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Lipid reorganization induced by membrane-active peptides probed using differential scanning calorimetry

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ABSTRACT

The overlapping biological behaviors between some cell penetrating peptides (CPPs) and antimicrobial peptides (AMPs) suggest both common and different membrane interaction mechanisms. We thus explore the capacity of selected CPPs and AMPs to reorganize the planar distribution of binary lipid mixtures by means of differential scanning calorimetry (DSC). Additionally, membrane integrity assays and circular dichroism (CD) experiments were performed. Two CPPs (Penetratin and RL16) and AMPs belonging to the dermaseptin superfamily (Drs B2 and C-terminal truncated analog [1–23]-Drs B2 and two plasticins DRP-PBN2 and DRP-PD36KF) were selected. Herein we probed the impact of headgroup charges and acyl chain composition (length and unsaturation) on the peptide/lipid interaction by using binary lipid mixtures. All peptides were shown to be α-helical in all the lipid mixtures investigated, except for the two CPPs and [1–23]-Drs B2 in the presence of zwitterionic lipid mixtures where they were rather unstructured. Depending on the lipid composition and peptide sequence, simple binding to the lipid surface that occur without affecting the lipid distribution is observed in particular in the case of AMPs. Recruitments and segregation of lipids were observed, essentially for CPPs, without a clear relationship between peptide conformation and their effect in the lipid lateral organization. Nonetheless, in most cases after initial electrostatic recognition between the peptide charged amino acids and the lipid headgroups, the lipids with the lowest phase transition temperature were selectively recruited by cationic peptides while those with the highest phase transition were segregated. Membrane activities of CPPs and AMPs could be thus related to their preferential interactions with membrane defects that correspond to areas with marked fluidity. Moreover, due to the distinct membrane composition of prokaryotes and eukaryotes, lateral heterogeneity may be differently affected by cationic peptides leading to either uptake or/and antimicrobial activities.

1. Introduction

Membranotropic peptides are of crucial importance in many biological processes such as membrane fusion, cell traffic or immunity. Particularly, two classes of membrane-active peptides are currently extensively studied: antimicrobial peptides (AMPs) and cell penetrating peptides (CPPs). Membrane translocation and the ability to reach the inner leaflet of the bilayer are critical for both CPPs and AMPs. Moreover, it has been reported that, at high concentrations, CPPs perturb membranes and may behave as AMPs [1], whereas AMPs may reach membrane targets before membrane permeabilization. As pointed out recently by Castanho [2], potentially all CPPs are AMPs and all AMPs are CPPs and, from a mechanistic point of view, there is no reason to study these two classes separately.

Different strategies have been employed to cross the membrane barrier and deliver hydrophilic molecules inside the cell for experimental and therapeutic purposes [3]. In this regard, a new class of

Abbreviations: AMP, antimicrobial peptide; CD, circular dichroism; CHO, Chinese hamster ovary; CL, cardiolipin; CPP, cell penetrating peptide; DMEM, Dulbecco's modified Eagle's medium; DMPC, 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine; DMPG, 1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]; DPPC, 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine; DSC, differential scanning calorimetry; DSPC, 1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine; DSPG, 1,2-Distearoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]; EDTA, Ethylenediamine-tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LUV, large unilamellar vesicle; MIC, minimal inhibitory concentration; MLV, multilamellar vesicle; ONPG, Ortho-NitroPhenyl-β-Galactoside; ONP, Ortho-NitroPhenol; PBS, phosphate buffer saline; POPG, 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]; POPC, 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine; RCB, red blood cells; LDH, lactate dehydrogenase; P/L, peptide/lipid ratio

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peptides, named cell penetrating peptides have been identified after the observation that some intracellular exogenous or endogenous proteins (Tat and pAnt) when added to the extracellular medium, were able to cross the membrane [4,5]. The CPP family includes all the peptides (natural, synthetic and chimeric) that are able to penetrate and transport cargos into the cell. Their general uptake mechanism is far from being understood, although the internalization was first considered to be endocytosis-independent [6,7], recent evidence suggests that both endocytic and non-endocytic routes are used [8,9].

The AMPs are characterized by a microbicidal activity against a large variety of pathogens [10,11]. These peptides share common properties with CPPs since they are usually short and cationic, interact strongly with cell membranes and selectively attack pathogens without disturbing host cell integrity [12,13]. Regarding the mechanism of membrane damage, AMPs can disrupt the membrane bilayer organization by forming pores composed of peptides alone or peptides and lipids, or by a carpet mechanism [13–16]. CPPs and AMPs represent two extreme behaviors of cationic peptides and there is probably a continuum between them regarding their membrane activity. Following the latter hypothesis, we have chosen to investigate in a systematic manner pure cell penetrating peptides (penetratin), peptides with both CPP and antimicrobial activities (RL16), classical antimicrobial peptides (dermaseptins) [17] and AMPs with more exotic properties such as chameleon-like (plasticins) [18].

Lateral heterogeneity in lipid distribution such as gel/fluid phase separation has been intensively investigated (*e.g.* [19]) and demonstrated to be relevant in biological functions ([20–23] to cite a few examples). Most interestingly, recent studies suggest a novel mechanism for breaching membrane permeability based on lateral membrane lipid segregation [24–26]. We investigated the effects of CPPs and AMPs on the lateral lipid distribution using DSC. Differential scanning calorimetry has emerged as a valuable tool to study peptide/lipid interactions and has been routinely employed to obtain important information regarding the level of interaction of various peptides with lipids [27]. This technique has been recently used to demonstrate the induction of lipid domain by AMPs [26,28–32].

Herein, the capacity of the selected CPPs and AMPs to reorganize binary lipid mixtures and to selectively recruit specific lipids is investigated by DSC together with membrane integrity assays and CD experiments. The lipid partners in the binary lipid mixture were selected on the basis of distinct and sufficient difference in phase transition temperatures (while still miscible) to be able to follow the possible preferential interaction or segregation of a given lipid component after peptide addition. Therefore the studies focus on: (i) the role of electrostatic forces on the peptide/lipid interaction by using DPPC and cardiolipin (7/3 mol/mol) vesicles, (ii) the role of the fatty acid chain length using DMPC/DSPC or DMPG/DSPG (1/1 mol/mol) vesicles and (iii) the role of the fatty acid chain unsaturation, which was investigated using DMPC/POPC and DMPG/POPG (1/1 mol/mol) vesicles, which although non-ideal, are completely miscible in both liquid and crystalline states.

2. Materials and methods

2.1. Materials

DMPC, DMPG, DPPC, DPPG, POPC, POPG, DSPC and DSPG were purchased from Genzyme (Switzerland) and were used without further purification. Cardiolipin from bovine heart was obtained from Avanti Polar Lipids (Alabaster, AL). The synthesis of the dermaseptin and plasticins analogs: DRP-PBN2, [$K^{8,\ 12}$, F^{18}]-DRP-PD3–6 (DRP-PD36KF), [33]; Drs B2, [1–23]-Drs B2 [34,35] was performed by Fmoc-solid phase strategy, while penetratin and RL16 were synthesized using Boc-solid phase strategy [36].

2.2. Cell culture

Chinese hamster ovary (CHO) K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 000 IU/L), streptomycin (100 000 IU/L), and amphotericin B (1 mg/L) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Cytotoxicity assays

Cytotoxicity assays were conducted using the Cell Counting Kit 8 (CCK-8) from Dojindo Molecular Technologies (Gaithersburg, Maryland, USA) with Chinese Hamster Ovary (CHO) cells. Briefly, a 96-well plate was inoculated with 100 μL/well of a suspension of CHO cells (10×10^3 cells/well). After 24 h of incubation (37 °C, 5% CO_2) 10 μL of different concentration of each peptide (final concentrations in the well being of 10 and 50 μM) were added and the plate was incubated overnight. Then, 10 μL of CCK-8 solution were added in each well and after 2 h of incubation the absorbance at 450 nm was measured using a microplate reader. Controls corresponded to untreated cells (negative control) and cells treated with 0.2% of Triton X-100 (positive control).

2.4. Antimicrobial activity

Gram-positive eubacteria (*Staphylococcus aureus*) and Gram-negative eubacteria (*Escherichia coli* 363) were cultured as described previously [33]. The minimal inhibitory concentrations (MICs) of peptides were determined in 96-well microtitration plates by growing the bacteria in the presence of 2-fold serial dilutions of peptide. Aliquots (10 μL) of each serial dilution were incubated for 16 h at 37 °C with 100 μL of a suspension of a midlogarithmic phase culture of bacteria at a starting absorbance $A_{630}=0.01$ in Poor-Broth nutrient medium (1% bactotryptone and 0.5% NaCl, w/v). Inhibition of growth was assayed by measuring the absorbance at 630 nm. The MIC was defined as the lowest concentration of peptide that inhibited the growth of ≥99% of the cells. To test for their bactericidal effects, bacteria incubated with the peptide at the MIC were plated out on solid culture medium containing 1% noble agar, the peptide was considered bactericidal after overnight incubation, the bacteria development was inhibited and non-bactericidal (bacteriostatic) when the bacteria was able to re-grow upon peptide incubation. All assays were performed in triplicate plus negative controls without the peptide and positive controls with 0.7% formaldehyde.

2.5. Hemolytic activity

Red blood cells (RBC) were isolated from rat plasma and washed three times with PBS (10 mM phosphate buffer, 140 mM NaCl, 3 mM KCl, pH 7.4). RBCs (10^8 cells/mL) in PBS were incubated with peptide concentrations ranging from 1 to 200 μM at 37 °C for 60 min. After centrifugation (15 min, 900 *g*, 4 °C), hemolysis was measured by monitoring the absorbance of the supernatant at 405 nm and compared with supernatants of lysed RBCs after addition of 1% (v/v) Triton X-100.

2.6. LDH leakage

Membrane integrity was measured using the Promega CytoTox-ONE™ assay. Briefly, CHO cells were seeded in 96-well plates 1 day before treatment with serum-free DMEM for 30 min. Untreated cells were defined as negative control (0% leakage) and lactate dehydrogenase (LDH) activity released by cell lysis in 0.2% Triton X-100 in the supplied buffer defined as positive control (100% leakage). Melittin, a peptide known to disturb the bilayer integrity, was employed as an additional positive control. The cells (20×10^3 cells/well) were

incubated with 1, 10, 50 and 100 μM of the peptide for periods of time ranging from 1–6 h. Release of LDH from cells with a damaged membrane lead to conversion of resazurin into resorufin that was monitored by measuring fluorescence intensity in a plate reader set with 550 nm excitation and 590 nm emission.

2.7. E. coli peptide-induced membrane permeabilization

The permeabilization of the cytoplasmic membrane of *E. coli* 363 by the peptides was assayed by measuring the β-galactosidase activity with the chromogenic substrate Ortho-NitroPhenyl-β-Galactoside (ONPG). *E. coli* 363 was grown in 10% Luria-Bertani broth, in the presence of 100 μg/mL IPTG, to induce the enzyme synthesis. Bacteria were washed twice with PBS (10 mM phosphate, NaCl 10 mM, pH = 7,0) and diluted to an absorbance $A_{630} = 0.5$. Aliquots (50 μL) of bacterial suspension (10^5–10^6 bacteria) were then mixed with various concentrations of peptides (10 and 50 μM). After 1 h of incubation 2 mM of ONPG were added and incubated 5 min. The hydrolysis of ONPG was stopped by boiling bacterial suspension and then monitored by measuring the absorbance at 405 nm of released Ortho-NitroPhenol (ONP). Complete permeabilization was assessed using bacteria treated with 1% Triton and assay was conducted in triplicate to ensure reproducibility.

2.8. Preparation of MLVs and LUVs

Lipid films were made by dissolving the appropriate amounts of lipid in a mixture of chloroform and methanol 2/1 (v/v), followed by solvent evaporation under nitrogen to deposit the lipid as a film on the wall of a test tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 3–4 h. Films were hydrated with 10 mM Tris, 0.1 M NaCl, 2 mM EDTA, pH 7.6 (Tris buffer) (for DSC experiments) or with 10 mM phosphate buffer, pH 7.6 (for CD experiments) and extensively mixed at a temperature superior to the phase transition temperature of the lipid to obtain MLVs. To better mimic the biological event, the peptide was added to the lipid after vesicle/liposome formation. To form LUVs, the lipid (MLVs) was subjected to five freeze/thawing cycles and the homogeneous lipid suspension passed (19 times) through a mini-extruder equipped with two stacked 0.1 μm polycarbonate filters (Avanti, Alabaster, AL). The size of the LUVs formed was monitored by light scattering and found to have a diameter of 90 ± 10 nm. Additionally, the UV absorbance was measured for the CD experiments to confirm that the solution did not scatter light.

2.9. Circular dichroism (CD) spectroscopy

CD spectra were recorded with a Jobin Yvon CD6 dichrograph linked to a PC microprocessor. The instrument outputs were calibrated with D(+)-10-camphorsulfonic acid. The spectra were scanned in a quartz optical cell with a 1 mm path length, unless specified otherwise, and recorded between 185–260 nm with 0.5 nm step. The measurements were performed at temperatures at which both lipid components are in fluid phase (well above the phase transition region), more specifically 55 °C for the DMPC/DSPC and DMPG/DSPG mixtures, 25 °C for the DMPC/POPC and DMPG/POPG mixtures and 40 °C for DPPC/CL. Typically, four or eight scans were accumulated and averaged after buffer (or LUV) spectra subtraction and baseline correction. The CD spectrum of each peptide was recorded in 10 mM phosphate buffer (pH 7.6), the peptide concentration was varied from 20 to 120 μM. Additionally, the spectrum was acquired in the presence of LUVs composed of the binary lipid mixtures: DMPC/CL (7/3 mol/mol), DMPG/DSPG (1/1 mol/mol), DMPG/POPG (1/1 mol/mol), DMPC/DSPC (1/1 mol/mol), DMPC/POPC (1/1 mol/mol) with a lipid concentration of 1250 μM to achieve a P/L ratio of 1/50. CD measurements are reported as $\Delta\varepsilon$ (M^{-1} cm^{-1}) per residue.

2.10. Differential scanning calorimetry

The calorimetry was performed on a high-sensitivity Differential Scanning Calorimeter (Calorimetry Sciences Corporation). A scan rate of 1 °C/min was used with a 15 min delay between sequential scans in a series that allow for thermal equilibration. Data analysis was performed with the fitting program Cpcalc provided by CSC and plotted with Igor (Wavemetrics). The total lipid concentrations used varied from 1 mg/mL for DMPC/DSPC and DMPG/DSPG to 4 mg/mL for DMPC/POPC and DMPG/POPG and to 6 mg/mL in the case of DPPC/cardiolipin. Samples containing the peptide alone, dissolved in buffer at peptide concentrations corresponding to those at the higher peptide/lipid molar ratios studied (P/L 1/10), exhibited no thermal events over the temperature range of 0–100 °C. This indicates that the endothermic events observed in this study arise solely from phase transitions of the phospholipids vesicles. A minimum of at least three to four heating and cooling scans were performed for each analysis depending whether or not the thermogram was reproducible. MLVs were used in this study because they exhibit much cooperative lipid phase transitions than LUVs. Indeed, the phase transition of LUVs is still quite good with single lipids but this is not the case when studying binary mixtures. Several heating/cooling scans were performed ensuring to reach equilibrium and to access to internal layers of MLVs. Different P/L ratios were used, to monitor the various events at membrane surface that depend on P/L ratio. In order to ensure homogeneity in the analysis of the effect of the selected peptides on the lipid phase transitions, we have chosen the simplest baseline correction to introduce the least amount of variability when comparing thermograms from different sets of experiments.

3. Results

The CPPs employed in this study were: penetratin, a non-amphipathic peptide (H-RQIKIWFQNRRMKWKK-NH_2) that has a sequence corresponding to the 16 residues of the third α-helix (residues 43–58) of the Antennapedia homeodomain protein of *Drosophila* [36] and RL16 (H-RRLRRLLRRLLRRLRR-NH2), a peptide designed from structure/penetration relationships of penetratin [37,38]. Four AMPs belonging to the dermaseptin superfamily (Drs B2 and its C-terminal truncated analog [1–23]-Drs B2 [35] and the two most potent plasticins (DRP-PBN2 and DRP-PD36KF) (Fig. 1 and Table 1) were also investigated. The dermaseptin superfamily includes peptides with very different structural characteristics: (a) the dermaseptins *stricto sensu* (Dermaseptins S and B) from *Phyllomedusa sauvagei* and *P. bicolor*, amphipathic α-helical peptides that all have a conserved tryptophan residue at position 3 and a positive net charge due to the presence of Lys residues punctuating alternating hydrophobic and hydrophilic residues [33], and (b) the plasticins, which are rich in Gly and Leu residues arranged in regular 5-mer motifs GXXXG (where X is any amino acid residue) [18,33]. Peptides of the dermaseptin family are prototypical members of a large class of membrane-damaging cationic peptides that undergo coil-to-helix transition upon binding to lipid bilayers. Plasticins distinguish themselves by their high structural malleability, and have thus been qualified as chameleon-like AMPs [18,39].

3.1. Cytotoxicity, antimicrobial and hemolytic activities

Penetratin and RL16 were not toxic at 10 μM to CHO cells neither were Drs B2 and [1–23]-Drs B2 whereas the more hydrophobic AMPs plasticin DRP-PBN2 and DRP-PD36KF were found to be cytotoxic on CHO cells (Table 2) and HeLa cells [33].

The antimicrobial activity of the selected peptides was assayed against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) for comparison (Table 2). RL16 inhibited the growth of both bacterial strains with minimal inhibitory concentrations (MIC) in the

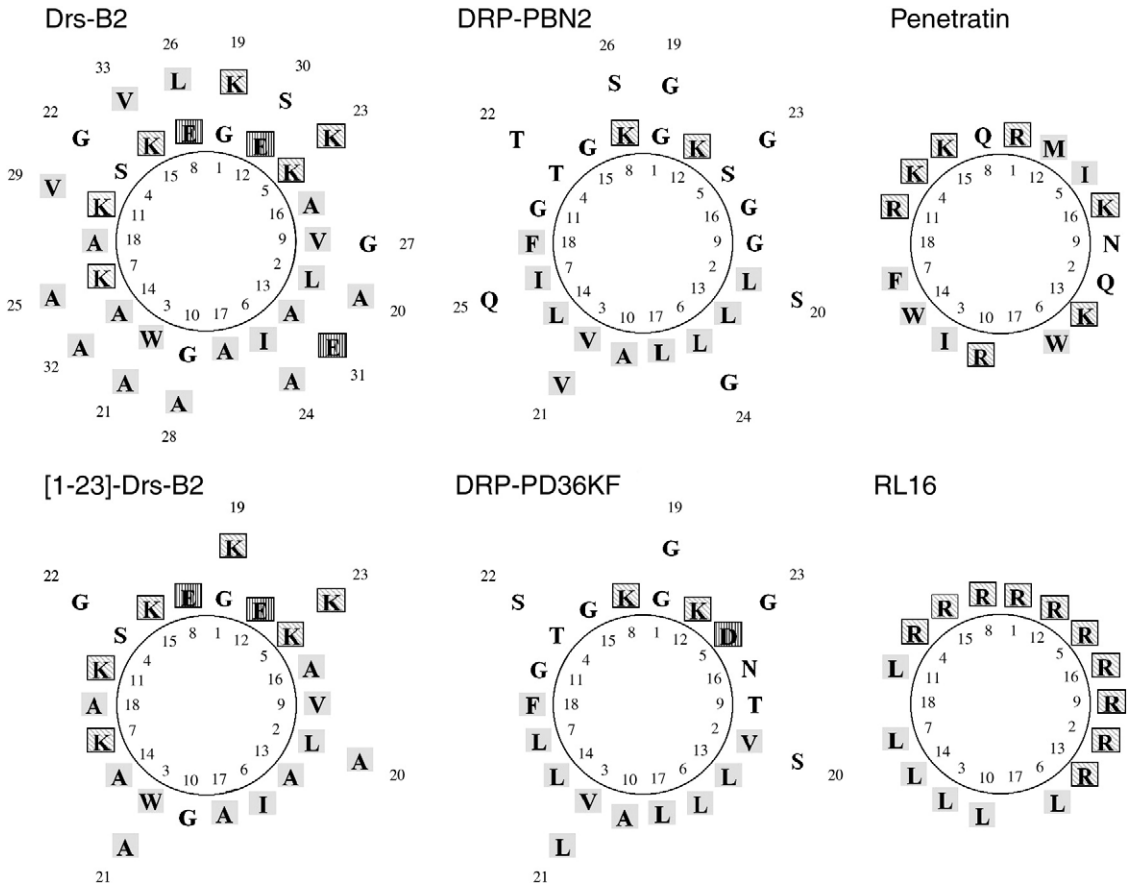

Fig. 1. Helical wheel projections of DRP-PBN2, DRP-PD36KF, Drs B2, [1–23]-Drs B2, RL16 and penetratin showing the distribution of amino acid side chains: nonpolar residues are on a grey zone, acidic residues on oblique stripes and basic residues on vertical stripes.

micromolar range, while penetratin has no inhibitory activity on bacteria growth. Penetratin did not show any hemolytic activity up to 250 μM, whereas RL16 induced moderate hemolysis, reaching 32% at 50 μM. The plasticins, DRP-PBN2 and DRP-PD36KF, were both active against *E. coli* with MIC values of 12 μM and 6 μM, respectively, and presented weak activity against *S. aureus*. They were slightly hemolytic over 100 μM (~30%). Like plasticins, dermaseptins were antimicrobial on *E. coli* with MICs of 0.3 and 25 μM for Drs B2 and [1–23]-Drs B2, respectively. Moreover, Drs B2 was active on *S. aureus* (MIC = 12 μM) while its truncated analog was not. Dermaseptins were not hemolytic even at 100 μM concentrations.

3.2. Perturbation of eukaryotic and bacterial membrane integrity

In order to test the perturbation of mammalian cell membrane, leakage of cytoplasmic lactate dehydrogenase (LDH) from CHO cells was measured after cell exposure to 1, 10, 50 and 100 μM of peptide during 1–6 h. Peptide concentrations were selected on the basis of the antimicrobial potencies and cell penetration properties of the investigated peptides, respectively. No leakage of LDH was observed with penetratin (Table 3), confirming previous studies [40,41]. LDH release from CHO cells was detected upon RL16 cell incubation over longer periods of time and higher peptide concentrations than those employed for melittin. Since RL16 is toxic for CHO cells [40], this may result from membrane cell lysis or pore formation. However, the level of membrane perturbation is much weaker than that observed for melittin, either because pores formed are of smaller size (so that LDH release is reduced) or of shorter duration. Dermaseptin B2 perturbs CHO cell membrane integrity starting at low concentrations (20 μM), which correlates well with its ability to form pores in planar lipid bilayers as reported [42]. The shorter analog, [1–23]-Drs B2 did not perturb the membrane integrity. Whereas the plasticins, DRP-PBN2

Table 1
Sequences and physicochemical properties AMPs and CPPs used in this study.

Name	Sequence	Net charge	Mean hydrophobicity
DRP-PBN2	GLVTSLIKGAGKLLGGLFGSVTGGQS	+2	0.33
DRP-PD36KF	GVVTDLLKTAGKLLGNLFGSLSG-CONH2	+1	0.5
Drs B2	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV-CONH2	+3	−1.97
[1–23]-Drs B2	GLWSKIKEVGKEAAKAAAKAAGK-CONH2	+4	−2.76
RL16	RRLRRLLRRLLRRLRR-CONH2	+10	−2.61
Penetratin	RQIKIWFQNRRMKWKK-CONH2	+7	−2.35

Note. mean hydrophobicity was calculated using the CSS scale with the HydroMCalc software by Alex Tossi and Luca Sandri (http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html).

Table 2
Cytotoxicity in mammalian cells (CHO), antimicrobial (in *E. coli* and *S. aureus*) and hemolytic activities of the peptides.

Peptides	Cytotoxicity CHO cells at 10/50 μM	MIC *E. coli* (μM)	MIC *S. aureus* (μM)	Hemolysis at 10/50 μM
DRP-PBN2	−/++	12	50	0/4
DRP-PD36KF	+/+++	6	>100	4/22
Drs B2	−/++	0.3	12	0/0
[1–23]-Drs B2	−/−	25	>100	0/0
RL16	−/+	50	9	0/32
Penetratin	−/−	>100	>100	0/0

Note. cytotoxicity values presented are for peptide incubation with the cells for a period of 4 h. The scale represents the following: − for 0%; + for 0–30%, ++ for 30–70%, +++ for 70–100%. Percentages of toxicity are expressed in percentages relative to the positive control (Triton X = 100% toxicity).

Table 3
Perturbation of eukaryotic (CHO cells) and bacterial (*E. coli*) membrane integrity.

Peptides	Eukaryotic membrane integrity perturbation 10/50 μM	Bacterial membrane integrity perturbation 10/50 μM
DRP-PBN2	−/++	++/++
DRP-PD36KF	+/+++	+++/+++
Drs B2	+/++	+++/+++
[1–23]-Drs B2	−/−	+++/+++
RL16	−/++	−/+
Penetratin	−/−	ND

Note. eukaryotic membrane integrity perturbation presented is after 4 h incubation and bacterial membrane integrity perturbation presented is after 1 h incubation with the peptides. The scale represents the following: − for 0%; + for 0–30%, ++ for 30–70%, +++ for 70–100%. Percentages of eukaryotic membrane integrity perturbation are expressed in percentages relative to the positive control (Triton X = 100% membrane integrity perturbation). Percentages of bacterial membrane integrity perturbation are calculated based on the melittin incubation (positive control) that represents 100%.

did not show any membrane integrity perturbation at 10 μM, DRP-PD36KF did. However, when cells were subjected to higher peptide concentrations (50 μM) and incubated for extended periods of time (4 h) both peptides greatly perturbed membrane integrity.

The permeabilization of bacterial membrane by the peptides was assayed by measuring the cytoplasmic β-galactosidase activity of *E. coli*. Both plasticin and dermaseptin analogs strongly permeabilized the bacterial membrane, an observation in good agreement with their antibacterial activity against this bacteria strain. Neither penetratin, nor RL16 showed any bacterial membrane perturbation, although an analog of RL16 in which all the leucines were replaced by Trp (RW16) displayed some perturbation and was found bactericidal against *E. coli* (MIC = 10 μM, data not shown).

3.3. Conformational transitions of the peptides upon binding to the various binary lipid mixtures

CD spectra were recorded to obtain structural information on the peptides both in solution and when bound to vesicles composed of different lipid binary mixtures. All experiments were performed at P/L of 1/50 in 10 mM sodium phosphate buffer and above the phase transition temperature of the lipid mixture (see Materials and methods section). This P/L ratio was chosen because it is a compromise between experimental possibilities and relevance for peptide–lipid interaction effects in relation with the lipid phase transition (DSC experiments). Investigations were not possible for higher P/L ratios due to peptide and/or lipid precipitation. For the peptides in which several P/L ratios were possible, no major changes

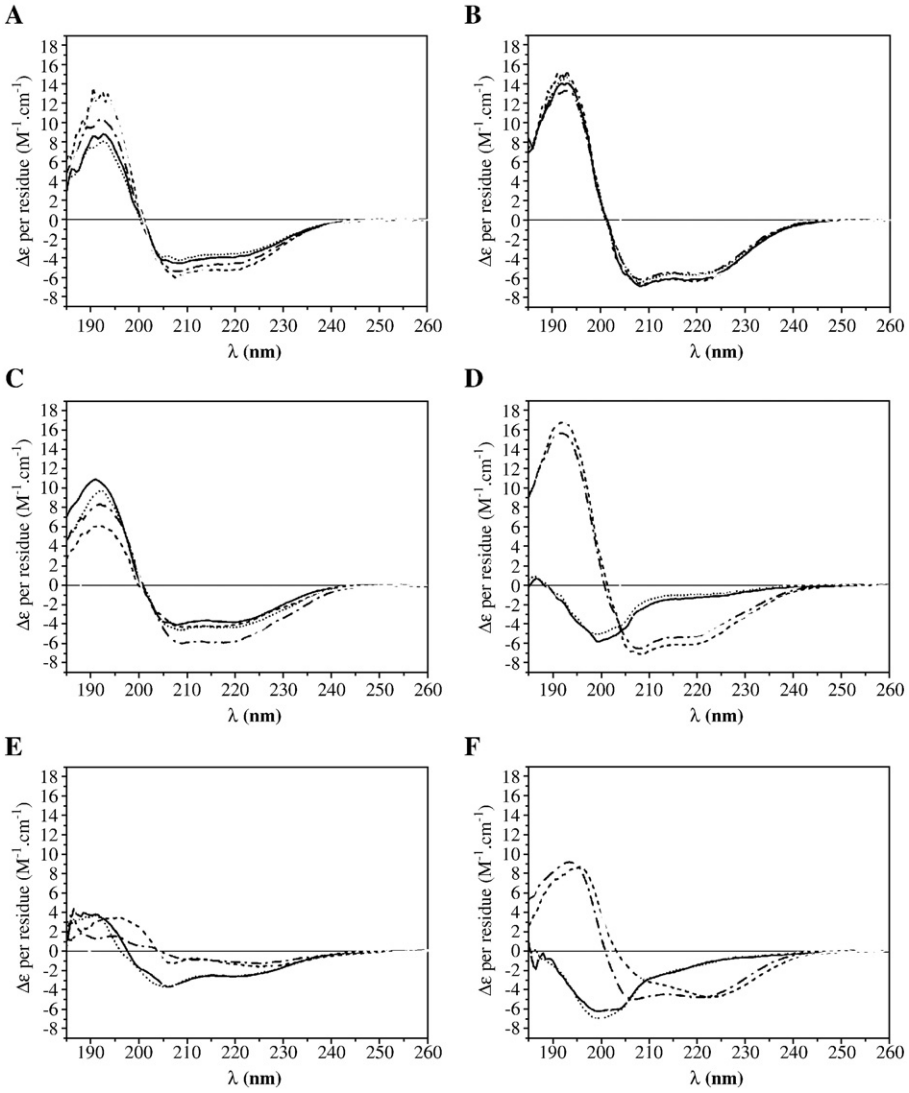

Fig. 2. Circular dichroism spectra of (A) DRP-PBN2, (B) DRP-PD36KF, (C) Drs B2, (D) [1–23]-Drs B2, (E) RL16 and (F) penetratin in the presence of LUVs composed of binary phospholipid mixtures above their respective T_m: DMPC:POPC (—), DMPG:POPG (…), DMPC:DSPC (▬▬), DMPG:DSPG (▪ ▪ ▪) and DPPC:CL (▪ ▪ ▪ ▪ ▪) or without any lipids (*i.e.* buffer ▬ ▪ ▬).

in the peptide secondary structure were observed. A first general overlook at the CD data denotes, generally, a marked presence of helical structure in the presence of lipids and a lack of structure in their absence (Fig. 2). Both plasticins were structured in α-helix, and, interestingly, in the case of DRP-PBN2, the helical content increased in the presence of anionic lipids. RL16 structured as α-helix in the presence of DPPC/CL and DMPG/POPG but was mainly random-coil in the presence of the other lipid mixtures with some various degree of structuration in both α-helix and β-sheet. Penetratin adopted a helical structure only in the presence of anionic lipids with the higher structure content observed in the presence of CL. It is noteworthy that the two CPPs as well as the C-terminal truncated analog [1–23]-Drs B2 were found unstructured in the presence of zwitterionic binary mixtures (DMPC/POPC and DMPC/DSPC) (Fig. 2).

3.4. Assessment of peptide interaction with lipid binary mixtures by DSC

The phase transition temperature and thermodynamics of lipid phase transitions are extremely sensitive to the presence of exogenously added compounds. Indeed DSC has been used in a variety of peptide–lipid studies [29,43–47]. Therefore, monitoring changes in these parameters can provide valuable information regarding the ability of peptides to interact and or disrupt the lipid acyl chain packing providing insight into their interaction mechanism. In the case of binary lipid mixtures, a careful selection of the lipids used in the mixture has to be considered because: i) not all the lipids are miscible, ii) the type of information obtained regarding peptide/lipid interactions will necessarily depend on the appropriate choice of the lipid partners. This will be further discussed in the following sections of the manuscript.

3.4.1. The role of electrostatic forces on the peptide/lipid interaction

Considering that the peptides investigated here are quite rich in positively charged amino acids (Table 1) the investigation of their interaction with negatively charged membranes is mandatory. Moreover, their antibacterial activities suggest that they may have a preferential interaction with charged lipids such as PG or cardiolipin that are especially present in the outer leaflet of bacterial membranes and often used in studies involving lipid model systems. Indeed, most of the peptides under study lead to a greater perturbation in the phase transition of DMPG *vs* DMPC, with the exception of RL16, suggesting a stronger interaction and perturbation of the fatty acid chain packing (data not shown) [33,36].

Herein, to get further insight into the role of electrostatics in the peptide/lipid interaction and possible alteration in terms of lateral

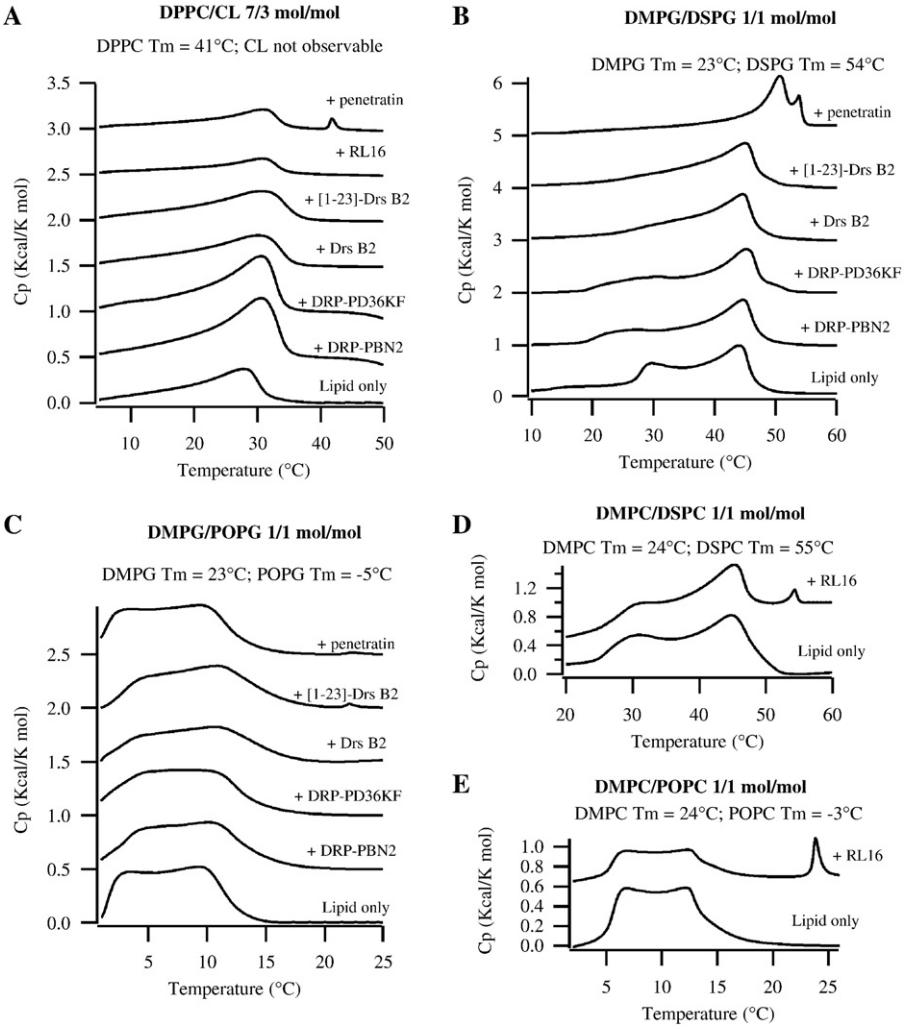

Fig. 3. High-sensitivity DSC heating scans illustrating the effect of the addition of DRP-PBN2, DRP-PD36KF, Drs B2, [1–23]-Drs B2, RL16 and penetratin on the thermotropic phase behavior of vesicles composed of DMPG/CL (7/3 mol/mol) (panel A), DMPG/DSPG (1/1 mol/mol) (panel B), DMPG/POPG (1/1 mol/mol) (panel C) at a P/L 1/20, DMPC/DSPC (1/1 mol/mol) (panel D) and DMPC/POPC (1/1 mol/mol) (panel E). Thermodynamic parameters are given in Tables 4, 5 and 6. The interaction of RL16 was studied with zwitterionic lipid mixtures only (DMPC/DSPC and DMPC/POPC) and not with the anionic mixtures (DMPG/DSPG and DMPG/POPG) because the lipid interacts strongly with the former lipids, the opposite was true for all the other peptides investigated in this study. The gel to fluid phase transition temperatures (Tm) of the lipids are indicated to aid interpretation [61]. For better readability, the zero value in Cp was incrementally added by 0.5, 1, 0.5, 0.4 and 0.6 U for panels A, B, C, D, and E, respectively.

lipid organization (lipid segregation), we have used binary lipid mixtures composed of DPPC and cardiolipin (7/3 mol/mol). While the role of electrostatics could be studied by just comparing the effect of the peptide on zwitterionic *vs* anionic lipid membranes (single lipid component), binary lipid mixtures containing lipids with sufficiently different phase transition temperatures are mandatory to evaluate the possible lipid recruitment induced by the peptide. Cardiolipin from bovine heart possess a high content of unsaturated fatty acids (which explains the lack of phase transition above 0 °C), in addition to the electrostatic effect investigated with the DPPC/CL there is also the impact of fatty acid chain unsaturation. The mixture of lipids often results in a reduction and broadening of the phase transition due to lipid intercalation, as a consequence of that, a relatively high lipid concentration was used (always with fixed P/L molar ratios) allowing a reasonable DSC signal to be observed. DPPC lipid phase transition occurs at 41 °C and CL has no observable transition between 0 and 100 °C. As it can be seen in panel A of Fig. 3, this lipid mixture produces a single (although broad) transition confirming good lipid miscibility (below and above the phase transition region). Very distinct effects on the thermogram were induced by the presence of the peptides with reductions or increases in the enthalpy of the transition accompanied or not by changes in T_m. In view of the broad transition observed, an accurate determination of T_m and ΔH is not straightforward. However, significant effects, such as the appearance of additional transition peaks at higher temperatures deserve consideration.

Both plasticin and dermaseptin analogs lead to changes in the enthalpy and T_m of the DPPC/CL transition suggesting that they interact with these lipids. Lack of change in the general form of the transition peak suggests the absence of lipid lateral redistribution in the presence of these AMPs. Similarly, RL16 leads to no change in the form of the transition but produces a strong decrease in the enthalpy of the transition, particularly at a P/L ratio of 1/20, which indicates a perturbation of the packing of the acyl chains. The most noticeable result was obtained with penetratin, with the appearance of a second transition around 41 °C, starting at P/L ratio of 1/50 and increasing in magnitude with peptide concentration. One should note that penetratin does not lead to any change in the phase transition temperature of DMPC [36]. This strongly suggests that penetratin preferentially binds and recruits CL, leaving a domain highly enriched in DPPC, which gives rise to the sharp transition at 41 °C.

3.5. The role of the fatty acid chain length

To investigate the potential role the lipid bilayer thickness plays in the lipid/peptide interactions, DSC studies were performed on binary lipid mixtures composed of lipids with distinct fatty acid chain length. We have chosen DMPC/DSPC or DMPG/DSPG (1/1 mol/mol) lipids with chain lengths differing by four carbons, the maximum chain length difference, that can be used before observing complete lateral phase separation [48,49]. This mixture is not ideal with a broad transition located between about 30 and 50 °C composed of two peaks, one towards 30 °C that corresponds to a DMPG rich region and another one around 44 °C that corresponds to a DSPG rich region (Fig. 3, panel B). Anionic lipid binary mixtures (DMPG/DSPG) were employed for all the peptides due to their preferential electrostatic interaction with this lipid (determined by previous studies [33,35]), except for RL16 that interacts preferentially with zwitterionic lipids [36]. Moreover, RL16 has a tendency to induce aggregation in the presence of anionic lipids [36]. Both plasticin and dermaseptin analogs change the overall shape of the transition with a strong decrease in the area under the peak located on the left side of the broad transition, which has been attributed to the DMPG rich region (see explanation above) (Fig. 3, panel B). For the dermaseptin analogs, the transition peak located on the left side of the broad transition was completely abolished at P/L ratio of 1/20. Studies performed in our laboratories have shown that both plasticins and dermaseptins lead to changes in both the T_m and the ΔH of DMPG [35,50]. The results suggest a stronger interaction of the peptides with the shorter lipid DMPG without observable DSPG segregation. Penetratin also leads to a decrease in the enthalpy of the transition and the appearance of a second transition at ~52 °C (starting at P/L

Table 4
Thermodynamic parameters obtained by DSC for the interaction of the peptides with DPPC/CL (7/3 mol/mol).

Peptides	P/L	T_m (°C)	ΔH (Kcal/mol)
Lipid only	No peptide	28	5.2
DRP-PBN2	1/100	29	8.6
	1/20	29.5	8.3
DRP-PD36KF	1/100	29	8.6
	1/20	29.5	7.8
Drs B2	1/200	28	5.2
	1/20	29	6
[1–23]-Drs B2	1/200	28	5.2
	1/20	29.5	5.2
RL16	1/100	29.5	6.8
	1/20	29.5	2.4
Penetratin	1/100	29	4.2
	1/20	29/41.8[a]	2.7

= No changes observed.
[a] Phase transition temperature of a second transition peak.

Table 5
Thermodynamic parameters obtained by DSC for the interaction of the dermaseptin and plasticin analogs as well as penetratin with DMPG/DSPG and for the interaction of RL16 with DMPC/DSPC (1/1 mol/mol).

Peptides	P/L	T_m (°C)	ΔH (Kcal/mol)
No peptide DMPG/DSPG	No peptide	29.8/44	15.6
DRP-PBN2	1/100	29.3/43.7	14.5
	1/20	27.9/44.7	11.8
DRP-PD36KF	1/100	29.9/44.5	15
	1/20	31.2/45.3	11.5
Drs B2	1/200	30/43.7	14.8
	1/20	[b]/44.6	12.5
[1–23]-Drs B2	1/200	29/43.2	12.9
	1/20	[b]/45	12.8
Penetratin	1/100	29/43.6	12.2
	1/20	[b]/51.8	6.2
No peptide DMPC/DSPC		31/44.7	15.6
RL16	1/100	31/44.7	17.8
	1/20	30.8/45/54.3[a]	10

= No changes observed.
[a] Phase transition temperature of a second transition peak.
[b] The phase transition disappears.

Table 6
Thermodynamic parameters obtained by DSC for the interaction of the dermaseptin and plasticin analogs as well as penetratin with DMPG/POPG (1/1 mol/mol) and for the interaction of RL16 with DMPC/POPC (1/1 mol/mol).

Peptides	P/L	T_m (°C)	ΔH (Kcal/mol)
No peptide DMPG/POPG		3.2/9.3	5.4
DRP-PBN2	1/100	4.1/9.1	6.7
	1/20	6.0/9.8	4.3
DRP-PD36KF	1/100	4.6/10.5	6
	1/20	ND	4.4
Drs B2	1/100	4.5/10.9	5.2
	1/20	4.6/10.6	4.5
[1–23]-Drs B2	1/100	6.3/9.8	6
	1/20	5/10.8/22[a]	4.4
Penetratin	1/100	2.8/9.3	5.2
	1/20	3.1/9.5/22.2[a]	4
No peptide DMPC/POPC		6.8/12.1	5.2
RL16	1/100	6.8/12.1	4.8
	1/20	6.9/12.3/23.8[a]	2.6

= No changes observed.
[a] Phase transition temperature of a second transition peak.

1/50) that coexists with the broad transition located between 30 and 50 °C. When increasing the P/L ratio the lipid phase transition occurs at higher temperatures and becomes less broad until it collapses into a single and very sharp transition at P/L 1/10, located around 50 °C. Similarly to penetratin effect on DPPC/CL, RL16 addition to DMPC/DSPC leads to an overall decrease in ΔH (Table 5) and to the appearance of a second transition ~54 °C starting from P/L 1/50 and increasing with peptide concentration (Fig. 3, panel D).

3.6. The role of the level of fatty acid chain unsaturation

Considering that CPPs and AMPs may insert into the fatty acid chain region, changes in the lipid bilayer fluidity may modulate their interactions. To investigate this point, a binary mixture of miscible lipids containing both saturated and unsaturated fatty acid chains was employed. To be miscible, lipids must have T_m values that differ by less than ~33 °C [51]. We have chosen DMPC/POPC (DMPC T_m = 23 °C, POPC T_m = −3 °C) and DMPG/POPG (DMPG T_m = 23 °C, POPG T_m = −2 °C), which, as their phase diagrams indicates, although non-ideal, are completely miscible in both liquid and crystalline states [51]. The 1/1 mol/mol lipid mixture produces a single and rather broad lipid phase transition occurring between 2 and 10 °C for both the zwitterionic and anionic lipids. The shape of this transition presents two shoulders, one at lower temperatures around 3 °C that should correspond to regions richer in POPG and a second one, around 9 °C corresponding to DMPG rich regions (Fig. 3, panel C). The analysis performed and conclusions drawn take into account the results obtained from DSC studies performed with single lipids DMPC or DMPG mentioned in the two previous sections. In terms of the transition enthalpy the effects of the peptides are minor except for penetratin and RL16 at P/L ratio 1/20, indicating that the peptides have a small effect on the fatty acid chain packing. Regarding the shape of the transition peak and the relative intensities of the lower and higher T shoulders, the peptides induce certain changes. DRP-PBN2, Drs B2 and [1–23]-Drs B2 lead to a slight enhancement of the transition at 9 °C therefore promoting lipid demixing with the peptide interacting more strongly with the unsaturated lipid (low temperature shoulder decreases). Moreover, [1–23]-Drs B2 and penetratin lead to the formation of an additional peak around 22 °C, which corresponds to the T_m of DMPG, indicating segregation of this lipid from binary mixture. Such effect was very evident in the case of RL16 where the enthalpy of the phase transition between 5 and 15 °C greatly decreases and concomitantly the transition at around 22 °C increases with the increase in P/L ratio.

4. Discussion

Most DSC studies, with the exception of a few recent ones [26,30,46], have been conducted on rather simple model systems, mainly composed of a single lipid species. Usually, zwitterionic lipids (such as PC) are used to model eukaryotic cell membranes and anionic lipids (like PG or cardiolipin) to mimic bacterial cell membranes. In this study, MLVs composed of two lipid mixtures were used to investigate the role of the electrostatic interactions and lipid bilayer fluidity on the peptide/lipid interactions. The idea behind the use of miscible binary lipid mixtures is to be able to examine an eventual preferential interaction of a peptide with one of the lipid components, called here segregation/demixing or lipid recruitment. One of our current working hypothesis is that a peptide may recruit specific lipids from the membrane leading to the formation of lipid phases/microdomains possessing specific physicochemical properties that will trigger the peptide biological activity on the membrane (*i.e.* penetrating, antimicrobial, fusion, etc…). Additionally, such lipid microdomains may work as recruiting platforms for the capture of assisting molecules that may be required for the process.

4.1. Plasticins

Plasticins, with a small positive net charge interact more strongly with anionic (DMPG) than zwitterionic (DMPC) lipids [33]. Such preference is not as strong as that observed for other AMPs [33,52,53] and may be related to the high hydrophobicity of plasticins which perturbs both bacteria and eukaryotic plasmatic membrane. From the comparison of Tables 2 and 3, one can see that cytotoxicity of these peptides arises, at least partially, from their capacity to perturb eukaryotic membrane. Similarly, their antimicrobial activity may arise from their capacity to perturb bacterial membrane. In the presence of MLVs composed of DPPC and CL, plasticins seem to interact at the phospholipid headgroup level. Since no substantial change in the thermodynamic parameters is observed in the case of zwitterionic lipids [50], the effects observed here indicate that electrostatic interactions may play a major role. Regarding lipid bilayer fluidity, plasticins interact preferentially with lipids with a lower T_m, (DMPG *vs* DSPG). This preferential interaction may be related to a better hydrophobic matching between the peptide length and the bilayer thickness, which is more favorable for shorter chains [54] and to the greater fluidity of the bilayer. No lipid demixing/segregation were induced by plasticins with the binary lipid mixtures investigated here.

4.2. Dermaseptins

In contrast to plasticins, dermaseptins are selective for anionic *vs* zwitterionic lipids, which translates in the lack of cytotoxicity (except for Drs B2 at 50 μM) and hemolytic activity of AMPs. DrsB2 perturbs eukaryotic membrane integrity, which explains the appearance of cytotoxicity at 50 μM. In contrast, [1–23]-Drs B2, which is lacking the C-terminal hydrophobic sequence of Drs B2, does not perturb eukaryotic membrane integrity and is not toxic. Noticeably, the presence of this hydrophobic tail in Drs B2 is responsible for its capacity to structure in α-helix whatever the liposome composition, while [1–23]-Drs B2 only adopts a helical structure in the presence of anionic lipids. The triggering of [1–23]-Drs B2 helical structure only by anionic lipid bilayers may explain its antimicrobial potency and lack of toxicity and eukaryotic membrane perturbation, since in the presence of zwitterionic lipids the peptide remains unstructured. A selective interaction of these peptides with lipids possessing a lower phase transition temperature is observed (DMPG *vs* DSPC and POPG *vs* DMPG). Such preferential interaction is quite evident for the shorter dermaseptin analog, for which a new transition is observed in the thermogram due to segregation of DMPG from the DMPG/DSPG mixture.

4.3. Cell penetrating peptides

The CPPs used in this study display opposite effects regarding their interaction with anionic *vs* zwitterionic lipids. Indeed, while penetratin preferentially interacts with anionic lipids (DMPG), having very minor effects on the phase transition behavior of zwitterionic lipids (DMPC) (as determined by DSC), RL16 had the opposite behavior [36]. In fact, while penetratin selectively interacts with CL in the DPPC/CL mixture leading to DPPC segregation, RL16 does not selectively interacts with CL. A similar behavior has been observed for α/β-antimicrobial peptide interactions with DPPE/CL mixtures, where the peptide preferentially interacts with CL leaving a domain enriched in PE [26]. This selective interaction may be related to the distinct peptide structuration observed, with penetratin adopting a helical structure only in the presence of anionic lipids and RL16 helicity increasing in the presence of zwitterionic lipids. Regarding fatty acid chain length, both peptides preferentially interact with short ones (possessing the lower T_m). This is in good correlation with the strong effect of penetratin and RL16 on the enthalpy of the phase transition of DMPG and DMPC, respectively [36]. The appearance of a new

transition at ~54 °C starting from P/L 1/50, which is accompanied by a decrease in the magnitude of the original broad transition observed in absence of peptide, is attributed to the segregation of DSPC from the binary lipid mixture (DSPC T_m is ~53 °C). Such lipid segregation is a consequence of the preferential interaction of the peptide with shorter aliphatic chains (DMPC). The observation that RL16 selectively interacts with shorter chains may be related to the formation of small vesicles (carpet type mechanism, proposed to occur for this peptide [36]) occurring more specifically with shorter than longer chains. Additionally, both peptides recruit unsaturated fatty acid chains, this effect being more noticeable in the case of RL16. A preferential interaction of the peptides with the unsaturated lipid may be a consequence of their higher fluidity arising from their lower phase transition temperature, correlating well with the favorable interaction with shorter *vs* longer fatty acid chains. The fact that such behavior was more substantially marked for RL16 than penetratin may be related with the different level of insertions into the lipid core region. In the presence of CL, both electrostatic and fatty acid chain unsaturation may play a role, but the peptide immobilization on the surface due to electrostatic interactions, may explain the lack of preferential interaction with the fatty acid chain (unsaturated *vs* saturated).

All the peptides investigated, except RL16, interact with the lipids, at a first level through electrostatic interactions established between the positively charged amino acids of the peptides and the negatively charged lipid headgroups. The present data clearly demonstrate that both CPPs and AMPs induce specific lipid segregation of binary lipid mixtures possessing lipid components with markedly different phase transition temperatures. It is noteworthy to mention that when selective lipid interactions were observed, the lipid being recruited from the binary mixture always corresponded to the one having the lowest phase transition temperature. A certain hierarchy in the demixing/segregation was also observed: the unsaturated lipids were preferentially recruited first, before lipids with shorter fatty acid chains, again this probably being a consequence of the lower T_m of unsaturated lipids. Such preference may be related to the higher fluidity (reduced fatty acid chain packing) of these lipids, which better adapt to lipid rearrangements taking place during the cell translocation and/or perturbation of the lipid bilayer. Regarding, the preferential interaction of the AMPs with anionic lipids (CL, data shown here; DMPG *vs* DMPC, data not shown) and their better antimicrobial activity for *E. coli vs S. aureus*, interesting observations can be drawn, taking into account the membrane lipid composition of *E. coli* (80% PE, 15% PG and 5% CL) and *S. aureus* (58% PG and 42% CL, no PE). Thus, a better antimicrobial activity would exist in bilayers where both anionic and zwitterionic lipids exist, rather than in bilayers solely composed of anionic lipids. A similar analysis has been reported by Epand et al. for the interaction of several antimicrobial peptides with binary lipid mixtures [26,30–32] and their antimicrobial activities in a large range of bacterial strains. Based both on the antimicrobial activities for the different bacterial strains and from lipid domain formation observed by DSC, the authors concluded that one of the causes for the differences in species selectivity with AMPs may come from clustering of anionic lipids and their segregation in domains. In contrast to dermaseptins and plasticins, RL16 which has no preferential interaction with CL nor DMPG, has low antimicrobial activity in *E. coli* (50 μM) but higher with *S. aureus* (9 μM).

Finally, the formation of lateral membrane heterogeneity in bilayers may have important consequences for the uptake and antimicrobial activities of CPPs and AMPs, respectively. Therefore, the lipid domains formed may present the necessary physicochemical properties to promote CPPs passage across the bilayer (by a non-endocytic pathway) *via* either the formation of inverted micelles [36–38,55,56] or other lipid structures such as tubes [40,57] or the recruitment of the necessary auxiliary proteins (*e.g.* clathrins, caveolins, etc…) and other molecules needed for their uptake (when an endocytic pathway is taken). Moreover, peptide-induced segregation of lipids into phases/domains can destabilize the membrane by introducing phase boundary defects between lipid domains [58,59]. Such boundary defects, offering reduced surface tension, may facilitate peptide penetration and/or leakage [24,25,60].

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