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Aggregation determines the selectivity of membrane-active anticancer and antimicrobial peptides: the case of killerFLIP

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Abstract

Host defense peptides selectively kill bacterial and cancer cells (including those that are drug-resistant) by perturbing the permeability of their membranes, without being significantly toxic to the host. Coulombic interactions between these cationic and amphipathic peptides and the negatively charged membranes of pathogenic cells contribute to the selective toxicity. However, a positive charge is not sufficient for selectivity, which can be achieved only by a finely tuned balance of electrostatic and hydrophobic driving forces. A common property of amphipathic peptides is the formation of aggregated structures in solution, but the role of this phenomenon in peptide activity and selectivity has received limited attention. Our data on the anticancer peptide killerFLIP demonstrate that aggregation strongly increases peptide selectivity, by reducing the effective peptide hydrophobicity and thus the affinity towards membranes composed of neutral lipids (like the outer layer of healthy eukaryotic cell membranes). Aggregation is therefore a useful tool to modulate the selectivity of membrane active peptides and peptidomimetics.

Keywords: aggregation, toxicity, host defense peptides, effective hydrophobicity

1. Introduction

Drug resistance is one of the most urgent problems in modern medicine. Both bacteria and cancer cells can become drug-resistant by several mechanisms, including protection or modification of the target molecule and reduction in the intracellular concentration of the drug (through reduced uptake, enhanced efflux or increased degradation) [1-5]. In this framework, development of drugs with novel mechanisms of action is crucial. Host defense peptides (HDPs) are a natural class of molecules endowed with activity against both cancer and bacterial cells, including those that are drug-resistant [3, 6]. Remarkably, being active against both bacteria and tumors, these peptides could help to fight cancer-associated infections, and to tackle the increased susceptibility to cancer resulting from chronic infections [6]. HDPs are short, amphipathic, cationic and usually kill pathogens very rapidly, by perturbing the permeability of their cell membranes, rather than by associating to a specific intracellular target, even if exceptions do exist [7]. Notably, HDPs are selective, i.e. they are active at concentrations significantly lower than those causing damage to cells of the host organism [8]. This selectivity is not receptor-mediated, but it is linked to differences in cell membrane properties. Even peptides acting on intracellular targets have to cross the plasma membrane to reach them, and therefore selectivity for the membranes of cancer cells versus those of healthy cells is essential also in this case.

The outer leaflet of healthy eukaryotic cells is essentially neutral, since negatively charged lipids are confined to the intracellular leaflet [8]. This membrane asymmetry is partially lost in cancer cells, which expose negatively charged lipids, such as phosphatidylserine (PS), on the extracellular surface [9-17]. In addition, cancer cells have a higher content of other anionic moieties on their surface, such as sialic acid and heparan sulphate [3, 18-20]. A net negative charge is a property shared by bacterial membranes, which contain significant fractions of the anionic lipids phosphatidylglycerol (PG) and cardiolipin (CL); additional negative moieties are present in other components of the cell envelope (teichoic and teichuronic acids and lipopolysaccharides) [8,21].

The cationic charge of HDPs is an obvious determinant of their selectivity. While the driving force for peptide binding to neutral membranes is only hydrophobic, in the case of anionic bilayers electrostatic forces contribute too. Indeed, a positive correlation between peptide positive charge and antimicrobial activity and selectivity has often been described [22-27]. However, a positive net charge is not sufficient for selective membrane association. Several artificial peptides were designed to have the cationic charge and amphipathic character of natural HDPs, but they often resulted to be highly toxic [28-30]. These findings provided a first indication of the complexities of peptide-membrane interaction, with multiple interconnected phenomena contributing in determining the final membrane-perturbing activity [8, 30-33]. Peptides in solution can have different conformations and aggregation states, once membrane-bound they can change

conformation, orientation, insertion depth, and aggregation state [8]. How these processes modulate the activity and selectivity of HDPs is not obvious.

The aim of this work is to characterize the role of aggregation in peptide selectivity: many HDPs have a strong tendency to aggregate, due to the hydrophobic interactions between their apolar residues. However, the effects of this phenomenon on peptide selectivity have received limited attention. In our study, we selected the killerFLIP peptide as a test case. This peptide was originally designed to interfere with apoptosis signalling [20]. Its sequence was derived from the C-terminal domain of c-FLICE-like inhibitory protein (c-$FLIP_L$), which inhibits apoptosis through its protein-protein interactions [34]. The active core of the sequence (FFWSLCTA) was fused (at the N-terminus) to the TAT penetrating sequence (GRKKRRQRRR), to favor cell uptake [20] (Scheme 1). This chimeric peptide, termed killerFLIP, induced cell-death in prostate and colon cancer and leukemia cells, at concentrations in the micromolar range. At the same time, no cytotoxicity was observed in normal epithelial and endothelial cells at the active concentrations. Moreover, killerFLIP was found to inhibit tumor growth in mice, without significant toxicity [20]. However, rather surprisingly, cellular assays demonstrated that the mechanism of action of killerFLIP-induced cell-death was not associated to the activation of apoptosis or necroptosis, but it was due to perturbation of cell membranes, which lost their integrity just a few minutes after treatment, as shown by Trypan blue assays and electron microscopy images [20]. It is worth mentioning that an analog where the FLIP sequence was scrambled conserved the anticancer activity of killerFLIP [20]. This finding confirms a mechanism of action based on the physicochemical properties of the peptide, rather than on the interaction with specific intracellular protein targets.

Scheme 1. Amino-acidic sequence of the investigated peptide. Hydrophobic, basic, and polar residues are reported in green, blue, and black, respectively.

Considering its mechanism of action, selectivity and physico-chemical properties, killerFLIP is a typical membrane-active, cationic, amphipathic peptide, just like natural and designed HDPs. KillerFLIP is amphipathic along its sequence, with a polar N-terminal segment and a hydrophobic C-terminal sequence, while most HDPs are spatially amphipathic, attaining a conformation (e.g. a helical structure) where most polar side chains are on one side, and the hydrophobic residues on the other. However, several examples of linearly-amphipathic HDPs are known [35-41].

The first characterization of killerFLIP showed that it forms aggregates in aqueous environment [20]. Therefore, we selected this peptide to investigate the possible relation between aggregation and selectivity. Our results demonstrate that the selectivity of

killerFLIP is strictly dependent on its aggregation state and indicate the propensity to aggregate as a parameter that must be taken into account in the design and development of selective membrane-active anticancer and antimicrobial peptides.

2. Materials and Methods

2.1. Materials

Phospholipids (POPC: 1-Palmitoil-2-Oleoyl-*sn*-Glycero-Phosphocholine; POPS: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) were purchased from Avanti Polar Lipids (Alabaster, AL, USA): Spectroscopic grade methanol, ethanol and chloroform were purchased from Carlo Erba Reagenti (Milano, Italy). 5(6)-carboxyfluorescein (CF), Triton-X 100 and Sephadex-G50 were purchased from Sigma Aldrich (St. Louis, MO, USA).

KillerFLIP (GRKKRRQRRRFFWSLCTA, with unmodified termini) was purchased from Biomatik. This sequence was termed killerFLIP-E in the original article, and it had an additional Tyr at the N-terminus. Control experiments showed that the biological activity of the shortened sequence is identical to that of the original peptide (data not shown).

2.2. Liposome preparation

Large unilamellar vesicles (LUVs) composed of POPC/POPS or POPC/POPG, in different molar ratios were prepared by dissolving in a 1:1 (v/v) $CHCl_3$/MeOH solution. The solvent was then evaporated in a rotary vacuum system, until a thin film was formed. Complete evaporation was ensured by applying a rotary vacuum pump for at least 2 h. The lipid film was hydrated with phosphate buffer 10 mM (pH 7.4) containing NaCl 140 mM and EDTA 0.1 mM. The liposome suspension was vigorously stirred and, after 10 freeze and thaw cycles, it was extruded through two stacked polycarbonate membranes with 100 nm pores, for 31 times. The final lipid concentration was determined by the Stewart phospholipids assay [42]. For CF leakage experiments, the lipid film was obtained as already described, and then hydrated with a 30 mM CF solution, titrated to pH 7.4 with NaOH, and containing 10mM phosphate buffer and 80 mM NaCl to make it isotonic to the dilution buffer (phosphate buffer 10 mM, pH 7.4, NaCl 140 mM, 270 mOsm). Liposomes were separated from unencapsulated dye by gel filtration on a 40 cm Sephadex G-50 column.

2.3. Spectroscopy and microscopy measurements

Absorbance experiments were carried out at room temperature with a Cary 100 Scan spectrophotometer (Varian, Middelburg, Netherlands) and killerFLIP concentration was determined from the absorbance values at 280 nm, considering that the molar extinction coefficient of Trp is ε_{280} nm= 5690 $M^{-1}.cm^{-1}$ [43].

CD spectra were collected using a Jasco J 700 spectropolarimeter (Tokyo, Japan) in the wavelength range between 195 and 250 nm, with bandwidth= 2 nm, sensitivity= 5 mdeg, time constant= 2 s. Spectra were obtained in phosphate buffer (10 mM, pH 7.4, 140 mM NaF) by titrating 0.5-30 μM of peptide in 1.0 cm path length cells for (0.5-1.5 μM), 0.5 cm for (2.0-7.0 μM) and 0.1 cm for higher concentrations. 8 scans were recorded during the acquisition of each spectrum, in order to maximize the signal to noise ratio, and an *a posteriori* correction for the background signal was performed, by subtracting the spectrum of a blank solution.

To characterize the size of peptide aggregates, dynamic light scattering (DLS) measurements were performed using a Horiba LB 550 particle size analyser, equipped with a laser diode of λ_{em}=650 nm, of 5mW potency. KillerFLIP was added (60 μM) to filtered phosphate buffer, pH 7.4 at 25 ºC, and the signal was acquired for 3 min.

All steady-state fluorescence measurements were performed on a Fluoromax-4 instrument (Horiba, NJ), or with an Infinite F200 PRO filter-based plate reader or fluorescence intensity top reading (Tecan, Austria). The temperature was thermostatted at 25 °C for all experiments.

Wide field fluorescence microscopy images were obtained with an Axio Scope A1 (Carl Zeiss, Oberkochen, Germany), using a 40x magnification air objective. A peptide concentration of 16 μM was analyzed, in the presence of 0.6 μM Nile Red. Images were analyzed with the software ImageJ [44]

2.4. Membrane-perturbing activity experiments

Studies on vesicle release kinetics were performed with the fluorimeter (λ_{ex}=490 nm bandwidth=0.2nm, and λ_{em}=520 nm bandwidth=1.5nm) at different peptide concentrations, using CF-loaded vesicles composed of POPC/POPS at different ratios. Peptide-induced leakage was investigated also for different peptide to lipid ratios at two fixed peptide concentration (0.2 μM and 5 μM), and varying concentrations of POPC/POPS liposomes (1:1 molar ratio) using the plate reader. A black 96-well, flat bottom, polystyrene, nonbinding plate (Model 655900, Greiner bio-one, Germany) was filled first with a fixed peptide concentration then adding various liposome solutions of different lipid concentrations in a total volume of 154 μl per well, recording 20 minutes after the addition of the liposomes (F). For the initial fluorescence of vesicles (F0), another well was filled at the same concentration of liposomes. The fluorescence intensity after total leakage (F100) was measured in all wells, after adding Triton X-100 (final concentration 1 mM). The fluorescence intensities of each well were measured three times using the following instrument settings: number of flashes 25, integration time 20 μs, settle time 10 ms, dichroic beam splitter 510 (i.e. optimized for fluorescein). The gain was optimized for each liposome concentration in a sample where liposomes had been disrupted by triton addition. The fraction of peptide-induced leakage was determined as [31, 45]

$$\text{Released fraction} = \frac{F - F_0}{F_{100} - F_0}$$

2.5. Peptide-membrane association

For peptide-membrane association experiments, the Trp fluorescence signal was followed titrating 1 μM peptide solutions with increasing amounts of POPC and POPC/POPS (1:1 molar ratio) liposomes. Spectra were collected between 300 and 450 nm, using $\lambda_{exc.}$= 280 nm and a 295-nm cut off filter in a 1 cm × 1cm quartz cell.

2.6. Critical aggregation concentration of peptide

Steady-state fluorescence measurements of pyrene (0.1 μM) were performed in phosphate buffer, pH 7.4, using 1 cm path length cuvettes. Samples were excited at 319 nm, bandwidth=1 nm, λ_{em}=360-460nm, bandwidth=2 nm, integration time=1 s. The concentration of the stock solution of pyrene in methanol was estimated from its absorbance, using a molar extinction coefficient of 54,000 $M^{-1}cm^{-1}$ at 335 nm [46]. The ratio of the intensities of the first (372 nm) and third (382 nm) vibronic peaks (I_3/I_1) in the pyrene emission spectra was calculated after blank subtraction. This ratio was then plotted as a function of peptide concentration and fitted with a Gaussian sigmoid fitting curve [47].

$$R = R_0 + (R_\infty - R_0)\frac{1}{1 + e^{\frac{-(C - C_{50})}{n}}}$$

where the variable *R* corresponds to the pyrene I_3/I_1 ratio value, the independent variable *(C)* is the total concentration of peptide, R_∞ and R_0 are the upper and lower limits of the sigmoid, respectively, C_{50} is the centre of the sigmoid, and *n* is a phenomenological parameter describing the steepness of the curve. The critical aggregation concentration was determined from the intercept of the two lines corresponding to the tangent to the center of the sigmoid and for concentrations approaching 0 (see Figure 4).

3. Results

3.1. KillerFLIP causes leakage in model membranes, with selective activity against anionic lipids

KillerFLIP was demonstrated to cause membrane disruption in cancer cells [20]. However, this effect could be caused directly by peptide interaction with the cell membrane, or it could be just a secondary event of cell death caused by another mechanism, although apoptosis and necroptosis and interaction with specific intracellular protein targets were ruled out [20]. To clarify this point, we studied peptide

permeabilization of artificial membranes mimicking those of cancerous and normal cells, by performing carboxyfluorescein (CF) leakage experiments, with liposomes forme d of POPC/POPS lipids in different molar ratios [9-19, 48]. A fixed amount of liposomes (lipid concentration 50 μM) was titrated with increasing concentrations of peptide and the release kinetics of the probe was recorded, (Fig.1a). Peptide-induced leakage was indeed observed, showing that killerFLIP is able to perturb the permeability of a simple lipid bilayer, and therefore supporting pore formation as its primary mechanism of toxicity towards cancer cells. The fraction of liposome contents released 20 minutes after peptide addition was used to directly compare the pore-forming activity of killerFLIP in membranes of different compositions (Fig.1b). Membrane leakage resulted to be strongly dependent on the content of negatively charged PS lipids, confirming also in model systems the selectivity of killerFLIP. To confirm that selectivity is due essentially to lipid charge, we performed experiments also on POPC/POPG lipids, obtaining results comparable to those of POPC/POPS vesicles (Fig.1b)

Control experiments with the TAT and FLIP fragments did not evidence any significant membrane-perturbing activity of these peptides, at least up to 50 μM, i.e. in the concentration range where killerFLIP causes vesicle leakage (data not shown). This finding is in agreement with previous observations, showing that TAT alone does not kill cancer cells, nor perturbs their membranes [20].

3.2. KillerFLIP aggregates in aqueous solution

KillerFLIP is linearly amphiphilic, with an apolar C-terminal region, and a charged N-terminal segment. Due to this amphipathic structure, a significant peptide tendency to form micelle-like aggregates in water was conceivable. In turn, killerFLIP aggregation might modulate the water-membrane partition equilibrium [31]. To test this hypothesis, we performed experiments directed to characterize peptide aggregation in aqueous solutions.

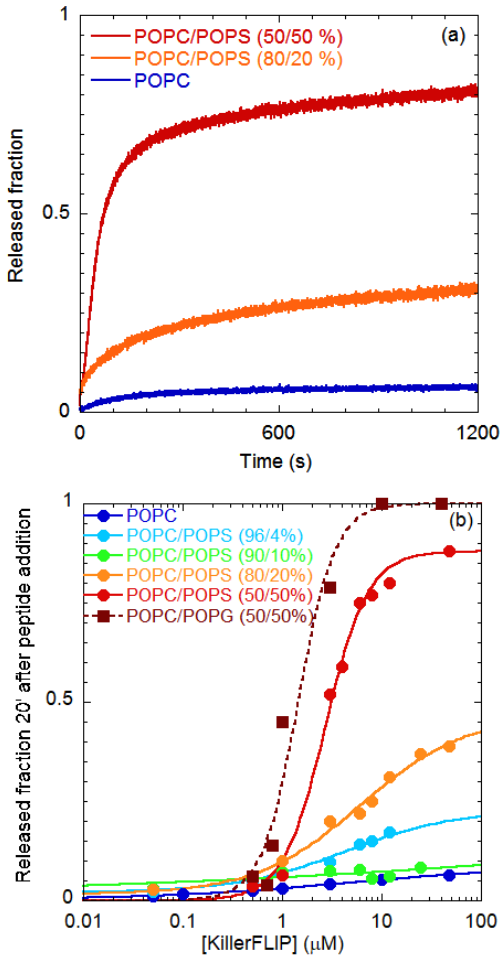

Fig. 1. Peptide-induced vesicle leakage, (a) kinetics of CF release after addition of peptide (10 μM) to POPC/POPS liposomes with different PS content (b) fraction of CF released 20 minutes after peptide addition in liposomes of varying composition. Liposomes concentration 50 μM

Dynamic light scattering (DLS) measurements at low peptide concentrations (*i.e.* below 1.5 μM) showed only one distribution of particle sizes (Fig.2), with diameters of 10 nm or below. This likely corresponds to the monomeric peptide as the size resolution of the light scattering technique is limited by the visible wavelength used by the instrument. At a concentration of 1.5 μM, the distribution became bimodal, with a population of much larger sizes (hundreds of nm), indicating the formation of aggregates. At higher concentrations, the monomer population was not observed anymore, indicating a highly cooperative aggregation process, reminiscent of micelle formation. Formation of large aggregates was previously reported for killerFLIP, but in that case experiments were performed at a single concentration [20]. DLS experiments performed in the presence of the reducing agent dithiothreitol (DTT), at a 10 mM concentration, had a very similar trend (data not shown), indicating that the possible formation of intermolecular disulphide bonds did not affect significantly the aggregation equilibria. For this reason, all

experiments were carried out without adding any reducing agent, *i.e.* under the same conditions used in the cellular studies.

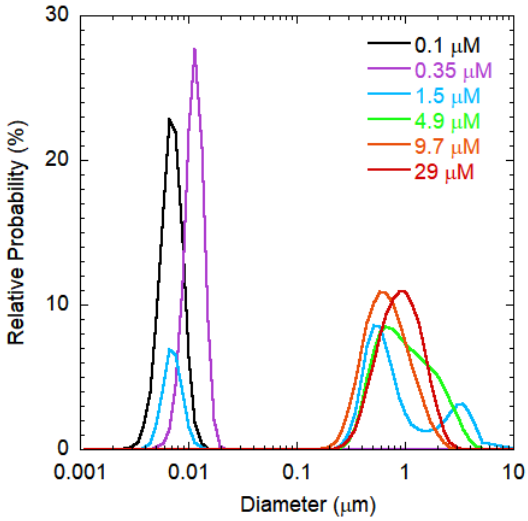

Fig. 2. Peptide aggregation, DLS measurements of the size of peptide aggregates, at different killerFLIP concentrations

We studied the aggregation process of killerFLIP more in detail by using an assay based on the vibronic structure of the fluorescence emission spectra of pyrene. The ratio of the intensities of the first (372 nm) and third (382 nm) vibronic peaks in the emission spectrum of this probe is sensitive to the polarity of the environment [49]. Pyrene is hydrophobic and its solubility in water is very limited. Therefore, when aggregates stabilized by the hydrophobic effect (such as detergent micelles, or peptide aggregates) are present in solution, it preferentially solubilizes in the interior hydrophobic regions of these supramolecular structures [50]. Fig. 3a shows the emission spectra of pyrene (0.1 μM) in the absence and presence of the peptide at a concentration corresponding to an aggregated state, according to the DLS data. Peptide addition caused a significant increase in the I_3/I_1 intensity ratio. Fig. 3b reports the changes of this ratio as a function of peptide concentration. At low concentrations, the pyrene fluorescence spectrum was very similar to that observed in water, with I_3/I_1 ~0.64 [49]. With increasing peptide concentration, a sudden, sigmoidal increase in I_3/I_1 was observed, then reaching a plateau. This trend is the behavior expected for the formation of aggregates formed by a high number of monomers, such as micelles, where the transition between the monomeric and the aggregated state takes place at a well-defined concentration, called critical aggregation concentration (CAC). Due to its high hydrophobicity, pyrene inserts into the aggregates as soon as they are formed. The fraction of pyrene molecules bound to micelles increases with the increasing concentration of aggregates, but when all pyrene molecules are inserted in micelles, a plateau is observed [50-53]. The critical concentration was determined by fitting the data with a phenomenological Boltzmann-type sigmoidal curve [47], obtaining 1.5±0.1 μM. It is important to note that this value is

lower than the active concentrations for all biological assays (anticancer activity and toxicity) [20]. Therefore, the biological effects of killerFLIP are determined by the aggregated peptide.

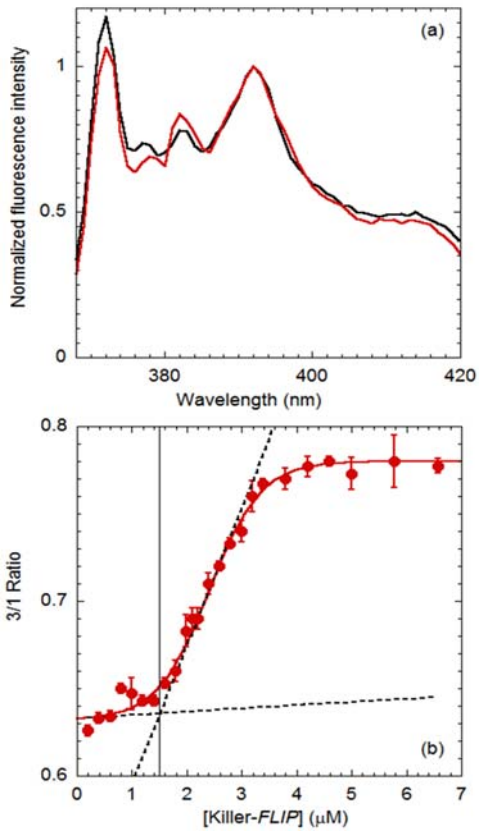

Fig. 3. Peptide critical aggregation concentration, (a) emission spectrum of pyrene (0.1 μM) in phosphate buffer, 10 mM (pH 7.4) containing NaCl 140 mM and EDTA 0.1 mM, in the absence (black line) and presence (red line) of the peptide (6.6 μM). (b) variation of the ratio between the intensities of the third (382 nm) and first (372 nm) vibronic peaks of pyrene fluorescence as a function of peptide concentration; error bars represent the standard error of the mean (± S.E.M). The continuous line represents a fit to the data [47], the dashed lines are defined in the materials and methods section, and their intersection defines the CAC, marked by the vertical solid line.

Finally, the morphology of killerFLIP aggregates was studied by fluorescence microscopy, using the dye Nile Red whose fluorescence is strongly enhanced after its spontaneous insertion in peptide aggregates [54]. The microscopic images show aggregates of globular shape, with a size distribution compatible with the DLS data (Fig.4).

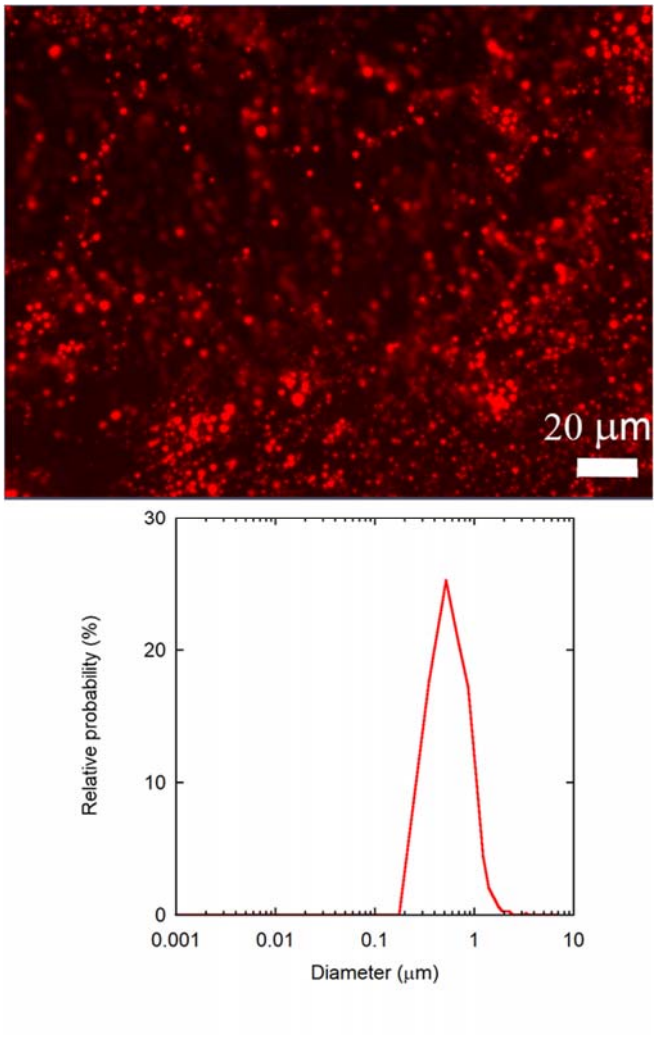

Fig. 4. Fluorescence microscopy images of Nile Red labelled killerFLIP aggregates and distribution of their radii.

3.3. KillerFLIP is unstructured both in the monomeric and in the aggregated state

To investigate possible structural changes induced by aggregation, circular dichroism experiments were performed. Fig. 5 shows the CD spectra of killerFLIP at peptide concentrations going from 0.5 to 7 µM, i.e. from concentrations below the CAC to values above this threshold. No variations in the CD spectra were observed by increasing the peptide concentration. At all concentration tested, the shape of the CD spectra was typical of a random coil conformation [55], indicating that killerFLIP is unstructured both in the monomeric and in the aggregated state.

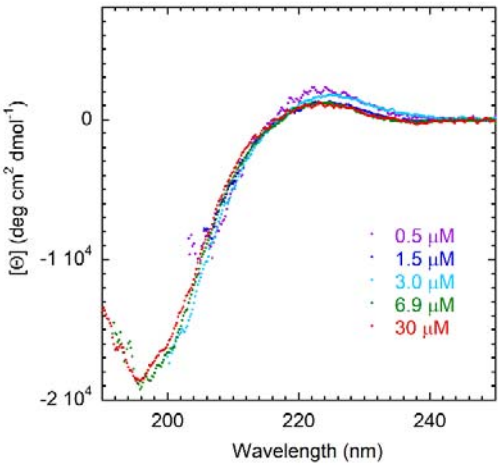

Fig. 5. Peptide secondary structure, circular dichroism spectra of killerFLIP in phosphate buffer (10mM, pH 7.4, NaF 140 mM), measured at different peptide concentrations. Per residue molar ellipticities are reported.

3.4. KillerFLIP is selective for anionic membranes only in the aggregated state

In principle, self-association can perturb the water-membrane partition equilibria, by shielding the hydrophobic residues from the water phase, thus reducing the hydrophobic driving force for association to membranes, and particularly to neutral bilayers [31].

To test this hypothesis, we studied the peptide-induced CF leakage at two peptide concentration values, 0.2 μM and 5 μM, which are below and above the threshold concentration for peptide aggregation determined above, respectively. To this end, we devised a novel method to follow peptide-induced vesicle leakage. Contrary to the typical leakage studies, where liposome concentration is maintained constant and the peptide concentration is varied, in this case we kept the peptide concentration fixed and varied the peptide to lipid ratio by performing experiments at different lipid concentrations. Fig. 6 shows the fraction of dye leakage observed 20 minutes after peptide addition, under different conditions of peptide and lipid concentration. In the experiments performed at a concentration where the peptide is aggregated (5.0 μM), we observed a high selectivity for membranes of different composition, just as reported in Fig. 1. Peptide-induced leakage was much higher for POPC/POPS liposomes than for pure POPC vesicles, as in the two cases 50% leakage was reached at approximately 2 μM and 60 μM lipid concentration, respectively. By contrast, measurements performed with monomeric peptide (0.2 μM) showed a very similar activity in both liposome compositions, reaching 50% leakage at the same concentration. Therefore, aggregation has a striking influence on peptide selectivity.

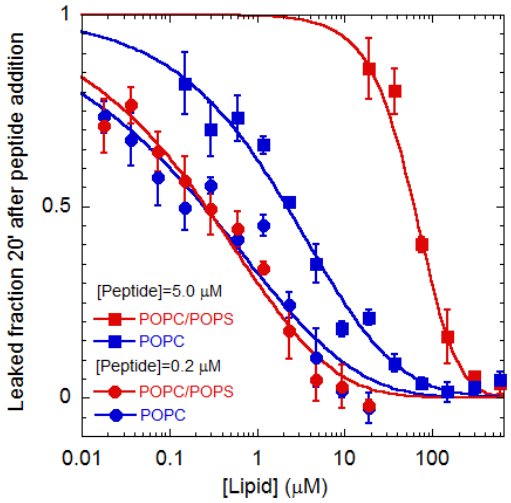

Fig. 6. Aggregation and membrane selectivity, effect of peptide concentration on selectivity for different membranes, [Peptide] = 5μM with POPC/POPS (1:1molar ratio): red square, POPC: blue square and [Peptide] = 0.2 μM with POPC/POPS (1:1molar ratio): red circle, POPC: blue circle. Each point is the average of three repetitions.

3.5. Selectivity of aggregated killerFLIP derives from differential membrane binding

Having demonstrated that killerFLIP acts directly on membranes, targeting bilayers containing negatively charged lipids, we investigated the determinants of peptide selectivity. The different pore-forming activities of killerFLIP on charged and neutral membranes could be due to a difference in affinity, or to a distinct behavior of the peptide once bound to the membranes. To discriminate between these possibilities, we assessed peptide affinity towards membranes of different compositions. Peptide association to a bilayer is typically followed by a significant blue shift of its fluorescence spectrum, as a result of the lower polarity of the membrane environment (Fig.7). The extent of the spectral changes was followed by determining the wavelength of maximum fluorescence emission. Titrating with POPC liposomes did not cause any significant shift of the fluorescence spectra, showing the lack of affinity for neutral membranes. These data showed that peptide selectivity was due to a much higher affinity of the peptide for charged than neutral lipid bilayers. This selectivity could be a simple consequence of the cationic nature of killerFLIP. However, previous studies performed in our laboratory and in other groups [28-30] demonstrated that charge is not sufficient to determine the selectivity of membrane-active peptides. Indeed, we studied peptide-membrane binding also at a concentration where it is monomeric. Peptide emission spectra were collected by titration of a 0.2 μM solution of killerFLIP with increasing amounts of POPC and POPC/POPS (1:1) liposomes. In contrast with the experiments performed with 10 μM peptide, in this case a blue shift of the fluorescence spectrum was observed with both lipid compositions. A plot of the wavelength of maximum fluorescence intensity as a

function of lipid concentration (Fig.8) shows that monomeric killerFLIP exhibits a comparable affinity for both charged and neutral lipid membranes.

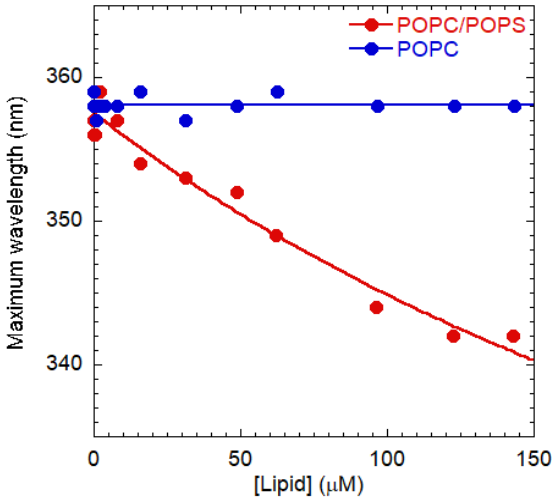

Fig. 7. Peptide-membrane association, Water-membrane partition as measured from the blue-shift in the emission spectrum of the Trp residue, [Peptide] = 10 μM, POPC, (blue symbols) and POPC/POPS, 1:1 molar ratio vesicles (red symbols).

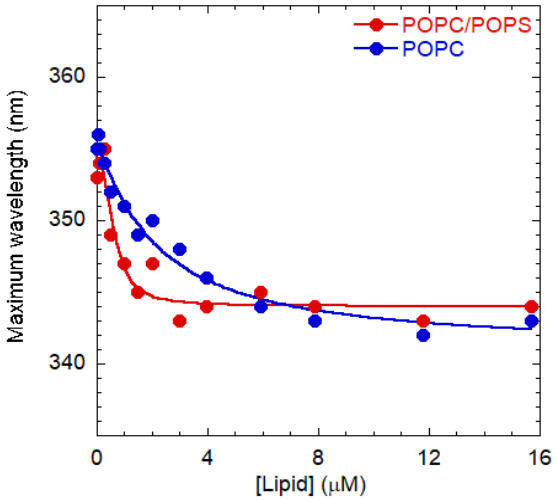

Fig.8. Peptide water-membrane partition for monomeric killerFLIP, the wavelength of maximum peptide fluorescence is reported as a function of lipid concentration for POPC (blue symbols) and POPC/POPS, 1:1 molar ratio (red symbols) liposomes. [Peptide] = 0.2 μM.

4. Discussion

Our data demonstrate that the monomeric and aggregated state of killerFLIP have a remarkably different selectivity for lipid bilayers mimicking the membranes of pathogens and those of eukaryotic cells. While the monomer is not selective, the aggregate is 30 times more active on anionic membranes than on neutral bilayers. This finding is caused

by a differential binding of the aggregated peptide to the two membrane types, while the monomer has a similar affinity for both lipid compositions. The CAC for killerFLIP is lower than the active concentrations for all biological assays (anticancer and toxicity) [20]. Therefore, the aggregated state is the relevant form regarding the biological effects of this peptide.

Given the amphipathic nature of HDPs, aggregation or self-assembly in the aqueous phase is a common finding for these molecules, and oligomerization, micellization, fibrillation of even gelation have been observed [8, 56]. Therefore, our data point to a previously unappreciated, possibly general role of supramolecular structure formation in determining HDP selectivity.

HDPs are typically cationic and amphipathic. Their association to membranes of any composition is driven by the hydrophobic effect, but affinity for negatively charged bilayers is enhanced by electrostatic interactions. Several studies concur to show that peptide hydrophobicity must be finely tuned to optimize activity and selectivity, and that two thresholds exist [8]. When the hydrophobicity is too low, peptide affinity for membranes might be insufficient, or the position in the bilayer might be too superficial to perturb the surface tension and lead to the formation of pores. Therefore, molecules of low hydrophilicity are often not membrane-active. However, if hydrophobicity surpasses a second, higher threshold, toxicity is observed, because binding to neutral membranes becomes significant. A high hydrophobicity often affects binding to neutral membranes more than to charged bilayers, and hemolysis more than bactericidal activity [57, 58]. This finding can be explained by considering that electrostatic and hydrophobic effects are not additive [59, 60]: an increased hydrophobic driving force causes a deeper embedding of the peptide inside the lipid bilayer, and therefore a reduction in its electrostatic interactions with negatively charged head groups.

Hydrophobicity is often calculated based on the sequence of a peptide. However, conformational equilibria affect the degree to which the hydrophobic side chains are exposed to the water phase, and therefore modulate the driving force for membrane binding, i.e. the “effective” peptide hydrophobicity [8, 30, 61]. Reversed-phase chromatography retention times have resulted to be an accurate measure of the effective peptide hydrophobicity of peptides [62]. Interestingly, strong correlation between RP-HPLC retention times and the hemolytic activity of AMPs has been reported [63-65]. For instance, many studies indicate that disrupting a perfectly amphipathic alpha helix by inserting helix-breaking residues [30], or by inserting polar residues on the hydrophobic face of the helix [65-68] increases peptide selectivity, by reducing the hydrophobic driving force for membrane binding [8]. The present study indicates that aggregation can be an additional mechanism for obtaining a similar effect.

Aggregation of amphiphilic peptides decreases their effective hydrophobicity by hiding the apolar moieties from the aqueous phase. As a consequence, the hydrophobic driving force for membrane binding is reduced in the aggregates [31-33, 69-71]. Considering the

two hydrophobicity thresholds discussed above, this effect can explain the increase in selectivity observed after aggregation for killerFLIP.

The effects of aggregation and self-assembly have received limited attention in the HDP literature, and the few available data are somewhat contradictory. Consistently with our findings, some studies reported an increase in selectivity with aggregation, due to differential effects on membrane binding [72, 73]. However, in other investigations a decrease in selectivity was observed upon the formation of supramolecular structures [69, 74]. Shankar et al. [75] suggested that toxicity of self-assembled lipopeptides depends on the specific structure of the fibrillar aggregates. These discrepancies are most likely due to the fact that often conclusions on the effects of aggregation are derived by modulating formation of supramolecular structures by altering the peptide properties, or by varying electrostatic interactions by changing the ionic strength of the solution. It is therefore difficult to discriminate between the direct effects of these changes (e.g. an increase in the hydrophobicity of the peptide sequence) and the consequence of the variations they induce in aggregation. By contrast, in our case, comparison of the same peptide in a monomeric and in an aggregated state allows a direct analysis of the effects of aggregation.

Of course, it should be mentioned that in cellular studies aggregation could affect selectivity through mechanisms that are not present in model membranes. For instance, preassembled HDPs might be unable to cross the LPS layer or the cell wall, and thus to reach the plasma membrane of bacteria. At the same time, they would still be able to interact with the "naked" membrane of host cells [74, 76-78]. However, this line of reasoning does not apply to selectivity between cancer and healthy cells (as in the case of killerFLIP), which both lack a cell envelope.

A computational study indicated that aggregation could lead to membrane selectivity both by thermodynamic and kinetic effects on membrane binding, with a prevalent role of time-dependent processes [73]: binding to host mammalian membranes will be slow and inefficient as long as the lipopeptides form micelles in solution, while binding to the bacterial surface will still be efficient, thanks to electrostatic interactions and to the higher fluidity of the membrane. However, in our case, peptide-membrane association was much faster than the time-resolution of our spectral measurements, and, most importantly, than the time-scale of peptide-induced leakage. Therefore, we can rule out that kinetic effects are at play in our system.

5. Conclusions

Overall, our data indicate that peptide aggregation should be taken into account as an additional method to vary the effective hydrophobicity of membrane-active HDPs, and therefore their cell-selectivity. Peptide aggregation or ordered self-assembly presents additional advantages, since it reduces susceptibility to proteolytic degradation, it affects the pharmacokinetics and pharmacodynamics [72, 79-81], and it allows the local release

of a high peptide concentration at a single site in the membrane [82-84]. Finally, aggregates of membrane-active peptides could be even considered for the entrapment of small-molecule drugs inside the hydrophobic core of the particles, for targeted delivery [85-86], leading to the development of new therapeutic tools based on synergistic effects [87].

Conflicts of interest

There are no conflicts to declare.

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