitterionic. The increased electrostatic interaction clearly plays a significant role in stabilizing peptide–PG complexes. Methanol addition (known to weaken hydrophobic interactions) disrupted binding between MPG/Pα and PC/PG that had been observed from 100% aqueous solutions; a stronger initial interaction required a higher percentage of methanol to destroy binding. These results indicate that detected MPG/Pα–lipid complexes were already formed in 100% aqueous solution, with the hydrophobic effect being a

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primary driving force promoting the interaction. The fact that the increased hydrophilicity of fibrinopeptide B resulted in a much weaker binding affinity to zwitterionic DMPC as compared to that of the fusion peptides MPG and Pα offered further evidence that hydrophobic interactions in solution contributed heavily to the formation of [MPG/α + PC] complexes. Fibrinopeptide B had moderate binding affinity for DMPG in 100% aqueous solution. Upon addition of methanol, however, it showed an initial slight decrease in binding to lipids that was followed by an increase in detected binding upon further addition of methanol. This behavior contrasts with that observed for the fusion peptides and PG, and thus offers additional evidence that hydrophobic interactions play a key role in allowing the mass spectrometric observation of the latter complexes. Furthermore, with a decrease in pH, detected Pα-DMPC complexes exhibited an immediate and steep drop in binding, whereas the detected MPG-DMPC binding was slightly augmented at pH 3.7 and then decreased gradually at even higher acidity. A comparison of lipid–peptide binding as a function of the degree of unsaturation offered the opportunity to test the effect of the hydrophobicity of lipid on binding. While both Pα and MPG exhibited affinities for unsaturated lipid, (18 : 1)PC bound slightly more strongly to MPG than the less hydrophobic (18 : 3)PC. Again, evidence for the importance of an initial solution hydrophobic effect in establishing binding between model fusion peptides and cell membrane phospholipids is offered. Study findings corroborate the notion that hydrophobic interactions between fusion proteins and cell membrane phospholipids can serve to initiate membrane perturbation in the early stages of viral fusion. At the same time, the ability of ES-MS to provide information regarding the strength of noncovalent interactions that originated from a hydrophobic effect is established [19].

Conformational Investigations in the Presence of Phospholipids

In water, the CD spectrum of MPG exhibits a single minimum centered at 198 nm, which is typical of that expected for a nonstructured peptide. Upon addition of phospholipid vesicles made of DOPG or DOPC/DOPG (80 : 20), dramatic changes in the CD spectra are observed. These modifications are characterized by a strong decrease in the intensity of the negative band at 198 nm associated with a slight increase in the contribution centered at 223 nm. The existence of a sheet structure as the major structural component is confirmed by FTIR observations which reveal, in the presence of phospholipids, the existence of a major amide I band around 1625 cm^{-1} associated with a broad shoulder at 1655 cm^{-1}. On the basis of NMR data obtained in solution in water, with and without micelles of SDS, it can be stated that the hydrophobic sequence of the peptide corresponds to the folded domain while the remainder corresponding to the NLS is not structured [20,21].

Peptide-induced Ionic Leakage

When the peptide (30 µl of a 10 µM peptide solution) in its free form or engaged in a complex at an oligonucleotide/peptide ratio of 0.1 was applied to voltage-clamped oocytes, marked increases in membrane conductance were observed. The presence of nucleic acids has no effect on the reversal potential (−12 mV) but appears to reduce the current amplitude. We cannot decide now whether this decrease in the current arises from an artificial decrease of the peptide concentration because it is engaged in a complex or to modification of the transmembrane current characteristics. Nevertheless, electrophysiological measurements strongly resemble those obtained for Pep-1 (see subsequent text) and other channel-forming CPPs [22], therefore suggesting that the peptide-induced membrane permeabilization properties are due to formation of an ion channel.

PEPTIDES OF THE PEP FAMILY

Another type of primary amphipathic peptides Pep, has recently been developed. It differs from MPG in the nature of the hydrophobic sequence (KETWWETWWTE) and was designed with the aim of delivering peptides, proteins or PNAs [7,23]. Pep-1, the leader peptide of the Pep family, forms complexes with proteic cargoes, which are rapidly delivered into a great variety of cell lines, and has been applied *in cellulo* and *in vivo* [24,25]. Pep-1 is now commercially available under the name of Chariot.

The ability of Pep-1 to penetrate into lipidic media was characterized by the monolayer approach as the MPG peptides. The variation of surface tension as a function of the peptide in the subphase showed a saturation at 5×10^{-7} M of Pep-1 and the corresponding surface tension was rather low (4 mN/m) compared to other CPPs (>15 mN/m) [20,26], indicating a weak amphipathic character for Pep-1. Characterization of the mechanism through which Pep-1 penetrates into membranes was performed through penetration experiments using phospholipids in the liquid-expanded (DOPC and DOPG) or liquid-condensed (DPPC and DPPG) states.

In the case of liquid-expanded monolayers, a strong increase in surface pressure is observed. The most important characteristics of these experiments are that (i) both DOPC and DOPG yielded identical cpi (45 mN/m) and that (ii) extrapolations at zero initial pressure were high (16 and 32 mN/m for DOPC and DOPG, respectively), and significantly different from those measured in the absence of lipid. For the other two phospholipids, DPPC and DPPG, again the cpi is high (33 mN/m) and does not depend on the nature of the headgroups (Figure 3) The high values found

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for the cpi suggest that the Pep-1 can spontaneously insert into natural membranes [28]. Extrapolations at zero initial pressure provide a good indication that strong peptide–lipid interactions can occur at least in monolayers, with all lipids except DPPC.

Structural Characterization of the Free and Detergent-bound Forms of Pep-1

We first examined the structural characteristics of Pep-1 peptide in its free form by CD and found that its conformation varied with its concentration. For a concentration ranging between 0.1 and 0.3 mg/ml, the CD spectrum showed a single negative band centered at 202 nm associated with a shoulder around 220 nm, suggestive of a nonstructured or poorly ordered structure. Interestingly, increasing the concentration of Pep-1 up to 3 mg/ml promoted dramatic modifications, which yielded a spectrum characterized by a minimum at 205 nm, a pronounced shoulder at 221 nm, and a maximum at 190 nm, representative of a helical conformation. This tendency for Pep-1 to adopt, at least in part, a helical structure was confirmed by observations in SDS-containing media. Indeed, above the CMC of the detergent, a spectrum with two minima at 207 and 222 nm and a maximum at 192 nm was obtained (data not shown), typically characteristic of a helical conformation. To further understand how Pep-1 was folded in solution and determine which residues were involved in helical conformations, we characterized the secondary structure of Pep-1 in solution by NMR. The sequential and medium range NOEs observed for Pep-1 in H_2O and Pep-1 in the presence of SDS are consistent with the existence of a helical secondary structure in this part of the sequence. In the presence of SDS, NOEs characteristic of a helix were observed for the same segment (residues 4 to 13). In addition, several NOEs detected at the beginning of the sequence are indicative of a 3_{10} helix. The extension of the helical structure to the *N*-terminus of Pep-1 in medium containing SDS is the only difference observed between the conformation of Pep-1 in H_2O and in SDS micelles. Interestingly, the NLS moiety of Pep-1 remained unstructured in both media [27].

Structural Characterization of Pep-1 in the Presence of Phospholipids

To determine whether the interaction of Pep-1 with an environment mimicking the cell membrane had any effect on its conformational state, we characterized the structural states of Pep-1 by CD in the presence of phospholipids. We found that successive addition of vesicles of DOPC/DOPG 80/20 to a dilute solution of Pep-1 in water induced a structural transition. Owing to the existence of isodichroic points around 220 nm, the various behaviors correspond to transitions between two states. The first state corresponds to a nonstructured form,

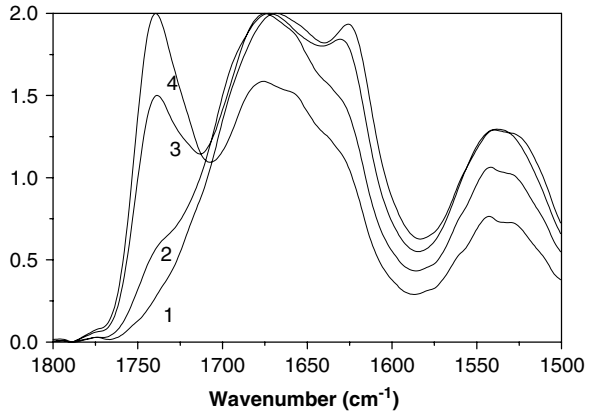

Figure 4 FTIR spectra (Amide I and II region) of Pep-1/phospholipid mixtures at various peptide/lipid ratios. Spectra 1–4 correspond to peptide/lipid ratios of 1/0, 1/1, 1/10, and 1/20, respectively. Reprinted from *Biochemistry*, **43**, Deshayes S, Heitz A, Morris MC, Charnet P, Divita G, Heitz F, insight into the mechanism of internalization of the cell penetrating carrier peptide Pep-1 through conformational analysis, 1449–1457, Copyright (2004), with permission from ACS.

while the second is more difficult to identify unambiguously because of the presence of 5 Trp residues, which are most likely involved in the contribution at 228 nm. Nevertheless, since the spectra always show a maximum at 191 nm and a minimum located in the 206–208 nm range, with no minimum between 215 and 220 nm, the presence of a sheet structure can be ruled out, and these data therefore also suggest that the conformation of Pep-1 is helical in the presence of phospholipids. The helical structure adopted by Pep-1 in the presence of phospholipids was confirmed by CD observations performed on peptide-containing transferred monolayers [29]. In all cases, with or without any type of lipid, whether neutral or negatively charged, spectra exhibit two minima at 206 and 222 nm and one maximum around 190 nm, thereby identifying a helical structure as the major structural component. To obtain more details on the structure of Pep-1 in the presence of phospholipids, an FTIR study was undertaken. As shown in Figure 4, all spectra showed a complex contour of the Amide I band. In the absence of lipids and at low lipid/peptide ratio (DOPC), two distinct contributions were observed, one at 1625–1630 cm^{-1} and the other centered at 1655–1660 cm^{-1}. An increase in the DOPC/peptide ratio generated a significant decrease of the 1625 cm^{-1} contribution associated with a broadening of the 1655–1660 cm^{-1} contribution [30]. Similar results were obtained with DOPG instead of DOPC. The low-wavenumber Amide I band contribution, which was only observed in the absence of lipids and for a low lipid/peptide ratio, can be unambiguously assigned to a β-sheet structure. However, the other contribution reflects the presence of a conformational mixture. The finding of a broad band is in full agreement with

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the NMR data obtained in SDS (see earlier text) and indicates that two helical forms are maintained in a lipidic medium (1655 cm^{-1}, α-helix; 1665 cm^{-1}, helix 3_{10}) [31,32].

Electrophysiological Measurements

When Pep-1 (10 μM) was applied to voltage-clamped oocytes, a marked increase in membrane conductance was recorded. This increase was best visualized by an increase in membrane current recorded during voltage ramps applied from – 80 mV (the usual holding potential) to +80 mV. The reversal potential for the Pep-1 induced current was close to −8 mV, similar to what has already been found for other pore-forming peptides selective for monovalent cations [22]. Taken together, these results suggest that the permeabilizing capabilities of Pep1 are due to the formation of membrane ion channels.

A final mechanism was recently suggested for internalization mediated by peptides belonging to the family of primary amphipathic peptides, namely MPG and Pep-1. On the basis of physicochemical investigations, including CD, FTIR and nuclear magnetic resonance spectrometries [12,21,27,33] associated with electrophysiological measurements and investigations dealing with the use of systems mimicking model membranes such as monolayers at the air–water interface and transferred monolayers, two very similar models have been proposed. Both are based on the formation of transient pore-like structures. The main difference between the model proposed for MPG and that proposed for Pep-1 is found in the structure giving rise to the pore structure. For MPG, it is formed by a β-barrel structure (Figure 5(A)), while that of Pep-1 depends on association of helices (Figure 5(B)). For both peptides strong hydrophobic phospholipid–peptide interactions have been detected, and in both models the folded parts of the carrier molecule correspond to the hydrophobic domain, while the rest of the molecule (linker + NLS) remains unstructured.

In conclusion, we have to emphasize the fact that the study of peptides or proteins interacting with membranes requires application of the use of a multidisciplinary approach involving spectroscopic methods associated with methods that are specific for membrane investigations. However, this has consequences on the interpretation of the data thus obtained. Indeed, such an approach requires that every piece of information be associated with the precise conditions of the experiments under which they were carried out. This is particularly the case when considering the concentrations, the peptide/lipid ratios, nature of the lipid and the consistency of the information. If, depending on the technique, conflicting conclusions are reached, a careful re-examination of the various experimental conditions is required.

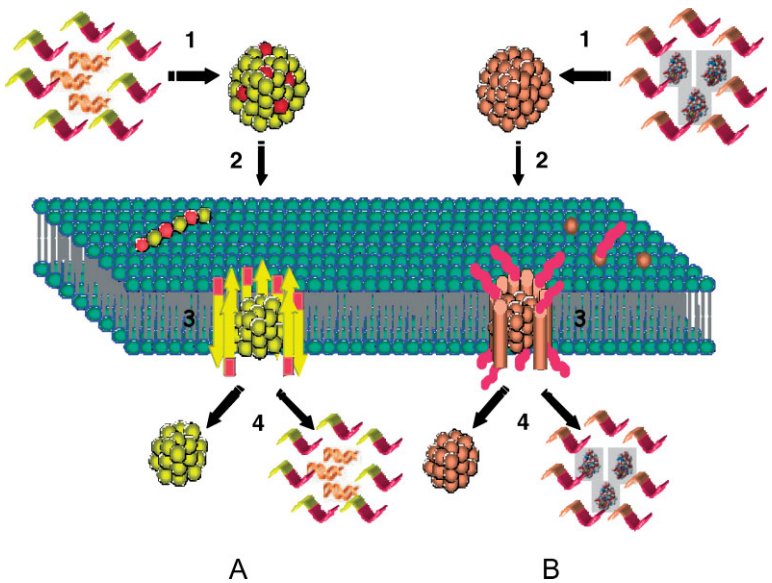

Figure 5 Model for the translocation process of Pep-1/cargo (right) and MPG/cargo (left) complexes through phospholipid bilayers. For both carriers, the four steps correspond to (1) formation of the complex, (2) membrane uptake of the complex, (3) translocation through the bilayer, and (4) release into the cytoplasmic side (color conventions: red corresponds to the NLS of both carrier, yellow and orange to the hydrophobic domain of MPG and Pep-1, respectively). Reprinted from *Biochemistry*, **43**, Deshayes S, Plénat T, Aldrian-Herrada G, Divita G, Le Grimellec C, Heitz F, primary amphipathic cell-penetrating peptides: structural requirements and interactions with model membranes, 7698–7706, Copyright (2004), with permission from ACS and Reprinted from *Biochim. Biophys. Acta*, **1667**, Deshayes S, Gerbal-Chaloin S, Morris MC, Aldrian-Herrada G, Charnet P, Divita G, Heitz F, on the mechanism of non-endosomial peptide-mediated cellular delivery of nucleic acids, 141–147, Copyright (2004), with permission from Elsevier.

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