Penetration of the Insect Defensin A into Phospholipid Monolayers and Formation of Defensin A–Lipid Complexes

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ABSTRACT Defensin A is an inducible cationic protein secreted in the hemolymph of fleshfly *Phormia terranovae* larvae in response to bacterial or septic injuries. Defensin A is known to permeabilize the bacteria cell membranes by forming voltage-dependent channels. The penetration of this small protein into lipid monolayers was studied as a function of the polar head and acyl chain length of phospholipids. The extent of penetration by defensin A is higher in monolayers made of anionic phospholipids than in monolayers made of zwitterionic phospholipids (phosphatidylcholines), because of electrostatic interactions. From the analysis of the compression isotherm parameters of mixed defensin A/phospholipid monolayers, it appears that defensin A interacts with phospholipid by forming 1:4 complexes. These complexes are not miscible in the lipid phase and induce microheterogeneity in the lipid membrane. These clusters might be related to the ion-channel structures responsible for the biological activity of defensin A.

INTRODUCTION

Insect defensins are inducible small proteins secreted in the insect hemolymph in response to bacterial or septic injuries. The proteins of this class present sequence homologies (among them six Cys residues, which form three disulfide bridges) and have the common characteristic of being highly basic (for a review see Maloy and Kari, 1995). Defensin A, a 4-kDa protein, isolated from the larvae of the fleshfly *Phormia terranovae*, belongs to this protein class. Its secondary structure includes a C-terminal antiparallel β-sheet linked to an amphiphilic α-helix via two S-S bridges, and an N-terminal loop linked to the β-sheet by the third S-S bridge (Cornet et al., 1995). The N-terminal loop is rather flexible, and the α-helical content has been shown to be sensitive to environmental conditions (Maget-Dana et al., 1995). The antibacterial activity of defensin A is directed principally against Gram-positive bacteria (Boman et al., 1991; Hoffmann and Hetru, 1992) and seems to be related to the formation of voltage-dependent channels in the cytoplasmic membrane of bacteria (Cociancich et al., 1993), strongly suggesting privileged interactions of defensin A with lipids.

In this report we used model monolayer systems to test the ability of defensin A to insert into a phospholipid model membrane. Interactions between the protein and lipids were studied by analyzing the compression isotherm parameters of the mixed films. The aim of this approach was to correlate the biological activity of defensin A with specific interactions between the protein and the membrane lipids of the target cell.

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MATERIALS AND METHODS

Recombinant insect defensin A was prepared by Transgène (Strasbourg, France). Phospholipids: L-α-Dimyristoyl phosphatidylcholine (DMPC), L-α-dimyristoyl phosphatidylethanolamine (DMPE), L-α-dipalmitoyl phosphatidylcholine (DPPC), beef brain phosphatidylserine (PS), diphosphatidylglycerol (cardiolipin), and egg lecithin were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents: Chloroform, ethanol, hexafluoroisopropanol (HFIP), and methanol were from Merck (Darmstadt, Germany). Pure water (resistivity: 18 MΩ · cm) was obtained from a Millipore (Milli Q) apparatus (Bedford, MA). Unillamellar egg lecithin vesicles were prepared under sonication in a bath by the injection method (Kremer et al., 1977). Their average diameter was ~50 nm, as shown by electron microscopy after negative staining.

Monolayer measurements

Penetration experiments were performed at constant area (19.6 cm^2) in a thermostatted ($T = 20 \pm 0.2$°C) glass dish containing 30 mM Tris (pH 7.4) as a subphase (volume 15 ml). The lipids were spread at the air/water interface from a chloroform/methanol 2:1 mixture to give the desired initial surface pressure π_i. Defensin A stock solution (7×10^{-5} M in the Tris buffer) was added to the subphase through a short vertical tube. The surface tension was measured by the Wilhelmy (platinum) plate method with an accuracy of ± 0.5 mN · m^{-1}.

For the mixed monolayer experiments, compression isotherms were recorded at 20 ± 0.2°C, using a Langmuir film balance system described elsewhere (Maget-Dana and Ptak, 1995). The defensin A stock solution (H_2O/methanol/HFIP 1:1:2, v/v/v) was mixed with the lipid stock solution (HFIP/methanol/chloroform 1:2:3, v/v/v) to achieve the desired composition. The mixture was then spread on a 30 mM Tris (pH 7.4) subphase with a 50-μl Hamilton syringe, and the solvent was allowed to evaporate for 30 min. The monolayer was then compressed at a rate lower than 0.1 nm^2 · $molecule^{-1}$ · min^{-1}. Each experiment was performed at least three times.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jobin-Yvon IV autodichrograph. Lipid vesicles were added to the defensin solution in a 1-mm-path length cell to obtain the desired defensin/lipid molar ratio. For all data shown, the corresponding defensin-free CD spectra (5 mM Tris, pH 7.4, buffer or lipid vesicle suspension in the same buffer) were substracted. The absorbance value of the defensin/lipid solution was always <1 in the

wavelength range of the CD experiment. Optical activities were reported as ellipticity per amino acid residue: Θ (deg · cm^2 · dmol^{-1}). The α-helix content was roughly estimated from CD data according to the formula % α-helix = $-\Theta_{222}/32{,}980$, given by Zhong and Johnson (1992) on the basis of a 26-protein data set (Θ_{222} is the ellipticity value at 222 nm).

RESULTS

Penetration of defensin A into phospholipid monolayers

The adsorption of defensin A at the air/water interface in the absence of lipid film is very slow. One cannot observe any detectable increase of the surface pressure before at least 1 h, and the plateau (~13 mN · m^{-1}) is reached only 3 h after the addition of defensin A (10^{-7} M) in the subphase (Maget-Dana and Ptak, 1996). Conversely, in the presence of a phospholipid film, the pressure increase, $\Delta\pi$, as a result of the penetration of defensin A molecules into the film, is instantaneous. This feature is indicative of the high affinity of defensin A for a membrane environment.

Fig. 1 illustrates the penetration behavior of defensin A into various lipid films as a function of their packing. At a similar initial pressure, π_i, of the lipid monolayer (Fig. 1 *A*), the penetration of defensin A is less effective in a DMPE than in a DMPC monolayer. However, the exclusion pressure (initial pressure of the lipid film at which the protein can no longer penetrate) is the same: ~32 mN · m^{-1}. Let us compare the behavior of defensin A in monolayers of phosphatidylcholine with various chain lengths: the penetration extent of defensin A varies in the order DMPC > egg lecithin > DPPC. The exclusion pressure is the same for egg lecithin and DPPC: ~22 mN · m^{-1} instead of 32 mN · m^{-1} for DMPC.

We can also analyze these experiments by taking into consideration the "initial molecular density," σ_i, of the phospholipids in the monolayer (Fig. 1 *A'*) instead of the "initial surface pressure" of the lipid film as a parameter of the lipid packing. (The molecular density of the lipid films is deduced from their compression isotherms: a molecular area, A_i, corresponds to a given initial pressure, π_i, and then $\sigma_i = 1/A_i$.) Owing to this representation, we can see that the penetration extent of defensin A is comparable in DMPC and DPPC monolayers at the same σ_i. The exclusion density (molecular density of the phospholipids at which the protein can no longer penetrate) is ~1.9 molecules/nm^2 for DMPC and DPPC films, but only 1.4 molecules/nm^2 when the films are made of egg lecithin, which possesses unsaturated acyl chains. The penetration extent of defensin A in DMPE monolayers does not exhibit large variation when the lipid density increases, in contrast with the situation encountered in phosphatidylcholine films. If we compare the defensin

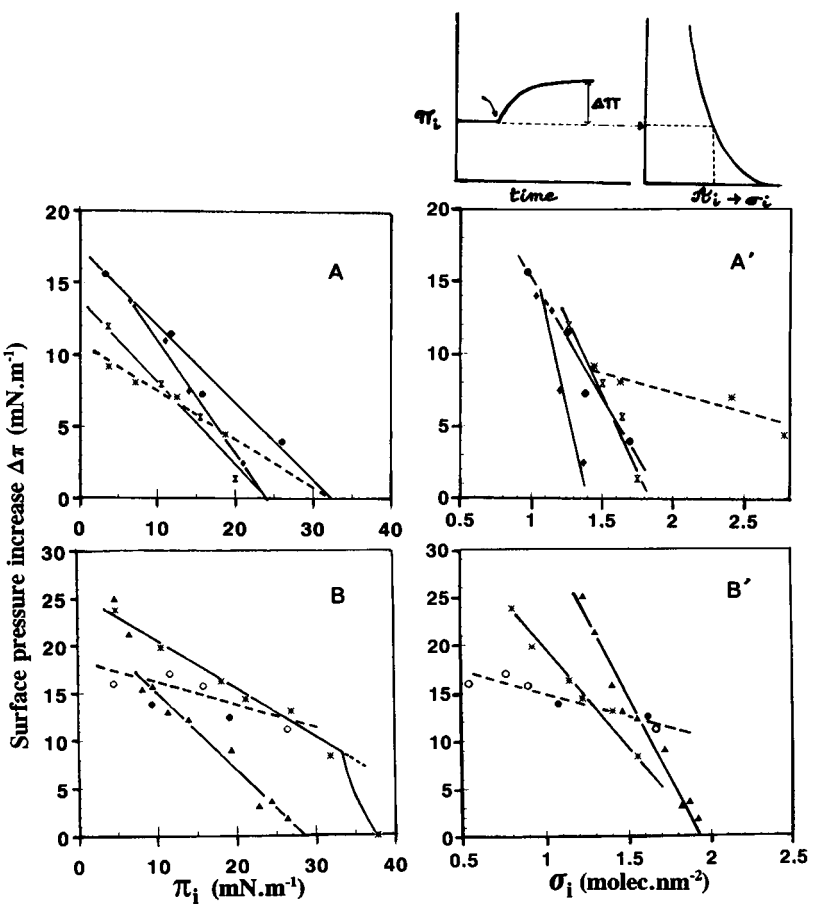

FIGURE 1 Penetration of defensin A into lipid monolayers as a function of the lipid packing. (*Left*) The lipid packing parameter is the initial surface pressure. (*Right*) The lipid packing parameter is the initial molecular density. (*A* and *A'*) Neutral lipid monolayers. ●, DMPC; ⧖, DPPC; ♦, lecithin; *, DMPE. (*B* and *B'*) Anionic lipid monolayers. *, PS; ▲, PS in the presence of 2 mM $CaCl_2$; ○, cardiolipin; ●, cardiolipin in the presence of 2 mM $CaCl_2$. A schematic representation of the experiment is drawn at the top of the figure: defensin A (10^{-7} M final concentration) was added (*arrow*) in the Tris 30 mM (pH 7.4) subphase, beneath the lipid monolayer, the initial pressure of which is π_i. The penetration of defensin A into the lipid monolayer induces a surface pressure increase $\Delta\pi$. The initial density of the lipid monolayer at π_i, $\sigma_i = 1/A_i$, is deduced from a previous compression isotherm.

insertion into DMPE and DMPC monolayers, we can see that in the common range of σ_i (1.4–1.9 molecules/nm^2), the penetration is more important in DMPE films, probably because of the smaller size of the polar headgroup.

As expected for a cationic protein, defensin A inserts very efficiently into anionic phospholipid monolayers. As shown Fig. 1 *B*, when the initial pressure of the PS monolayer is low (~5 mN · m^{-1}), $\Delta\pi$ reaches 24 mN · m^{-1} instead of 14 mN · m^{-1} in the case of lecithin. The exclusion pressure is 37 mN · m^{-1} (~23 mN · m^{-1} in the case of lecithin monolayers). The addition of calcium ions (2 mM) to the subphase causes a reduction in $\Delta\pi$, except when the initial pressure of the PS monolayer is low (~5 mN · m^{-1}), and the exclusion pressure is now less than 30 mN · m^{-1}. The penetration extent of defensin A into diphosphatidylglycerol (cardiolipin) monolayers does not vary extensively with π_i from 5 to 30 mN · m^{-1}. Furthermore, the presence of calcium ions in the subphase does not affect the penetration degree of defensin A in cardiolipin monolayers.

Analyzing the results as a function of σ_i (Fig. 1 *B'*) leads to the conclusion that at a given PS molecular density, the penetration degree of defensin A into the lipid monolayer is enhanced by the presence of calcium ions in the subphase, although the exclusion density is the same in both cases (1.9 molecules/nm^2).

Mixed defensin A/lecithin monolayers

Fig. 2 shows the compression isotherms of mixed defensin A/lecithin monolayers plotted at different defensin molar fractions, $X_{Def} = N_{Def}/(N_{Def} + N_L)$, where N is the number of molecules spread at the interface; the subscripts Def and L refer, respectively, to defensin A and lipid.

The compression isotherm of a pure defensin A monolayer presents a slope change near π = 13 mN · m^{-1} called "transition pressure," π_t, between two states: a liquid-expanded (LE) state and a more condensed one. The collapse pressure, π_c, is ~30 mN · m^{-1} (Maget-Dana and Ptak, 1996). π_t is defined as the intersection of the tangents to the steep and the flattened parts of the isotherm, respectively (see Fig. 2, *inset*). The composition dependence of π_t is shown Fig. 3. The fact that the transition pressure of the mixed films is different from that of the pure defensin film indicates that the two film components are miscible on the whole range of composition. The π_t–ln X_{Def} plot can be divided into two regions. In region I ($X_{Def} \leq 0.2$), the variation of π_t (π_t = 17.4 ± 0.4 mN · m^{-1}) is in the range of experimental error, whereas in region II ($X_{Def} \geq 0.2$), π_t varies linearly with ln X_{Def}. This result suggests the formation of a defensin-lecithin complex with a stoichiometry corresponding to X_{Def} = 0.2, the transition pressure of which occurs near 17 mN · m^{-1}. From the application of the two-dimensional phase rule $F = 3-\varphi$ (F, number of freedom degrees of the system; φ, number of phases at the interface) (Crisp, 1949), we can deduce that along the horizontal line *ab* three phases (because there is no variation in π_t, F = 0 and φ = 3) are in equilibrium with each other, which are lecithin in the LE state, the defensin-lecithin complex in the LE state that is not miscible with the excess lecithin, and the same complex in a more condensed state. The coexistence regions of these phases are indicated in the legend of Fig. 3. In region II ($X_{Def} \geq 0.2$), the data are well described by the first-order relation π_t = 13.7–2.3 ln X_{Def}

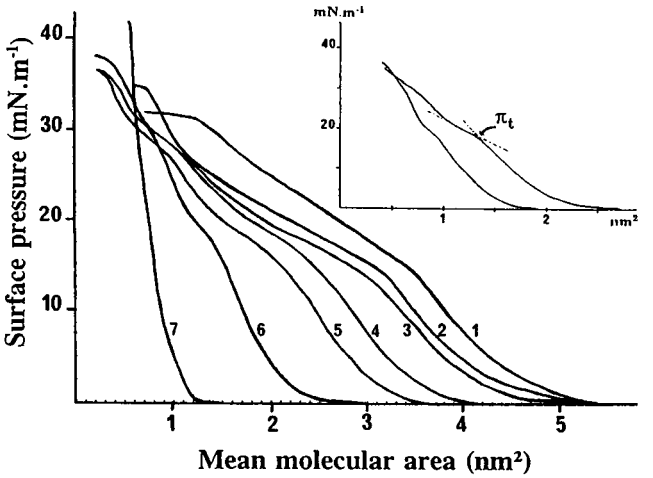

FIGURE 2 Compression isotherms of mixed defensin A/lecithin monolayers. Subphase: 30 mM Tris (pH 7.4); T = 20 ± 0.2°C. (*1*) X_{Def} = 1 (pure defensin A monolayer); (*2*) X_{Def} = 0.8; (*3*) X_{Def} = 0.67; (*4*) X_{Def} = 0.5; (*5*) X_{Def} = 0.4; (*6*) X_{Def} = 0.2; (*7*) X_{Def} = 0 (pure lecithin monolayer). (*Inset*) Isotherms obtained for (*left*) X_{Def} = 0.1 and (*right*) X_{Def} = 0.2 recorded with an enlarged scale. The transition pressure π_t is defined as the intersection of the tangents to the steep and the flattened parts of the isotherm, respectively.

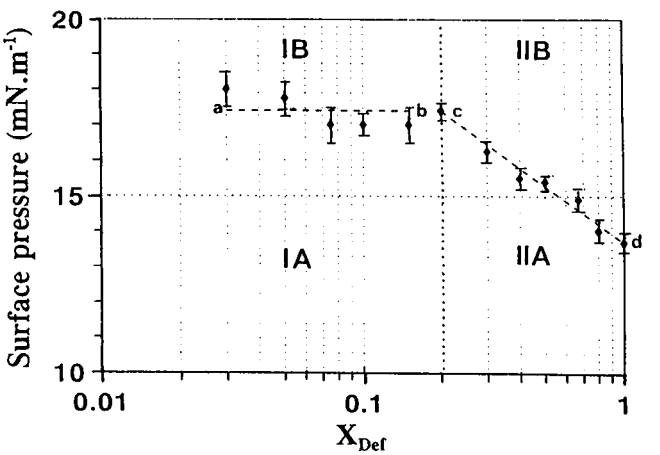

FIGURE 3 Transition pressure-composition diagram for mixed defensin A/lecithin monolayers. The dotted line *ab* is drawn according to π_t = 17.4 ± 0.4 mN · m^{-1}; the dotted line *cd* is drawn according to π_t = 13.7–2.3 ln X_{Def}. IA region: Lecithin in the LE state, defensin-lecithin 1/4 complex in the LE state (not miscible with the excess lecithin). IB region: Lecithin in the LE state, defensin-lecithin complex in a condensed state. IIA region: Defensin-lecithin complex in the LE state (miscible with the excess defensin). IIB region: Defensin and the complex in a condensed state (miscible).

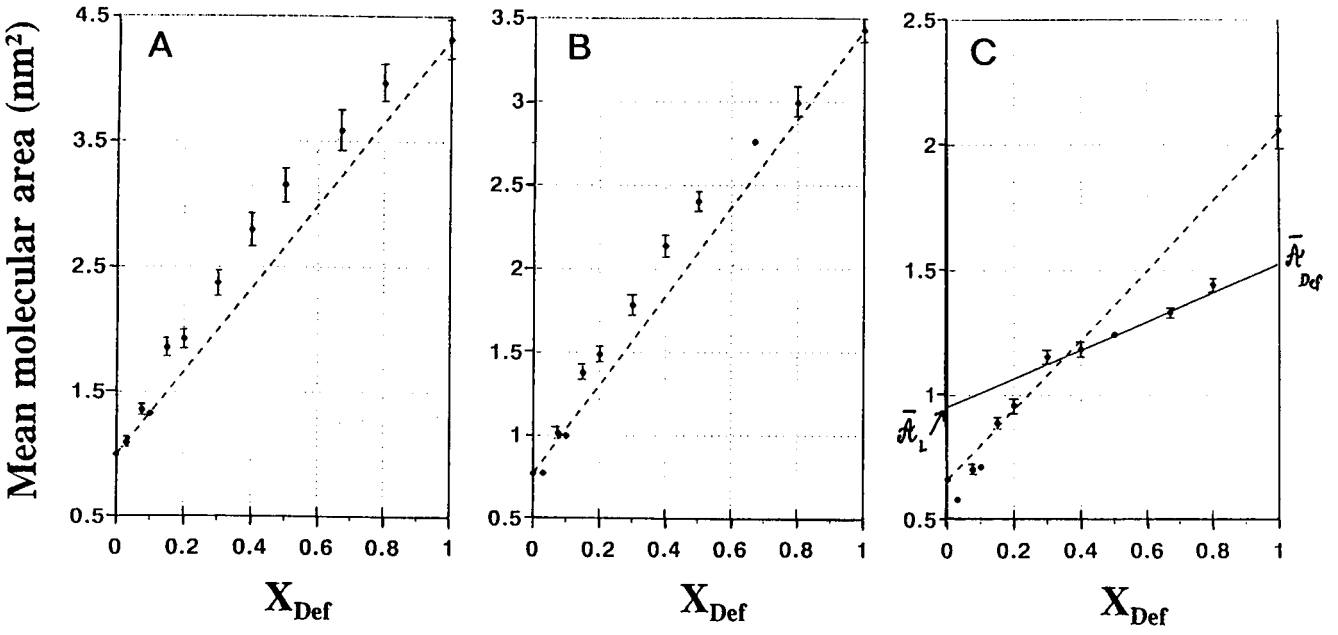

FIGURE 4 Mean molecular areas of mixed defensin A/lecithin monolayers as a function of composition. (*A*) At 5 mN · m^{-1}; (*B*) at 15 mN · m^{-1}; (*C*) at 25 mN · m^{-1}. A_{Def} is the partial molecular area of defensin A, and A_L is the partial molecular area of lecithin for $X_{Def} > 0.2$. The broken line corresponds to the additivity rule.

(linear regression coefficient $r = 0.987$). For an ideal miscibility, the slope of this plot should be $kT/A_t \approx 1.1$ mN · m^{-1} (where $A_t = (3.6 \pm 0.3) \times 10^{-18}$ m^2 is the molecular area of defensin A at π_t in a pure monolayer). This result is indicative of a nonideal mixing, and we can deduce that along the line *cd*, two phases (because there is a first-order relation between π_t and X_{Def}, $F = 1$ and $\varphi = 2$) are in equilibrium with each other: a LE phase of the defensin-lecithin complex and a condensed phase of the defensin-lecithin complex that is miscible with the excess defensin.

In Fig. 4 are plotted the values of the mean molecular area of the mixed monolayers at various surface pressures as a function of their composition. At pressures lower than 20 mN · m^{-1}, the areas follow the additivity rule for $X_{Def} \leq 0.1$, whereas in the $X_{Def} > 0.1$ range the areas present positive deviations. Conversely, when $\pi \geq 20$ mN · m^{-1}, we observe negative deviations for $X_{Def} \geq 0.4$.

Mixed defensin A/phosphatidylserine monolayers

Fig. 5 shows the compression isotherms of mixed defensin A/PS monolayers at different X_{Def}. In contrast to mixed defensin/lecithin monolayers, we cannot detect any kink below the collapse pressure when $X_{Def} < 0.2$. This fact implies an intimate mixing of defensin A and PS, which form a preferred array collapsing around 36 mN · m^{-1}, as seen in the pressure-composition phase diagram (Fig. 6). For $X_{Def} > 0.2$, the collapse pressure varies with composition roughly according to the relation $\pi_c = 31.7 - 4.5 \ln X_{Def}$ ($r = 0.918$). For an ideal miscibility, this slope would be $kT/A_c \approx 3.4$ mN · m^{-1} (where $A_c \approx 1.2 \times 10^{-18}$ m^2 is the molecular area at the collapse in a pure defensin monolayer). Concurrently, the transition pressure decreases linearly with $\ln X_{Def}$ from $X_{Def} = 0.2$, and the data are distributed along the first-order line $\pi_t = 13.3 - 4.2 \ln X_{Def}$ ($r = 0.980$). For an ideal miscibility, the slope of the plot would be 1.1 mN · m^{-1}, as seen above.

The plot giving the mean molecular area of the mixed defensin/PS films as a function of their composition can be divided into three parts (Fig. 7). In the $X_{Def} < 0.2$ range, the deviations from the addivity rule are slightly negative. In the $0.2 \leq X_{Def} < 0.5$ range, the deviations are slightly positive

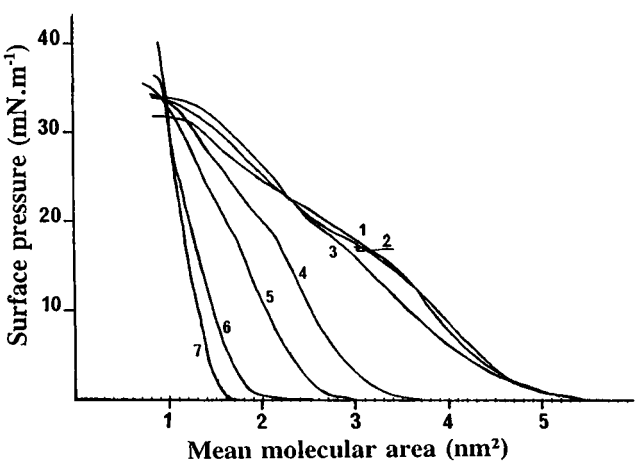

FIGURE 5 Compression isotherms of mixed defensin A/PS monolayers. (*1*) $X_{Def} = 1$ (pure defensin A monolayer); (*2*) $X_{Def} = 0.8$; (*3*) $X_{Def} = 0.5$; (*4*) $X_{Def} = 0.4$; (*5*) $X_{Def} = 0.2$; (*6*) $X_{Def} = 0.1$; (*7*) $X_{Def} = 0$ (pure PS monolayer). Subphase: 30 mM Tris (pH 7.4); $T = 20 \pm 0.2$°C.

and the area values follow the additivity rule at pressures higher than 15 mN · m^{-1}. In the $X_{Def} \geq 0.5$ range, the deviations are straight positive, and the partial molecular area of the lipid ($\bar{A}_{PS}$) is comparable to the area of defensin A in a pure defensin monolayer.

Interaction of defensin A with phospholipid vesicles

The CD spectrum of defensin A in a Tris buffer solution is affected by the presence of DMPC vesicles (Fig. 8). The intensity of the negative peak near 208 nm increases according to the amount of vesicles added. We observe an isodichroic point at 197 nm, indicating an equilibrium between two forms of defensin A, which can be a free form and a form bound to lipids. When the defensin molecule is inserted into the lipid bilayer, its helical content increases; at the protein-to-lipid ratio $R = 0.25$ (corresponding to $X_{Def} = 0.18$), the magnitude of $|\Theta_{222}|$ goes from 3500 to 6440 deg · cm^2 · dmol^{-1}. This result agrees with that found in the CD spectra of transferred defensin A/lecithin monolayers (Maget-Dana et al., 1996). It has not been possible to study the interaction of defensin A with vesicles containing PS (even at the PS/DMPC ratio of 0.1), because vesicle disruption occurred immediately, indicating strong defensin A–PS interactions.

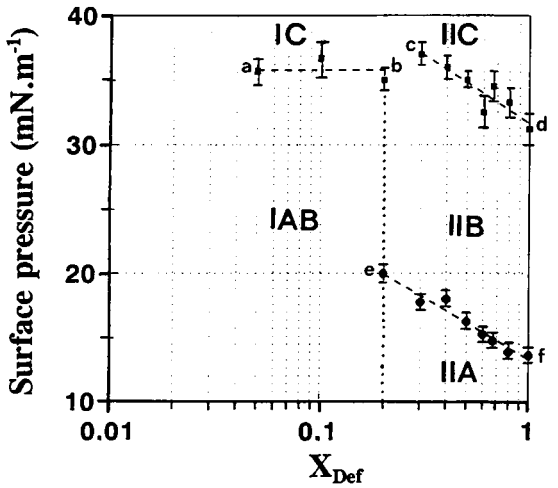

FIGURE 6 Pressure-composition phase diagram for defensin A/PS monolayers. ●, Transition pressure π_t; ■, collapse pressure π_c. The dotted line *ab* is drawn according to $\pi_c = 35.8 \pm 0.7$ mN · m^{-1}; the dotted line *cd* is drawn according to $\pi_c = 31.7–4.5 \ln X_{Def}$; the dotted line *ef* is drawn accordind to $\pi_t = 13.3–4.2 \ln X_{Def}$. IAB region: PS in the LE state and the defensin A–PS 1:4 complex in the LE state (not miscible); IC region: PS in the LE state and complex in the collapsed state; IIA region: one phase in the LE state containing the defensin A-PS complex and the excess defensin (miscible); IIB region: one phase in the condensed state containing the complex and the excess defensin A (miscible); IIC region: one collapsed phase containing the same entities.

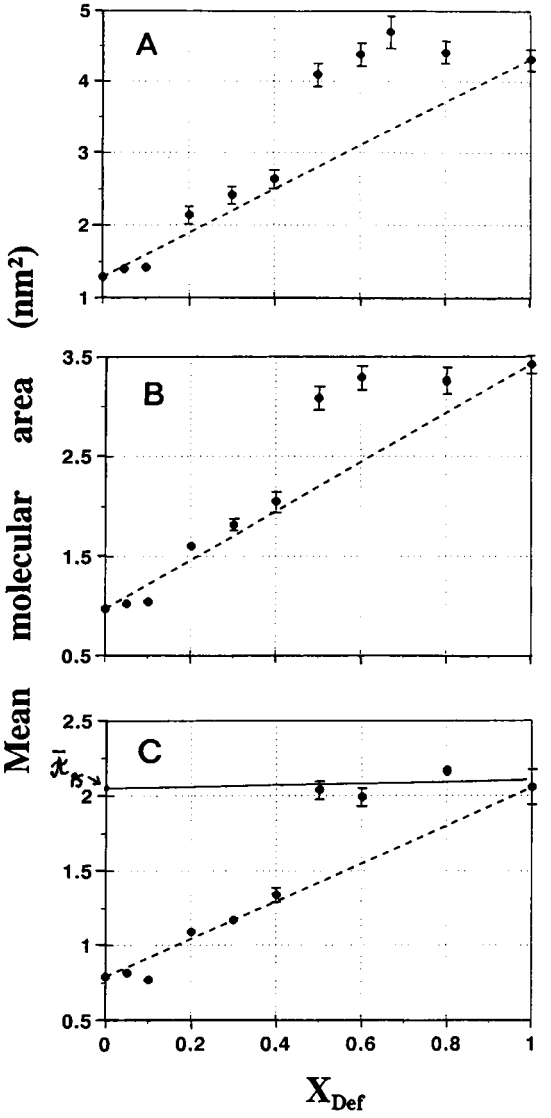

FIGURE 7 Mean molecular areas of mixed defensin A/PS monolayers as a function of composition. (*A*) At 5 mN · m^{-1}; (*B*) at 15 mN · m^{-1}; (*C*) at 25 mN · m^{-1}. $\bar{A}_{PS}$ is the partial molecular area of PS for $X_{Def} > 0.4$.

DISCUSSION

Defensin A penetration into lipid films

The experiments described in this paper gives evidence of the affinity of defensin A for lipid films. The results have been analyzed as a function of two parameters that symbolize the lipid packing: the "initial surface pressure" of the lipid monolayer, which is macroscopic data, and the "initial molecular density" of the lipid in the monolayer. This last parameter allows us to analyze the results on the molecular scale. As a matter of fact, the pressure-area isotherm may vary greatly from one phospholipid type to another as a function of its polar head and acyl chains. For example, the physical state of DMPE is very different from that of cardiolipin at the same surface pressure, whereas it is sim-

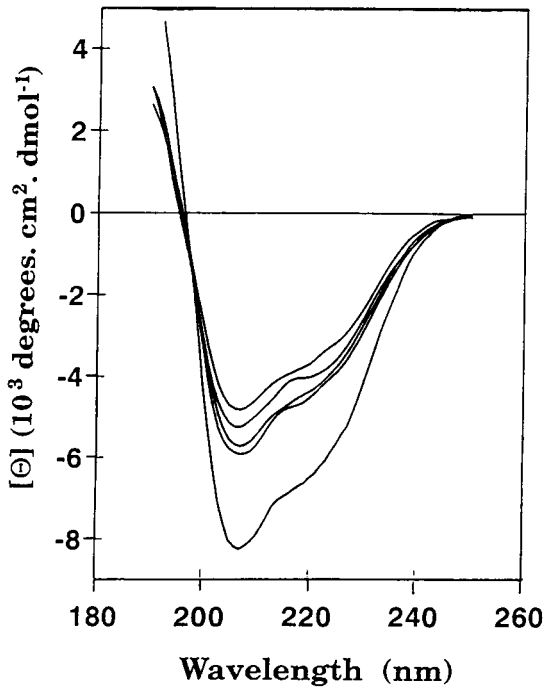

FIGURE 8 Circular dichroism spectra of defensin A in the presence of preformed lecithin vesicles. *Upper spectrum*: Defensin A (2×10^{-5} M); then in the presence of lecithin vesicles at the protein-to-lipid ratio (*from top to bottom*) $R = 0.025$; $R = 0.05$; $R = 0.1$; $R = 0.25$. The experiments were done in 5 mM Tris (pH 7.4) at $T = 21 \pm 0.5$°C.

ilar at the same molecular density. We believe these two lipid packing indicators give complementary information.

Except in the case of DMPE and cardiolipin monolayers, the penetration of defensin A is shown to be strongly dependent upon the molecular packing of the lipids. The penetration extent of defensin A into phosphatidylcholine monolayers is more sensitive to the lateral hindrance of the acyl chains (the unsaturation gives rise to bends in the general orientation of the chains) than to their length. This is illustrated, in the $\Delta\pi$-σ_i curve, by the comparable insertion profile of defensin A into DMPC (14:0) and DPPC (16:0) monolayers, whereas the difference is more pronounced between DPPC and egg lecithin (predominantly 16:0 and 18:1). The higher penetration into anionic (as compared to neutral) phospholipid monolayers indicates that electrostatic interactions play a crucial role in this process. This is illustrated by the comparison of the insertion profiles of defensin A into the monolayers of two natural phospholipids: lecithin and PS. Whatever the type of representation (as a function of either π_i or σ_i), it is clear that defensin A inserts much more effectively into PS than into lecithin films. The penetration of defensin A into PS monolayers at the same π_i is reduced by the presence of 2.5 mM calcium ions. This result agrees with the inhibition by calcium of the biological activity of defensin A at the membrane level (Cociancich et al., 1993). Near the membrane interface the free Ca^{2+} concentration is strongly enhanced (Seelig, 1990) and contributes to increased ordering of the lipid acyl chains (Traüble et al., 1976). At the same time, the charge neutralization of the PS headgroups by Ca^{2+} ions induces a condensation of the film. This is why analyzing the results on the molecular scale (as a function of the molecular density of PS) leads to the conclusion that, at the same σ_i, the surface pressure increase is higher in the presence of calcium. We do not observe such an effect of Ca^{2+} ions on the penetration of defensin A into monolayers made of cardiolipin, an anionic phospholipid widely distributed in bacteria membranes (Shaw, 1974). This indicates that the nature of the phospholipid polar head plays a role in the interaction with defensin A. It is also important to notice that our experiments show that defensin penetration may occur in the actual situation encountered in vivo. As a matter of fact, the upper limit of the molecular packing in a cellular membrane was estimated to ~ 1.3 nm^{-2}, a value found for egg lecithin in an aqueous dispersion (Cornell and Separovic, 1983) as well as in a bilayer vesicle (Huang and Mason, 1978).

Formation of defensin A–lipid complexes

The formation of defensin-lipid complexes is attested to by the analysis of the mixed monolayer parameters and by the results of the CD experiments.

The pressure-composition diagrams of the mixed defensin A/lipid monolayers clearly indicate that the two film components are not ideally miscible and form a cluster, the stoichiometry of which is 1:4, in both lecithin and PS. This is in agreement with the results of the CD experiments, which show the existence of an isodichroic point in the spectra recorded as a function of the defensin-to-lecithin ratio. This isodichroic point is indicative of an equilibrium between two forms of defensin: one free and the other complexed with lecithin. Increased helicity in the presence of lipids has already been suggested to explain the ion-channel activity of cecropins, another class of insect defense peptide (Merrifield et al., 1982).

From the application of the phase rule, it appears that neither the defensin-lecithin nor the defensin-PS complexes are miscible with the lipid phase. This feature is corroborated by the positive deviations from ideality observed in the area-composition diagrams. From a thermodynamic point of view, positive deviations indicate that mutual interactions between the two film components are weaker than the interactions between the pure component molecules themselves, inducing some kind of microheterogeneity in the mixed film. This agrees well with previous results demonstrating the high tendency of defensin A to self-associate when its concentration in solution increases (Maget-Dana and Ptak, 1996).

To explain the contraction of the mixed defensin/lecithin monolayers in the $X_{Def} \geq 0.4$ range at pressures up to 15 mN · m^{-1}, we first analyzed the composition of the mixed film after it was compressed. To this end, we used the approach developed by Tavena and Keough (1994). We

evaluated the change in the initial composition of the mixed monolayer corresponding to $X_{Def} = 0.5$ at 25 mN · m^{-1}. By the "method of the tangents" we can calculate the partial molecular areas, $(dA/dX)_{T,\pi}$, of lecithin ($\bar{A}_L = 0.95$ nm^2) and defensin ($\bar{A}_{Def} = 1.52$ nm^2). The apparent area per lecithin molecule, $A'_L = 2.46$ nm^2, is obtained by dividing the total trough area by the number of spread lecithin molecules only. The difference between the apparent and the partial molecular areas of lecithin, $\Delta A_L = \bar{A}'_L - \bar{A}_L = 1.51$ nm^2, corresponds to the area occupied by defensin molecules. For one molecule of lecithin in the film, there is $\Delta A_L/\bar{A}_{Def} \approx$ one molecule of defensin. Then, at 25 mN · m^{-1}, the monolayer has the same composition as the spread monolayer. If the contraction of the mixed monolayer up to $X_{Def} = 0.4$ is due to a desorption phenomenon, we can conclude that the desorbed molecules have the same molar fraction as in the initial film. We can estimate the amount of squeezed-out molecules by considering that if the desorption did not occur, the mean molecular areas would follow the additive rule, as in the 0–0.4 X_{Def} range: for $X_{Def} = 0.5$, the mean molecular area is 1.25 nm^2 instead of 1.35 nm^2 (corresponding point on the additivity line). The proportion of squeezed-out molecules is then (1.35–1.25)/1.35 ≈ 8%. This amount reaches ~20% in mixed defensin/lecithin monolayers rich in defensin ($X_{Def} \geq 0.67$). It is also possible to explain this sudden contraction of the mixed defensin/lecithin monolayer by an organization or an orientation change of the film components. Such an orientation change probably occurs in mixed defensin/PS monolayers rich in defensin ($X_{Def} > 0.4$), where the partial molecular area of PS is the same as the molecular area of defensin A. In other words, in the presence of a defensin excess, 1) the defensin-lipid complexes present the same orientation as the defensin molecules and 2) in the complex the PS molecules are intimately bound to defensin and occupy the same surface as the defensin molecule.

CONCLUSION

This study clearly shows that defensin A is able to interact with naturally occurring phospholipids and to penetrate into lipid membranes. We have also demonstrated the formation of defensin-lipid complexes with a defined 1:4 stoichiometry. The microheterogeneity induced in the mixed defensin/lipid films may correspond to the formation of oligomeric structures responsible for the increased permeability of bacteria membranes upon defensin attack. Modeling of the defensin-lipid interactions is now in progress in our laboratory, which will allow us to precisely determine the arrangement of defensin A-lipid complexes.

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REFERENCES

Boman, H., I. Faye, G. Gudmundsson, J.-Y. Lee, and D.-A. Lidholm. 1991. Cell-free immunity in Cecropia. A model system for antibacterial protein. *Eur. J. Biochem.* 201:23–31.

Buldt, G., and J. Seelig. 1980. Conformation of phosphatidylethanolamine in the gel phase as seen by neutron diffraction. *Biochemistry.* 19: 6170–6175.

Conciancich, S., A. Ghazi, C. Hetru, J. A. Hoffman, and L. Letellier. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus. J. Biol. Chem.* 268: 19239–19245.

Cornell, B. A., and F. Separovic. 1983. Membrane thickness and acyl chain length. *Biochim. Biophys. Acta.* 733:189–193.

Cornet, B., J. M. Bonmatin, Ch. Hetru, J. A. Hoffmann, M. Ptak, and F. Vovelle. 1995. Refined three-dimensional solution structure of insect defensin A. *Structure.* 3:435–448.

Crisp, D. J. 1949. A two dimensional phase rule. I. Derivation of a two dimensional phase rule for plane interface. II. Some applications of a two dimensional phase rule for a single surface. *In* Surface Chemistry. Butterworths, London. 17–22, 23–35.

Gaines, G. L. 1966. Mixed monolayers. *In* Insoluble Monolayers at Liquid-Gas Interfaces. Prigogine, editor. Interscience, New York. 281–300.

Hoffmann, J., and Ch. Hetru. 1992. Insect defensins: inducible antibacterial peptides. *Immunol. Today.* 13:411–415.

Huang, C., and J. T. Mason. 1978. Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc. Natl. Acad. Sci. USA.* 75:308–310.

Kremer, J. M., M. W. Esker, C. Pathmamaoharan, and P. H. Wiersema. 1977. Vesicles of variable diameter prepared by a modified injection method. *Biochemistry.* 16:3932–3936.

Lambert, J., E. Keppi, J. L. Dimarq, C. Wicker, J. M. Reichart, B. Dunbar, P. Lepage, C. Van Dorsselaer, J. Hoffman, J. Forthergill, and D. Hoffmann. 1989. Insect immunity: isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. *Proc. Natl. Acad. Sci. USA.* 86:262–266.

Maget-Dana, R., J. M. Bonmatin, Ch. Hetru, M. Ptak, and J. M. Maurizot. 1995. The secondary structure of the insect defensin A depends on its environment. A circular dichroism study. *Biochimie.* 77:240–244.

Maget-Dana, R., Ch. Hetru, and M. Ptak. 1996. Surface activity of the insect defensin A and its interactions with lipids in mixed monolayers. *Thin Solid Films.* 284/285:841–844.

Maget-Dana, R., and M. Ptak. 1995. Interactions of surfactin with membrane models. *Biophys. J.* 68:1937–1943.

Maget-Dana, R., and M. Ptak. 1996. Behavior of insect defensin A at the air/water interface. *Colloids Surfaces B Biointerfaces.* 7:135–143.

Maloy, W. L., and U. P. Kari. 1995. Structure-activity studies on magainins and other host defense peptides. *Biopolymers.* 37:105–122.

Merrifield, R. B., L. D. Vizioli, and H. G. Boman. 1982. Synthesis of the antibacterial peptide cecropin A (1–33). *Biochemistry.* 21:5020–5031.

Seelig, J. 1990. Interaction of phospholipids with Ca^{2+} ions. On the role of the phospholipid head groups. *Cell Biol. Int. Rep.* 14:353–360.

Shaw, N. 1974. Lipid composition as a guide to the classification of bacteria. *Adv. Appl. Microbiol.* 17:63–108.

Tavena, S., and K. M. W. Keough. 1994. Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air-water interface. I. Monolayers of pulmonary surfactant protein SP-B and phospholipids. *Biophys. J.* 66:1137–1148.

Traüble, H., M. Teubner, P. Wooley, and H. Eibl. 1976. Electrostatic interactions at charged lipid membranes. I. Effects of pH and univalent cations on membrane structure. *Biophys. Chem.* 4:319–342.

Zhong, L., and W. C. Johson. 1992. Environment affects amino acid preference for secondary structure. *Proc. Natl. Acad. Sci. USA.* 89: 4462–4465.