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Interactions of Primary Amphipathic Vector Peptides with Membranes. Conformational Consequences and Influence on Cellular Localization

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Abstract. The conformations of two peptides produced by the combinations of a nuclear localization sequence and a sequence issued from the fusion protein gp41 of HIV 1 have been analyzed both in solution and in membranes or in membrane mimicking environments. Both are shown to be nonordered in water, α-helical when incorporated into SDS micelles where the helical domain concerns the hydrophobic part of the peptides. Interactions with lipids induce the formation of β-sheet and the lipid-peptide interactions are governed by the nature of the lipid polar headgroups. A monolayer study shows that replacement of the sequence separating the two sequences with an arginine favors the lipid-peptide interactions which may contribute to the understanding of the different, nuclear and membrane associated, cellular localizations of the peptides.

Key words: Amphipathic peptides — Conformations — Lipid-peptide interactions

Introduction

To facilitate the cellular internalization of informational molecules such as drugs or nucleic acids we have designed and synthesized a series of peptides containing both a hydrophobic and a hydrophilic sequence. Their role is respectively to facilitate the membrane anchoring and intracellular addressing. One of these was designed by linking a hydrophobic sequence derived from the fusion sequence of gp41 of HIV 1 (Gallaher, 1987; Slepushkin et al., 1992) to the hydrophilic fragment derived from the nuclear localization sequence (NLS) of SV40 large antigen T (Kalderon et al., 1984; Goldfarb et al., 1986). The sequence of gp41 was selected on the basis of the properties already described for peptides of the same family and which were shown to form aggregates and to interact with lipids (Rafalski, Lear & Degrado, 1990; Slepushkin et al., 1992). We have recently shown that this peptide can be internalized by fibroblasts in less than 3 minutes. In addition, this peptide can form complexes with nucleic acids (Morris et al., 1997; Vidal et al., 1997*a*); these complexes can enter cells and the presence of the peptide does not prevent the expression of DNA or mRNA. With the aim of understanding the mechanism leading to the internalization process and of identifying the role of each sequence we have synthesized two analogues with the following sequences: Ac-Gly-Ala-Leu-Phe-Leu-Gly-Trp-Leu-Gly-Ala^{10}-Ala-Gly-Ser-Thr-Met-Gly-Ala-Arg-Lys-Lys^{20}-Lys-Arg-Lys-Val-Cym and Ac-Gly-Ala-Leu-Phe-Leu-Gly-Trp-Leu-Gly-Ala^{10}-Ala-Gly-Ser-Thr-Met-Gly-Ala-Trp-Ser-Gln^{20}-Pro-Lys-Lys-Lys-Arg-Lys-Val-Cym where Ac is an acetyl group and Cym a cysteamide group (-NH-CH_2-CH_2-SH) which allows post synthesis modifications of the peptides (Méry, Brugidou & Derancourt, 1992; Méry et al., 1993). In these peptides which are designated hereafter as [1] and [2], respectively, the 17 N-terminal residues correspond to the gp41 sequence while the 6 C-terminal residues arise from the NLS sequence and the main difference between [1] and [2] lies in the presence

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Abbreviations: NLS, nuclear localization sequence; Cym, cysteamide; AEDI, aminiethyldithio 2-isobutyric acid; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DOPG, dioleoylphosphatidylglycerol; TFE, trifluoroethanol; FTIR, Fourier transform infrared; DQF-COSY, double quantum filtered correlation spectroscopy; ROESY, 2D nuclear Overhauser enhancement spectroscopy in the rotating frame; 1D, one dimensional; SUV, small unilamellar vesicle.

of a structural breaking Trp-Ser-Gln-Pro sequence in [2]. In addition we introduced a Phe → Trp substitution in position 7 in order to provide an intrinsic fluorescent probe. After linking the peptides to a fluorescent probe, namely lucifer yellow, through their cysteamide moiety and incubation with fibroblast cells we observed that the fluorophore localized to various cellular domains in a sequence-dependent manner. Peptide [1] induced a membrane-associated localization (Vidal et al., 1996) of the probe while peptide [2] localized the probe mainly to the nucleus.

To understand the mechanism(s) leading to the various cellular localizations and determine the origin of these differences, we undertook a conformational study of both peptides in different media focusing on these which are considered as mimicking membrane environments. Here, we describe the various conformational states we detected for the peptides depending on the nature of their environment and also show that their interactions with phospholipids may govern the final localization of the peptides.

Materials and Methods

Materials

Peptide [1] has the same origin as reported by Vidal et al. (1996). Peptide [2] was synthesized by the same procedure: solid phase peptide synthesis using the Fmoc strategy with AEDI-Expansin resin on a 9050 Pepsynthetizer Milligen (Millipore, UK). Both peptides were purified by semi-preparative HPLC with a Nucleosil 300, C8, 5 μm column, 200 × 20, SFCC (Neuilly-Plaisance, France). They were identified by Electrospray mass spectrometry in the positive ion mode using a trio 2000 VG Biotech Mass Spectrometer (Altringham, UK) and amino acid analysis performed on a High Performance Analyzer (Model 7300, Beckman Instruments, Fullerton, CA).

Chemicals

DOPC, DOPS, DOPG and TFE were purchased from Sigma (St. Louis, MO), SDS, SDS-d_{25} and TFE-d_3 from Interchim Molecular Probe (Montluçon, France).

Preparation of Vesicles

Small Unilamellar Vesicles (SUV) were prepared from DOPG, DOPS or DOPC by sonication. Briefly, dry lipids were dissolved in $CHCl_3$: MeOH (3:1 v/v). The solvent was evaporated and desiccated under high vacuum for at least 3 hr to remove the residual solvent. Lipids (at a concentration of 5 mg/ml) were resuspended in the buffer by sonication in a water bath. The resulting lipid dispersion was sonicated in one heating-cooling cycle (~20 min at 80% pulse cycle at 30°C and 20 min in an ice/water bath) with a probe sonicator. All SUV preparations were centrifuged for 10 min at 6500 t/min and used on the day after their preparation.

Study at the Air-Water Interface

Surface tension measurements were made by the Wilhelmy method using a Prolabo (Paris) tensiometer recorded to a X - Y Kipp and Zonen (Delft, The Netherlands) recorder, model BD 91. All measurements were made after injection of aliquots of aqueous solutions of the peptides into the subphase (0.154 M NaCl). The subphase was then gently stirred with a magnetic stirrer and measurements were made when the surface tension remained constant with time.

Spectroscopic Measurements

CD measurements were carried out on a Jobin-Yvon Mark V dichrograph at a scan speed of 20 nm/min and using quartz cells with 0.1 or 1 mm path length according to the peptide concentration. All spectra presented in this study correspond to an averaging of five spectra and were corrected by the base line obtained for peptide free preparations.

UV spectra were recorded with a Cary 3 E (Varian) spectrophotometer and analyzed according to Lange et al. (1996).

Fluorescence spectra were recorded on a spectrofluorimeter Spexfluorolog Model 1681 (Jobin-Yvon). The excitation wavelength was 280 nm and emission spectra were recorded in the 300–420 nm range with a band pass of 4 nm.

FTIR spectra were obtained on a Bruker IFS 28 spectrometer equipped with a liquid nitrogen cooled MCT detector. The Bruker OPUS/IR2 program was used for spectrum analysis (second derivative). Spectra (1,000 scans) were recorded at a spectral resolution of 4 cm^{-1}. Samples were prepared from peptide containing vesicles at various lipid/peptide ratios which were deposited onto fluorine plates and the spectra were recorded after evaporation of the water.

NMR experiments were recorded on 400 or 600 MHz Bruker AMX spectrometers. Data were acquired at 280 and 305 K and TSP-d_4 was used as internal reference. All 2D experiments were performed according to the standard procedures (Wüthrich, 1986) using quadrature detection in both dimensions with spectral widths of 11.7 ppm in both dimensions. The carrier frequency was centered on the water signal and the solvent was suppressed by continuous low power irradiation during the relaxation delay and during the mixing time for NOESY spectra. The 2D spectra were obtained using 2048 or 4096 points for each t1 value and 512 t1 experiments were acquired for TOCSY, ROESY experiments. 800 t1 increments were used for DQF-COSY. TOCSY spectra were recorded with spin lock times of 30 and 60 msec. The mixing time was 200 and 300 msec in NOESY spectra and 300 msec in ROESY spectra. Prior to Fourier transform, the time domain data were multiplied by $\pi/8$ and $\pi/4$ phase-shifted sine bell functions for t2 and t1 domains respectively.

NMR studies were performed on 1 to 6 mM solutions of the peptides in H_2O/D_2O (90/10). For micelles bound peptides, 1 to 2 mM peptide solutions were used and mixed with 80 or 200 mM SDS-d_{25}. The pH was adjusted by HCl or NaOH to 3.6–3.7.

Chemical shifts assignments for the peptides were achieved by the well-known method developed by Wüthrich (1986) using TOCSY and DQF-COSY to identify spin systems and NOESY to connect the adjacent spin systems by sequential NOEs.

Results

Conformations of the Peptides in Various Media

The conformations of both peptides were examined in various media and although they adopt different confor-

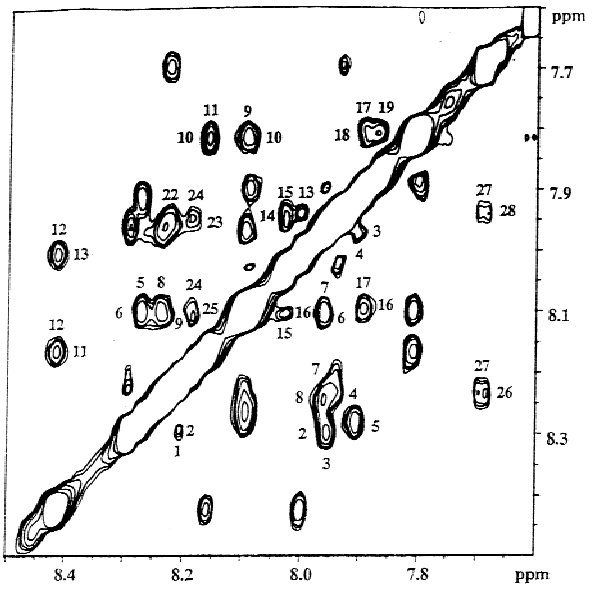

Fig. 1. N-N region of the NOESY spectrum of peptide [1] in the presence of micelles of SDS d_{25} at a SDS/peptide ratio (mole/mole) of 100.

mational states depending on their environment, they are not or nearly not distinguishable one from the other. Similar to the parent peptide (Vidal et al., 1997*b*), they are nonstructured in pure water while they are mainly α-helical in the presence of TFE or micelles of SDS as commonly found (Rozek, Buchko & Cushley, 1995). In the latter case the localization of the helical domain was achieved by NMR. Strong dNN NOEs (Fig. 1) are detected in the N-terminal part of the peptides and examination of the dαN(i, i + 3) and dαβ(i, i + 3) NOEs (Fig. 2) shows that the helical part is restricted to the hydrophobic domain of the peptides. The NLS sequence remains disordered resembling thus other primary amphipathic peptides (Chaloin et al., 1997). It must be noted that the extension of the α-helix is not identical for both peptides and the introduction of the Trp-Ser-Gln-Pro sequence, although lengthening the peptide, generates a shortening of the α-helical domain (from residue 1 to residue 11 which has to be compared with 1 to 15) indicating that this linking sequence acts as structural breaker.

In the presence of acidic phospholipids (DOPS or DOPG) the situation differs from that described just above and indicates that in these media the peptides rather adopt a β-sheet structure. This conformational state was characterized by CD and FTIR observations. In the presence of vesicles of DOPS the CD spectrum revealed a negative band centered at 218 nm (Fig. 3) identifying the presence of β-sheet. The presence of a lipid induced β-like structure was confirmed by infrared observations which revealed a major Amide I band contribution at 1628 cm^{-1} (Dong, Huang & Caughey, 1990) (*data not shown*).

Interactions with Phospholipids and Insertion into Membranes

Spectroscopy

To discriminate between superficial and embedded positioning of the peptide, we used the spectroscopic properties arising from the presence of Trp residues. For peptide [1] upon binding to the micelles the maximum of the Trp fluorescence emission shifts from 355 to 330 nm and this shift was accompanied by an enhancement of the fluorescence intensity (*data not shown*). This indicates that the Trp residue is embedded in the micelle core (Lakowicz, 1983) and therefore that the hydrophobic domain of the peptides is also embedded in the micelle core. This conclusion was enforced by an UV study based on the 4th derivative method (Lange et al., 1996) (Fig. 4) which confirmed that the polarity of the Trp environment is strongly reduced upon binding of the peptide to the micelles.

When both peptides were examined in the presence of lipids in the vesicular form, two different situations occurred according to the specific fluorescence emission spectra of the tryptophane residues. The first situation corresponded to that obtained with negatively charged phospholipids (DOPG and DOPS) and was very similar to that obtained with the micelles. When vesicles of one or the other of these two lipids were added to solutions of the peptides, the maximum of the Trp fluorescence emission shifted from 355 nm to 330 nm accompanied by a 3-fold enhancement of the fluorescence intensity (Fig. 5*A*). Therefore, similar to the micellar situation it can be concluded that, at least the hydrophobic domain of the peptides is embedded in the lipid bilayer.

The second situation was encountered when the headgroup of the lipid was zwitterionic. Indeed, when using DOPC, no significant shift of the maximum of the fluorescence spectrum could be detected and only a small increase of the fluorescence intensity occurred (Fig. 5*B*). This small effect indicates that the Trp residues remain in a polar environment, probably close to the lipid headgroups and therefore that the peptides do not insert the bilayer and remain at the lipid-water interface.

Air-Water Interface

Since the conformational study did not evidence any major structural difference between peptides [1] and [2] which could be responsible for their different cellular localizations, we attempted to identify a difference in their lipid binding properties using a monolayer approach where the peptides were allowed to insert the

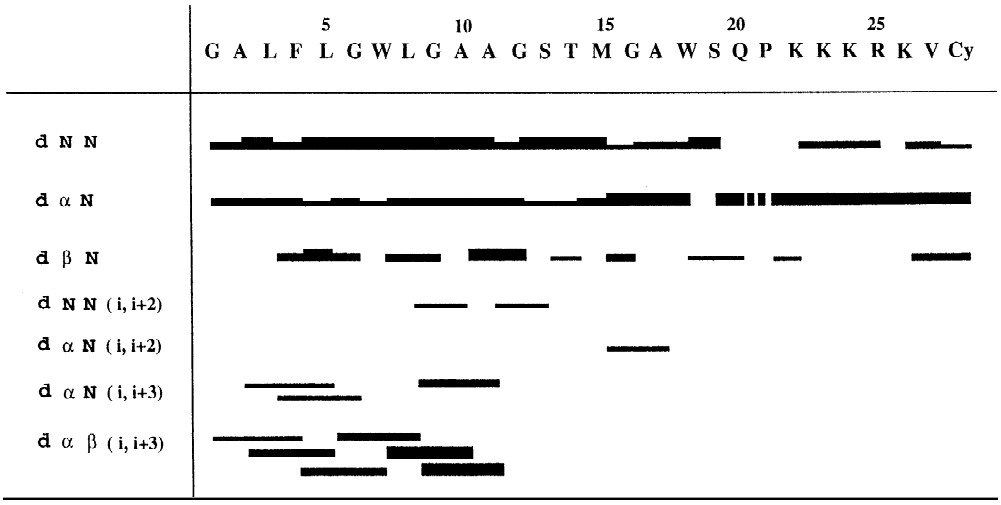

Fig. 2. Medium-range NOE contacts observed in the NOESY spectrum of peptide [2] in SDS d_{25} at a SDS/peptide ratio (mole/mole) of 100. The thickness of the horizontal bars is indicative of the NOEs relative intensities.

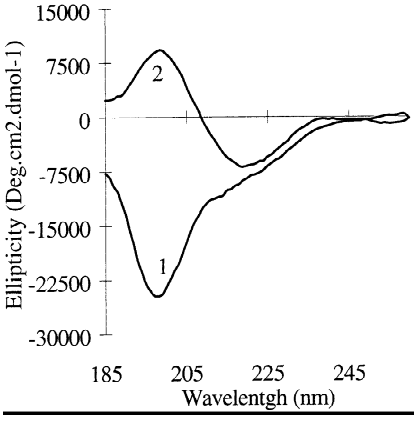

Fig. 3. Far UV CD spectra of peptide [2] in the absence (1) and in the presence of vesicles of DOPG (2) at a lipid/peptide ratios (in mole/mole) of 50 in water at 25°C. (peptide concentration 10^{-5} M, cell path 1 mm).

lipid monolayer. For this type of experiment it was essential to determine the peptide concentration required to obtain the maximum effect on the surface tension of a lipid-free air-water interface. Figure 6*A* shows the variations of the surface pressure of the air-water interface induced by both peptides when injected into the subphase. Clearly, both peptides decrease the surface tension of the air-water interface. However, an important difference between the two peptides was noticed since [2] generated a larger tension lowering than [1]. For the experiments dealing with penetration into lipid monolayers, on the basis of the experiments reported just above, we selected a lipid pressure close to that obtained for peptide [2] at equilibrium (21.5 mN/m) (Rafalski et al., 1990). This pressure has also the advantage of representing a state that is sufficiently condensed to interpret the pressure increase as arising from peptide-lipid interactions and not from coadsorption of the peptide. Figure 6*B* reveals that two distinct behaviors were observed depending on the nature of the phospholipid headgroups, in accordance with the fluorescence experiments described above. Indeed, with DOPC the relative variations of the surface tension at constant surface area are rather low (≈7 mN/m). Owing to the fluorescence experiments and to the relatively low variation of the surface tension it can be stated that these variations reflect electrostatic interactions occurring between the lipid headgroups and the charged residues of the NLS sequence. With DOPG the variations induced by both peptides are similar both in their amplitudes and in the required concentrations and the amplitudes of the pressure variations. The strong amplitude of the pressure variation (about 14 mN/m) indicates that, upon insertion into DOPG monolayers, beside the electrostatic interactions hydrophobic interactions also occur. All these observations are in line with the spectroscopic data and confirm that the peptides insert into DOPG monolayers leading to both hydrophobic and electrostatic interactions and that in the case of DOPC only the polar headgroups are engaged in the peptide-lipid interaction process. Interestingly, both peptides induce very similar variations of the surface pressure when in the presence of lipid. Owing to the data reported in Fig. 6*A*, and since the surface pressure is strongly enhanced for [1] compared to [2] it can be stated that the lipid-peptide interactions are more favored for peptide [1] than for peptide [2] (Verger & Pattus, 1982). This difference may be related to the membrane-associated localization of [1] and the intracellular one of [2]. It is tempting to attribute this difference to the replacement of the Trp-Ser-Gln-Pro sequence which separates the hydrophobic and the hydrophilic do-

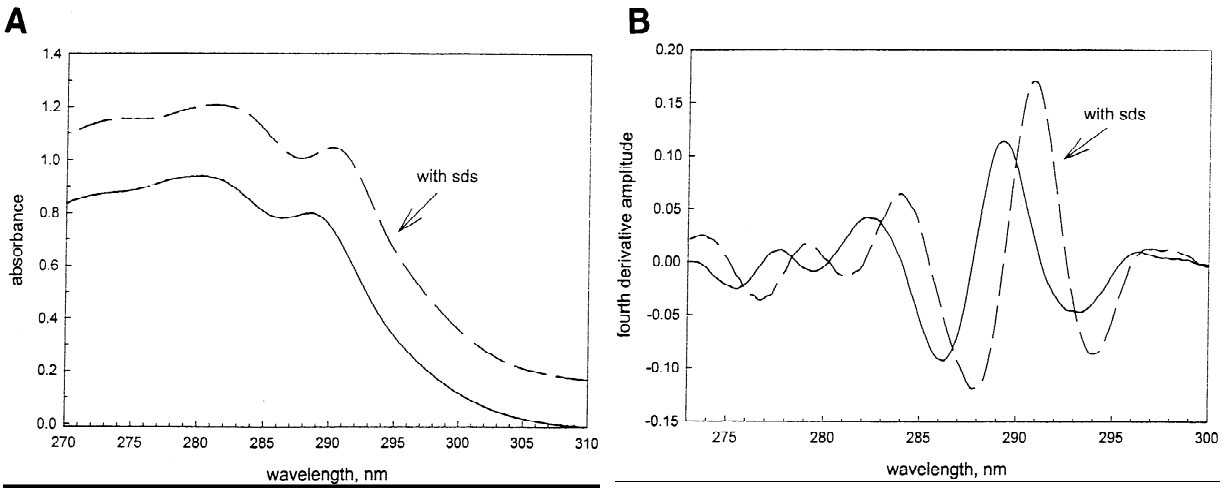

Fig. 4. (*A*) UV absorption spectra of peptide [1] in the ide chain absorption region; — in water; – – in the presence of micelles of SDS (shifted upwards for clarity reasons). (*B*) Fourth derivatives of the spectra shown in *A*.

mains by an arginine, since it was shown that the introduction of even a single arginine can block translocation (Whitley, Gafvelin & von Heijne, 1995).

Conclusion

The present work was mainly devoted to the understanding of the mechanism(s) leading to the different cellular localizations of two primary amphipathic peptides composed of a hydrophobic sequence issued from the fusion protein gp41 of HIV 1 associated with the hydrophilic NLS sequence of SV40, the two being separated or not by a structural breaker. When examined under identical experimental conditions, both peptides showed the same conformational properties. Indeed, both were mainly α-helical when in SDS micelles while in the presence of lipids they instead adopt a β-sheet structure. From the structure identified in SDS micelles it is likely that the structured domains concern the hydrophobic parts of the peptides while the hydrophilic ones remain disordered. On the basis of monolayer experiments we propose that the origin of the various cellular localizations lies in the difference in the affinities for the negatively charged lipids due to the replacement of the Trp-Ser-Gln-Pro sequence between the hydrophilic and the hydrophobic domains by an arginine, that generates peptide-phospholipid interactions which are energetically favorable. As to the particular role of the sequence issued from the fusion protein gp41 of HIV 1, the fact that it exists in a β-sheet form when in the presence of lipids and that it is membrane embedded or not according to the lipid headgroups questions its role in the viral fusion process. Nevertheless, it appears that the β-sheet form is the conformational state which very probably plays the major role in the membrane translocation process.

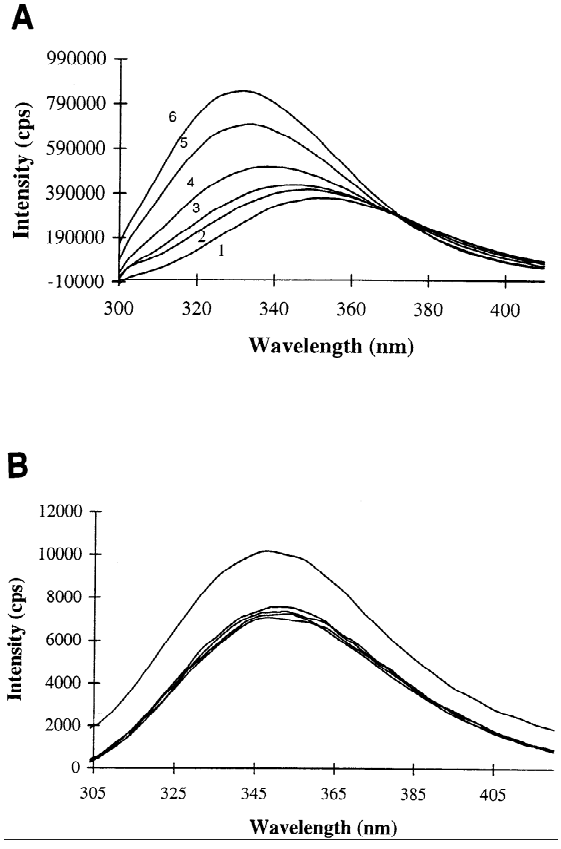

Fig. 5. (*A*) Tryptophane fluorescence spectra of peptide [1] obtained upon additions of vesicles of DOPS at different lipid/peptide ratios R (mole/mole) (25°C, peptide concentration 10^{-5} M). R = 0, 0.5, 1, 2, 4 and 8 correspond to spectra 1 to 6 respectively. (*B*) As for *A* but using DOPC vesicles. The spectrum at R = 8 was shifted upwards to show the very small modifications which occur upon binding of the peptide to DOPC.

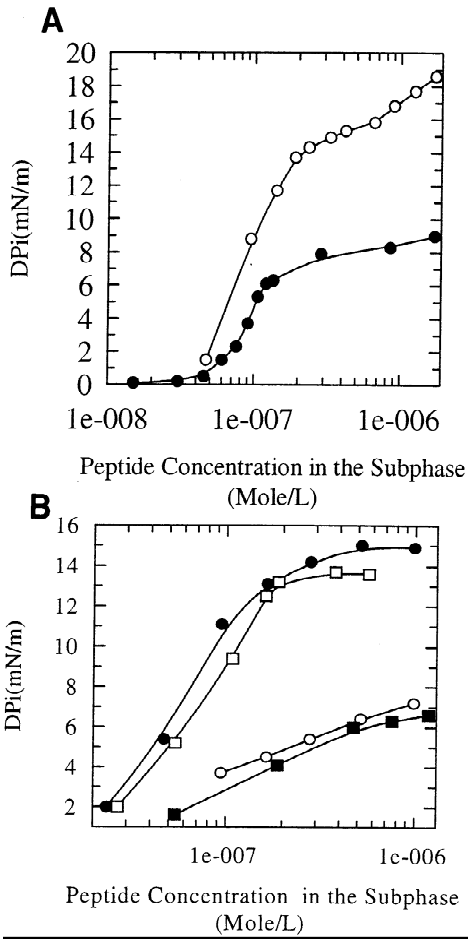

Fig. 6. Adsorption experiments at the air water interface. (*A*) Relative variations of the surface tensions as a function of the peptide concentration in the subphase in the absence of lipid. ● peptide [1], ○ peptide [2]. (*B*) As for *A* but in the presence of lipid at an initial pressure of 21 mN/m. ● peptide [1] with DOPG, □ peptide [2] with DOPG, ○ peptide [1] with DOPC, ■ peptide [2] with DOPC.

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