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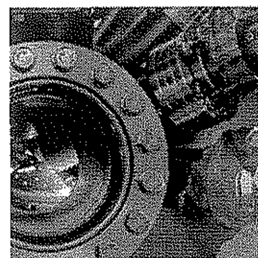

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INTERACTION OF THE N-TERMINAL DOMAIN OF γ-ZEIN $(VHLPPP)_8$ WITH PHOSPHATIDYLCHOLINE LIPOSOMES. TOWARD A BETTER UNDERSTANDING OF THE BIOLOGICAL ROLE OF THIS DOMAIN

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INTRODUCTION

γ-Zein is a major constituent of maize endosperm and is found lining the membrane of endoplasmic reticule (ER) derived protein bodies[1]. The N-terminal domain of this protein that contains a highly repetitive sequence $(VHLPPP)_8$ is necessary for sorting γ-zein into the ER and this sequence suggests the formation of a long helix that may be related to the peripheral location of γ-zein into the protein body. In a previous work we demonstrated that the peptide $(VHLPPP)_8$, which adopts a polyproline II conformation (Figure 1a), has amphipatic properties and self-assembles in aqueous solution giving cylindrical micelles[2] (Figures 1b and 1c). In order to contribute to the better understanding of the peptide interaction with vegetal biological membranes we studied the interaction of $(VHLPPP)_8$ with phosphatidylcholine sorbitan liposomes (which were used as a membrane model). The results presented here (transmission electronic microscopy, light dynamic scattering and the immunological detection of the peptide) put in evidence the interaction between the peptide and the phospholipidic membrane.

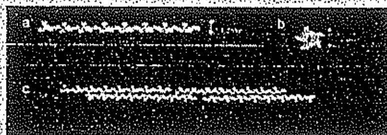

Figure 1. a) The peptide $(VHLPPP)_8$ in aqueous solution adopts an amphipathic helical PPII conformation. The charged histidine side-chains (blue) being hydrophilic and the valine and leucine (yellow) alkyl side-chains being strongly hydrophobic. b) Schematic representation of a micelle formed by the molecules of $(VHLPPP)_8$. This micelle has a diameter of 3 nm (frontal view). c) Diagram summarizing the diameter and longitudinal growth of a cross sectioned micelle. Notice the lateral interaction between the hydrophobic residues in yellow (diameter growth). Longitudinal growth occurs by superposition of coherently stacked molecules.

RESULTS

We studied the interaction of $(VHLPPP)_8$ with liposomes of soybean's phosphatidylcholine. A peptide $(VHLPPP)_8$ solution (0.033 mM) was incubated with extruded phosphatidylcholine (0.5 mM) vesicles (190 nm diameter). The interaction between the peptide and the phospholipidic bilayers was studied by Dynamic light scattering, immunologically and by Transmission Electron microscopy. All determinations were carried out after 24 h of incubation at room temperature.

☆ DYNAMIC LIGHT SCATTERING EXPERIMENTS

Dynamic Light scattering is a non-destructive technique that can provide information on size and shape in solution conditions. The measurements carried out showed a slight increase in the size of the liposomes incubated with the peptide respect to the control (195 nm vs. 190 nm, respectively).

☆ SEPARATION AND IMMUNODETECTION OF THE COMPLEX PHOSPHOLIPID-PEPTIDE

In order to separate the free peptide (non-bonded to the liposome) from the complex peptide-phospholipid, the suspension was ultracentrifuged at 30000 r.p.m for 2 h at 7 °C. The pellet was resuspended in water and recentrifuged. The detection of the peptide at the pellet was carried out through a dot blot. The presence of the phospholipids at the pellet was detected chemically (Marshal's reagent).

☆ TRASMISSION ELECTRONIC MICROSCOPY (TEM)

Freeze fractured technique have now proved to be the best method to prevent drying artifacts in the study of liposomes[3]. Platinum-carbon replicas of liposome samples (0.5 mM phosphatidylcholine in water), which were freeze-fixed and freeze-fractured, were observed by transmission electron microscopy (TEM). Micrographs 2a and 2b show sharp steps (red arrows) in the membrane of freeze-fractured liposomes, which had been incubated with a peptide solution of $(VHLPPP)_8$ 0.033 mM. In contrast the liposomes control do not present alterations (figure 2c).

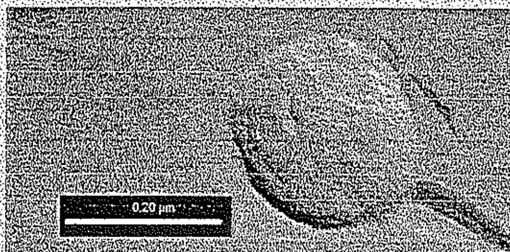

Figure 2 (a, b, c): TEM images of replicas of freeze-fixed and freeze-fractured of phosphatidylcholine (0.5 mM in water) liposomes: a,b) Liposomes incubated with an aqueous solution of $(VHLPPP)_8$ 0.033 mM, and c) Control

DISCUSION

- The peptide $(VHLPPP)_8$, which adopts an amphipathic conformation in aqueous solution, interacts with phosphatidylcholine liposomes.
- The formation of a lipo-peptide complex is evidenced by immunological detection of the peptide at the phospholipidic pellet (obtained after ultracentrifugation).
- The slight increase in the size of the liposomes incubated with the peptide respect to the control can be attributed to an interaction of the peptide with the bilayer.
- The presence of steps (observed in TEM micrographs) in the membrane of the freeze-fractured liposomes can be interpreted with the model depicted in the scheme. First, molecules of the amphipathic peptide intercalates in the external face of the liposome's bilayers. After that, the peptide self assembles giving extended domains (blue areas in the scheme). The presence of such domains possibly induce different fracture planes represented in the scheme.

Scheme of the complex peptide $(VHLPPP)_8$-phosphatidylcholine bilayer and possible fracture planes observed by TEM (the amphipathic molecules of the peptide are represented by cylinders in which the yellow part is hydrophobic and the blue is hydrophilic. The hydrophilic heads of the phospholipids are represented in green and in black the hydrophobic bodies. In purple continued lines are represented the fractures produced at the contact areas between the phospholipids and the peptide, and in red dotted lines are represented the fractures produced at the bilayers control).

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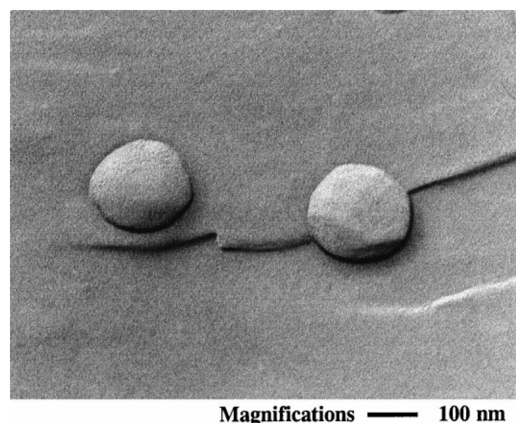

Fig. 1. Freeze-fracture electron micrograph corresponding to two PC vesicles. The magnification is given in the micrograph.

1965) and was shown to be pure by thin-layer chromatography (TLC). The nonionic surfactant *n*-octyl-β-D-glucopyranoside (OG) was purchased from Sigma Chemical Co. (St. Louis, MO). Tris(hydroximethyl)-aminomethane, TRIS buffer, (Merck) was prepared as 5.0 mM TRIS and 100 mM NaCl (pH 7.4).

PC liposomes of about 200 nm diameter were prepared by extrusion of large unilamellar vesicles previously obtained by reverse phase evaporation (de la Maza et al., 1998). The hydrodynamic diameter of the liposomes was determined by means of a dynamic light-scattering technique using a photon correlator spectrometer (Malvern Autosizer 4700c PS/MV) equipped with an Ar laser source (wavelength 488 nm). All the experiments were performed at 25°C, and at a reading angle of 90°. The data analysis was performed using the version of the program CONTIN provided by Malvern Instruments, England.

The morphological characterization of liposomes was performed using freeze-fracture electron microscopy. About 1 μl of liposomes was sandwiched between two copper platelets using a 400-mesh gold grid as spacer. The sample was then frozen in a propano-jet at −180°C and fractured at −150°C and 2×10^{-7} mbar in a Balzers BAF 300 freeze-fracturing apparatus (BAL-TEC, Liechtenstein). The replica was obtained by unidirectional shadowing with 2 nm of Pt/C and 20 nm of C, floated on distilled water and then examined in a Philips EM 301 electron microscope at 80 kV (Egelhaaf et al., 1996).

The amount of PC incorporated into the SC tissue was calculated as the difference between the PC concentration present in the incubation medium before and after incubation with the liposomes. These PC concentrations were determined using TLC coupled to an automated flame detection system (TLC–FID) (Ackmam et al., 1990).

The separation of hairless pig epidermis and the isolation of SC sheets were based on the method of Wertz and Downing (1983). The SC tissue was incubated with 3 ml of PC liposomes 5.0, 10.0 and 15.0 mM, at 25°C for 18 h and then it was removed from the liposome solutions and immediately treated with a solution of 20 mM OG. To this end, mixtures of SC (15 mg) and OG (2.5 ml) were sonicated at 25°C for 15 min in a bath sonicator (514 ECT, Selecta, Spain) and then incubated at the same temperature for 18 h in nitrogen atmosphere (Dencher and Heyn, 1982; Kragh-Hansen et al., 1993; López et al., 1997). The native SC, without previous incubation with liposomes, was subjected to the same treatment with OG in order to evaluate the protective effect caused by the PC liposomes on the structural alterations induced by the OG in the SC tissue.

Five samples were analyzed using high-resolution, low-temperature scanning electron microscopy: native SC, SC treated with OG and SC incubated with different concentrations of liposomes (5.0, 10.0 and 15.0 mM PC) and further treated with OG. Cylindrical pieces of each sample (diameter of about 2 mm and a length of about 1–2 mm) were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were cryoprotected with 30% (v/v) glycerol and mounted on a 3 mm aluminum holder. Thereafter, the SC samples were frozen by plunging them into liquid propane and fractured with a microtome knife in a Balzers BAF 300 freeze-etching device (Bal-Tec., Liechtenstein) at 10^{-7} mbar and a temperature of −110°C. After etching for 2 min, the fracture plane was coated with 2 nm Pt/C (unidirectional at an angle of 45°) followed by 5–7 nm C at an angle of 90°. Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in lents field emission scanning electron microscope equipped with a highly sensitive annular YAG-detector for back scattered electrons. The specimens were investigated at −110°C. The beam current was $1–3 \times 10^{-11}$ A as measured with a Faraday cage. The primary accelerating voltage was 10 kV. Images were obtained with the back scattered electron signal and recorded digitally with a Gatan Digiscan 688 connected to an Apple Quadra 950 (Walther et al., 1995).

3. Results and discussion

PC liposomes showed a monomodal size distribution curve with a hydrodynamic diameter of 180 nm and a polydispersity index ranging from 0.10 to 0.13 by dynamic light scattering. Fig. 1 shows a freeze-fracture electron micrograph of two PC vesicles that exhibit the typical rounded shape and a diameter of about 190 nm.

The use of high-resolution, low-temperature scanning electron microscopy technique was suitable for visualizing native and treated SC. The main advantage of this technique is that the replica-cleaning process is not necessary and, consequently, the risk of replicas breaking into small pieces is avoided. In order to prevent structural changes during the freezing process, the samples were prefixed with glutaraldehyde and cryoprotected with glycerol prior to cryofixation in propane (Section 2). To check if this prefixation process caused artifacts in the final visualization of the samples,

dehydrated, control samples delipidized SC were incubated in water alone to evaluate the possible changes induced by rehydration during incubation with the liposome suspension.

2.2. High-resolution low-temperature scanning electron microscopy studies

Three different samples were analyzed: native SC, delipidized SC and SC treated with liposomes after delipidization. Cylindrical pieces of each sample (diameter of about 2 mm and a length of about 1–2 mm) were fixed with 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were cryoprotected with 30% (v/v) glycerol and mounted on a 3 mm aluminium holder. Thereafter, the SC samples were frozen by plunging into liquid propane and fractured with a microtome knife in a Balzers BAF 300 freeze-etching device (Bal-Tec., Liechtenstein) at 10^{-7} mbar and a temperature of −110°C. After etching for 2 min, the fracture plane was coated with 2 nm Pt/C (unidirectional at an angle of 45°) followed by 5–7 nm C at an angle of 90°. Afterwards, the cold samples were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in lens field emission scanning electron microscope equipped with a highly sensitive annular YAG-detector for back scattered electrons [21]. Specimens were investigated at −110°C. The beam current was $1–3 \times 10^{-11}$ A as measured with a Faraday cage. The primary accelerating voltage was 10 kV. Images were obtained with the back scattered electron signal and recorded digitally with a Gatan Digiscan 688 connected to an Apple Quadra 950.

In order to study in detail the pictures obtained by HRLTSEM, the corresponding image analyses was performed. One-dimensional optical density profiles corresponding to the intensity variations vs distance in the images (nm) were processed in six different areas. In addition, to obtain statistical information the Fourier-transform of these selected areas were computed and the array sizes corresponding to the intensity peaks distance were determined.

Table 1
Lipid composition (wt%) of the material extracted from stratum corneum forming liposomes

Lipids	%wt
Ceramides	36
Cholesterol	27
Free fatty acids	24
Cholesteryl esters	4
Triglycerides	3
Cholesteryl sulfate	5

3. Results and discussion

The lipids extracted from the SC were able to form liposomes (lipid composition is shown in Table 1). The dynamic light scattering technique showed for these vesicles a bimodal distribution curve, in which the size varied in a range between 80 and 150 nm. FFEM was a useful technique to visualize these bilayers, in agreement with previous studies [4,24]. Fig. 1 shows some of these vesicles with sizes around 100 nm of diameter in accordance with the dynamic light scattering measurements.

The use of HRLTSEM was suitable to visualize SC samples according to a method previously described [21]. This improved SEM technique showed similar resolution than the conventional freeze-fracture TEM [25] and has one additional advantage: The replica-cleaning process is not

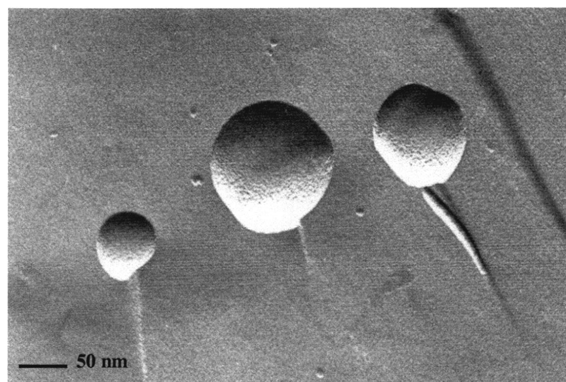

Fig. 1. Freeze-fracture electron micrograph corresponding to SC lipids liposomes. The magnifications are given in the micrograph.