Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1668 (2005) 41–52
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

c-Fos and phosphatidylinositol-4,5-bisphosphate reciprocally reorganize in mixed monolayers

Graciela A. Borioli*, Beatriz L. Caputto, Bruno Maggio

CIQUIBIC-Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Pabellón Argentina-Ciudad Universitaria, 5000 Córdoba, Argentina

Received 10 August 2004; received in revised form 4 November 2004; accepted 9 November 2004
Available online 26 November 2004

Abstract

The transcription factor c-Fos has surface thermodynamic properties that allow it to differentially interact with phospholipids, especially PIP_2. It regulates phospholipid metabolism both in vivo and in vitro, and modulates degradation of phospholipid monolayers by phospholipases in a way that depends on the membrane intermolecular packing (i.e., surface lateral pressure). With the aim to understand details of the interactions of c-Fos at the membrane level, we studied the surface packing, dipole potential, compressibility and topography of mixed films of the protein with PIP_2. We show that c-Fos changes the packing of liquid-expanded PIP_2 monolayers, in a different manner with respect to its effect on the similarly liquid-expanded dilauroylphosphatidylcholine monolayers. The changes at the local molecular level are transduced to long-range inhomogeneities of the surface, detected by Brewster angle (BAM) and epifluorescence microscopy (EFM). Our results highlight the capacity of c-Fos to alter the packing and dipole potential of the lipid–protein interface. This involves variations of the surface in-plane elasticity and lateral segregation of phase domains. These dynamic, reversible alterations of surface organization provide a basis by which c-Fos may transduce molecular information at the membrane level.

Keywords: Lipid–protein interaction; PIP_2; Brewster angle microscopy; Epifluorescence microscopy; Surface topography

1. Introduction

c-Fos, the protein product of the immediate early homonymous gene c-*fos*, has been extensively characterized in its role as a transcription factor that is translocated very rapidly upon stimulus into the nucleus, regulating the expression of other genes. More recently, the participation of c-Fos in phospholipid synthesis and its association to the endoplasmic reticulum have been demonstrated [1,2]. This new function and cellular localization prompted us to study its surface activity. Thus, we reported that c-Fos is amphitropic, highly surface active, and capable of undergoing interfacial reversible reorganization depending on surface lateral pressure (π), which is a way to sense and transduce the state of the membrane. It also establishes favorable interactions with specific phospholipids, preferentially phosphatidylinositol-4,5-bisphosphate (PIP_2), phosphatidylserine and phosphatidylglycerol [3]. These results concerning the surface properties of c-Fos clearly place it in a membrane scenario and pose the study of possible effects of this protein in biointerfaces.

The capacity of c-Fos to participate in lipid-mediated membrane signaling processes, especially those linked to lipid metabolism, is illustrated by its fine modulation of phospholipases A_2, C and sphingomyelinase [4]. These enzymes participate in transduction events by generating second messengers like fatty acids, lysophospholipids, diacylglycerol (DAG) and ceramide, which couple signals

Abbreviations: π, surface lateral pressure; MARCKS, myristoylated alanine-rich C kinase substrate; GAP-43, growth-associated protein; CAP-23, cortical cytoskeleton-associated protein; PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; NBD-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); PLA_2, phospholipase A_2; PLC, phospholipase C; PC, dilauroylphosphatidylcholine; EFM, epifluorescence microscopy; BAM, Brewster angle microscopy

* Corresponding author. Tel.: +54 351 4334171; fax: +54 351 4334074.
E-mail address: gborioli@dqb.fcq.unc.edu.ar (G.A. Borioli).

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.11.007

originated at the membrane with metabolic pathways in the cell [5]. Regulation of these and other phospholipase activities result from interfacial changes in molecular organization, some of which can modify substrate availability through altered surface topography [6]. Several lines of evidence support that lipase activities are modulated, and in turn are affected, by lateral phase separation and variation of membrane topology [7–12]. This level of regulation of lipid degradation is especially relevant because lipid sequestration in domains appears to be an important mechanism for signal transduction [13]; thus, proteins that can induce lipid domain reorganization become an inherent part of the signaling mechanism. It has long been known that interaction of amphitropic proteins with lipid interfaces causes changes of lipid organization with formation of segregated clusters of altered phase state [14,15]. However, few proteins have been studied regarding the implication of those effects on membrane information transduction [11,16,17]. Among these proteins, the major PKC substrates MARCKS, GAP43 and CAP23 induce PIP_2 clustering in rafts [18], which constitute hotspots for both protein and lipid signaling pathways [19]. In this context, we hypothesized that c-Fos could directly modify interactions with PIP_2 to induce alterations of molecular organization at the membrane interface. To approach this possibility, we employed binary lipid–protein monolayers of c-Fos and PIP_2 at the air–buffer interface to directly determine interactions, compressibility, dipole potential and surface topography of the planar interface. The use of lipid–protein monolayers has been validated over the years by a large number of reasons, one of the most important being that it is the only method allowing to obtain measurements in layers of oriented molecules under precisely controlled intermolecular conditions [20]. The average surface pressure at which the monolayer behavior corresponds to that of molecules in a bilayer organization is about 30 mN/m [21] but, due both to the thermal energy available and to the surface compressibility, the lateral pressure can fluctuate by more than ±15 mN/m [22]. We covered this full range of pressures in our study. Thus, the monolayer approach is unique for investigating molecular interactions in biomembranes [20,23], especially when combined with techniques that enable the optical inspection of the surface film, allowing the study of both short- and long-range changes over the cross-sectional areas and lateral surface pressures known to occur in biomembranes, while avoiding spontaneous relaxation to topological rearrangements due to curvature tensions.

2. Materials and methods

Binary mixtures with different mol fractions of protein were made with recombinant N-terminal His-tagged c-Fos, purified by affinity chromatography as previously described [4], and dilauroylphosphatidylcholine (PC) or porcine brain PIP_2 from Avanti Polar Lipids Inc., Alabaster, AL.

Langmuir monolayers of the binary mixtures or of the pure components were spread as previously described [24,25]. Premixed solutions of lipids and c-Fos were usually in chloroform–methanol–H_2O 2:1:0.3 (for some of the solutions with the highest proportion of protein the amount of methanol was increased up to 1:1, with respect to chloroform, in order to achieve a single mixed solvent phase). The surface behavior of c-Fos is the same whether spread from aqueous or solvent solution [3]. The apparent molecular mass taken for c-Fos was 57 kDa. The ideal mean molecular area in mixed films was calculated on the basis of the mol fraction of c-Fos in the mixture, according to the mean areas of the pure lipid and protein at a given π [4]. Surface pressure and surface potential versus area isotherms were obtained at room temperature by spreading less than 50 μl of lipid–protein solution on the surface of 74 ml of 145 mM NaCl in a 80-cm^2 compartment of a specially designed circular Monofilmmeter teflon trough (Mayer Feintechnik, Germany). Similar interactions of c-Fos with PIP_2 and with PC were found using a buffer of 64 mM HEPES/140 mM NaCl/4.5 mM KCl/0.5 mM $MgCl_2$/5.6 mM glucose at pH=7 [3]. Monolayers for simultaneous observation by Brewster angle microscopy (BAM; mini-BAM, Nanofilm Technologies, Göttingen, Germany), and epifluorescence microscopy (EFM; Zeiss Axiovert, Carl Zeiss, Oberkochen, Germany) were spread from the same premixed solutions with 2% egg NBD-PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[7-nitro-2-1,3-benzoxadiazol-4-yl], Avanti Polar Lipids) over the surface of a rectangular 332-cm^2 teflon trough (KSV minitrough, KSV Helsinki, Finland) with 150 ml of 145 mM NaCl; NBD-PE is a fluorescent probe that preferentially partitions into surface phases having a liquid-expanded character [26,27]. BAM experiments and determination of surface reflectance were performed as described previously [26]. Surface pressure was measured by a platinized-Pt sensing plate connected to a surface pressure transducer. Surface potential was measured by a high impedance millivoltmeter (Corning ionalyzer 250) connected to a surface ionizing ^{241}Am electrode positioned 5 mm above the monolayer surface, and to a reference calomel electrode connected to the aqueous subphase through a saline bridge. Absence of surface-active impurities in the subphase and in the spreading solvents was routinely controlled as described previously [25]. At least duplicate monolayer isotherms were obtained and averaged at a compression rate of 0.45–0.60 nm^2 $molecule^{-1}$ min^{-1}; reducing the compression speed produced no change in the isotherms. Reproducibility was within maximum S.E. of ±1 mN/m for surface pressure, ±30 mV for surface potential, and ±0.04 nm^2 for molecular areas. The monolayers of the pure components and of all mixed films were stable and reproducible, with hysteresis, after several compression–expansion cycles. Less than 10% of molecules were lost from the interface during those cycles as indicated by negligible variations in the limiting mean molecular area and dipole potential at the collapse

pressure. Deviations of mean molecular area and surface potential per unit of molecular surface density from the additive behavior [28] were ascertained as published elsewhere [29,30]. Monolayer compressibilities were obtained from the surface pressure–mean molecular area isotherms as $C_s=-(1/A)(dA/d\pi)$; the interfacial elastic modulus of area compressibility, reflecting variations of the film in-plane elasticity, was calculated as $K=C_s^{-1}=-A(d\pi/dA)$, where C_s is the compressibility and A the molecular area at each surface pressure point of the isotherm. It was shown by Li et al. [31] that experimentally obtained K values at surface pressures above 30 mN/m are highly reproducible and, together with variations of the dipole potential, can be reliably used to detect monolayer phase transitions and pre-collapse behavior. Monolayer reorganizations were located more precisely from second or fourth derivatives [29] of the compression isotherms and from inflexion points of the K versus molecular area curves. The excess free energy of mixing in the liquid expanded state was calculated as the difference between the area under the experimental and the ideal surface pressure–molecular area isotherms, integrated between 2 and 40 mN/m [30]. These conditions reduce errors derived from the rather variable gaseous region of the isotherms below surface pressures of 2 mN/m that are markedly dependent on technical artifacts during spreading. Thus, our comparisons include only the liquid-expanded and/or condensed state of coherent films, omitting the important contribution from the gaseous states.

3. Results and discussion

We have previously shown [3] that c-Fos reversibly changes its own intermolecular packing according to π. The conformation of a protein depends on its environment, and the interface is a very special one because its bidimensionality imposes energetic conditions not present in solution. Such restrictive conditions result in an ordered orientation of all molecules, including both lipids and proteins, at the interface. This is a concept that must be kept in mind when working with monolayers since such ordered organization means that the protein usually adopts a different conformation than that found in bulk, but not necessarily has to be considered denatured. Moreover, in most cases this conformation is not irreversible. As a consequence of the conformational change, some amphitropic proteins may lose or gain biological activity depending on the interfacial composition and/or organization, as clearly shown for several membrane-associated enzymes [4,6,8,16,18].

When a monolayer of c-Fos is compressed, a marked reorganization takes place between 8 and 18 mN/m as indicated by three independent surface parameters, namely the variations of the molecular area under compression, the surface compressibility, and the density of dipole potential perpendicular to the interface [3]. Such reorganization provides the basis to correlate possible effects of c-Fos with some processes occurring at the interface. Consequently, we explored the hypothesis that some key phospholipids intervening in a variety of membrane phenomena, such as PIP_2, could be affected in their packing through interactions with c-Fos, and in turn affect the protein surface behavior. In order to study comparatively the interactions with c-Fos, we selected two lipids, PIP_2 and PC, that have a similar liquid-expanded character, surface compressibility, collapse pressure, mean cross-sectional area and dipole potential at different surface pressures. Furthermore, c-Fos readily penetrates and becomes incorporated into films of both lipids up to cut-off pressures above 32 mN/m while exhibiting clearly marked preferences for penetration into PIP_2 at lower surface pressures [3].

Fig. 1A shows the surface pressure–mean molecular area compression isotherms of pure PIP_2, c-Fos, and of mixed lipid–protein films in different molar proportions; the

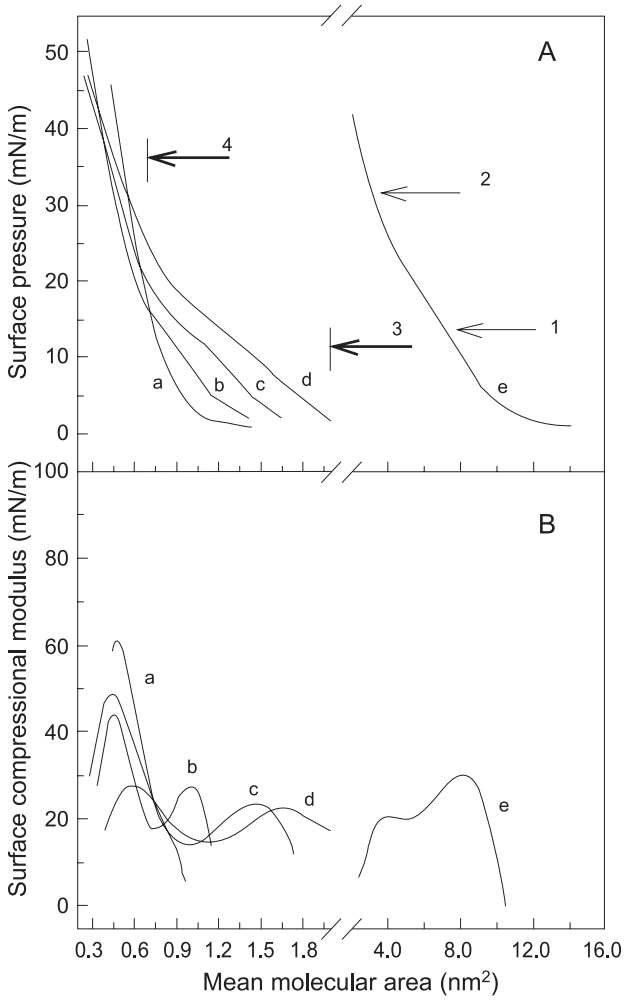

Fig. 1. Molecular packing of PIP_2/c-Fos films. Surface pressure (A) and surface compressional modulus (B) as a function of the mean molecular area are shown for films of pure PIP_2 (a), mixed films with a mol fraction of c-Fos of 0.03 (b), 0.06 (c), 0.09 (d) and pure c-Fos (e). In panel A, arrows indicate the surface pressure points (or ranges) at which pure c-Fos (arrows 1 and 2) or the mixed films (arrows 3 and 4) undergo molecular reorganization.

corresponding variation of the interfacial elastic modulus of area compressibility (K) with packing is shown in Fig. 1B. As reported before [3], pure c-Fos shows a marked reversible reorganization at molecular areas between 5.5–8.5 nm^2 over a range of ~10 mN/m, centered at a surface pressure and mean molecular area of ~13 mN/m and 7.4 nm^2, respectively (arrow 1, Fig. 1A). This is accompanied by a preceding change of the surface dipole potential per unit of molecular surface density ($\Delta V/n$, [27]), centered at 8.4 nm^2 and 9 mN/m (Fig. 2, arrow 1), indicating a reorientation of the overall protein resultant dipole moment in the direction perpendicular to the interface. At this point a progressive electrostatic depolarization of the interface initiates (arrow 1 in Fig. 2), which increases under reduction of the molecular area and marks the maximum reduction of the in-plane elasticity of the c-Fos monolayer (maximum of the curve e in Fig. 1B). The variation of the surface elasticity reveals still another, highly reproducible, reorganization of c-Fos that is difficult to detect in the surface pressure–area isotherm, centered at a mean molecular area of 3.3 nm^2 at ~32 mN/m (arrow 2 in Fig. 1A). This is not due to a decrease of film stability (see Materials and methods) but reflects pre-collapse changes of organization, as also described for pure lipid systems [31]. Fig. 2 shows that this additional high pressure-induced condensation of c-Fos is again accompanied by further enhancement of depolarization, as indicated by the surface potential per unit of molecular surface density, centered at 4 nm^2 and 26 mN/m, again in coincidence with a reduction of in-plane elasticity (see arrow 2 in Fig. 2). It should be noted that depolarization becomes already apparent at the mean molecular areas corresponding to the maximal change of

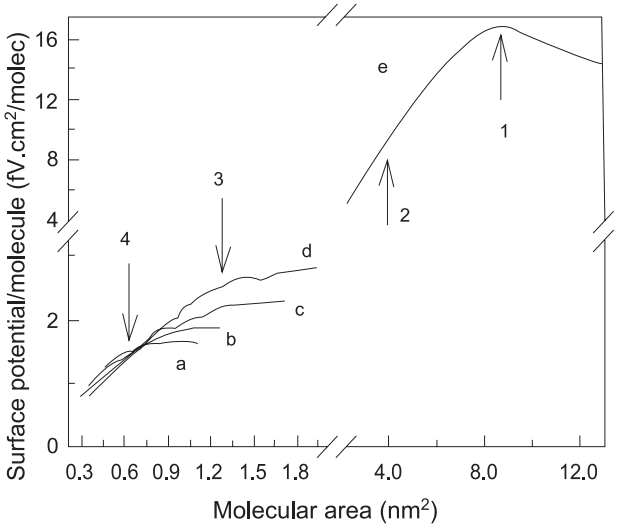

Fig. 2. Interfacial electrostatics of PIP_2/c-Fos films. Surface potential/molecule (surface potential per unit of molecular surface density) as a function of the mean molecular area is shown for films of pure PIP_2 (a), mixed films with a mol fraction of c-Fos of 0.03 (b), 0.06 (c), 0.09 (d) and pure c-Fos (e). Arrows indicate the mean molecular areas at which pure c-Fos (arrows 1 and 2) or the mixed films (arrows 3 and 4) undergo dipolar reorganization.

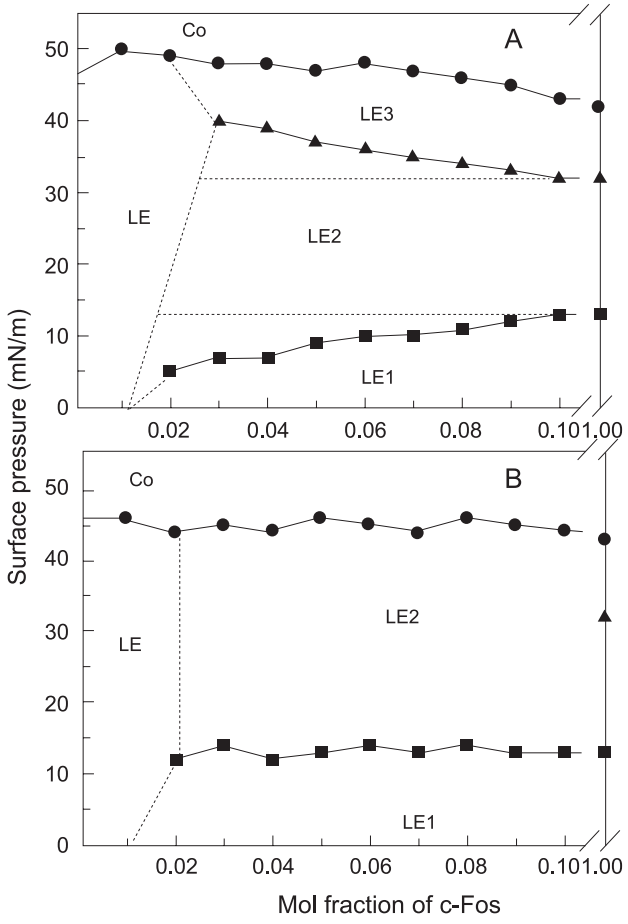

Fig. 3. Surface pressure-I and composition-induced phase transitions of mixed films. Two-dimensional partial phase diagram of mixtures of PIP_2 (A) or PC (B) with different proportions of c-Fos showing surface pressure-dependent presence of different liquid expanded (LE, LE_n with n=1–3) phases; Co represents the collapsed phase. The circles correspond to the collapse point, and the squares and triangles to the first and second molecular reorganization of c-Fos, respectively.

the in-plane elasticity, suggesting that the measurement of surface potential is more sensitive than surface pressure to detect interfacial reorganization. The compression of pure PIP_2 does not show noticeable two-dimensional transitions in the surface pressure–area isotherm, but the variation of K reveals a smooth change centered at ~25 mN/m and mean molecular area smaller than 0.61 nm^2, from a higher to a lower liquid expanded state of the film as the surface pressure is increased (curve a in Fig. 1B). Also, the surface electrostatics of PIP_2 reveals variation under compression, indicating only slight depolarization as the surface pressure is increased; the slope of $\Delta V/n$ shows that maximal changes occur at the mean molecular area (~0.7 nm^2) corresponding to the maximum variation of K (Figs. 1B and 2).

3.1. The mixed interface of c-Fos and PIP_2 evidences phase coexistence with condensation and depolarization

The behavior of mixed monolayers was studied in films containing a small proportion of c-Fos not exceeding 0.1

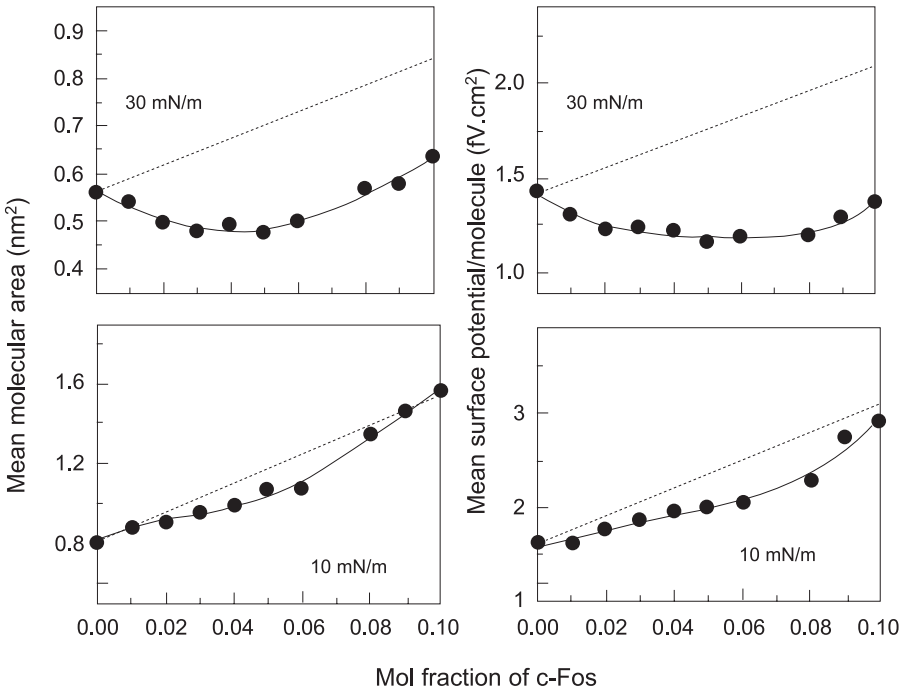

Fig. 4. Molecular interactions in mixed films. Mean molecular area (left panels) and mean surface potential/molecule (right panels) as a function of composition for mixed films of PIP_2 and c-Fos at the surface pressure indicated. Dashed lines indicate the values for ideally mixed monolayers; continuous lines indicate the best polynomial fitting to the experimental points (●).

mol fraction. The compression isotherms become gradually expanded and tend to resemble the protein isotherm as the mol fraction of c-Fos is increased in the mixed films. Also, the changes of surface elasticity and electrostatics under compression show features that become progressively similar to those of the pure protein at mol fractions of the latter above 0.03 (Figs. 1 and 2). However, the points of reorganization that for pure c-Fos are centered at ~13 mN/m and 32 mN/m are respectively displaced downwards (to 5–11 mN/m), or upwards (to 34–39 mN/m) in the mixtures, depending on the proportion of c-Fos (better illustrated in Fig. 3A). This indicates that the low- and high-surface pressure threshold points for inducing protein reorganization (arrows 3 and 4 in Fig. 1A) are respectively facilitated and impaired by PIP_2 since less and more amount of two-dimensional compression work is respectively required for the reorganization to take place.

The transition points between the different film states and their variation with surface pressure and film composition are clearly indicated in the partial two-dimensional surface pressure–composition phase diagram shown in Fig. 3A. From ~0.02 mol fraction of c-Fos, the phase diagram reveals surface pressure-dependent coexistence of protein-enriched and lipid-enriched phases. It should be noticed that, although the surface pressure–area isotherm of films containing below 0.02 mol fraction of c-Fos does not reveal inflexions, the variations of surface elasticity and $\Delta V/n$ with the mean area clearly show transition points already apparent in the mixture with 0.1 mol fraction of c-Fos (not shown). The minimum mol fraction of c-Fos at which the behavior of the film becomes dominated by features observed in the pure protein film is ~0.08. Above that proportion the low surface pressure transition point remains isobaric (within ±1 mN/m) with that of the pure protein film. Briefly, the surface pressure-dependent phase changes at a fixed composition are as follows: a film with 0.06 mol fraction of c-Fos at a surface pressure under 10 mN/m

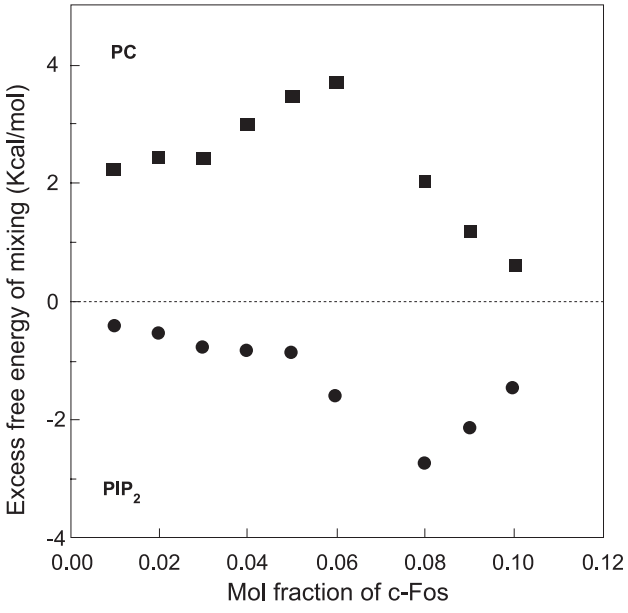

Fig. 5. Excess free energy of mixing. The compression excess free energy as a function of composition is given for mixed films of c-Fos with PIP_2 (●) or with PC (■).

constitutes a liquid expanded phase (LE1). Increasing the pressure above 10 mN/m causes the film to become biphasic, with a lipid-enriched LE phase containing between 0.01 and 0.02 mol fraction of protein coexisting with the LE1 phase progressively enriched in c-Fos up to the limiting composition of 0.08 mol fraction or more. Between the low and high surface pressure transition points, the 0.06 mol fraction mixture represents a biphasic film constituted by the protein-enriched LE2 phase containing above 0.08 mol fraction of c-Fos and the lipid-enriched LE phase containing up to ~0.03 mol fraction of protein. Above 36 mN/m, the LE3 phase coexists with the collapsed phase, and c-Fos undergoes its second interfacial reorganization (see Figs. 1 and 2). Again, the second pressure threshold point remains isobaric (within ±1 mN/m) at 32 mN/m in mixed films containing above 0.08 mol fraction of c-Fos. The maximum amount of c-Fos that can form a single LE phase in a mixed monolayer with PIP_2 varies between 0.01 and 0.03 mol fraction depending on the surface pressure. The collapse pressure of mixed films progressively decreases as the proportion of c-Fos increases, and is higher than that of the pure components, indicating mutual stabilization at the interface.

The lipid–protein monolayers are clearly non-ideal. Fig. 4 shows negative deviations of the mean molecular area as a function of composition with respect to the additive behavior expected for ideally mixed films. The interactions are characterized by area reduction, with the negative deviations increasing with the surface pressure, and by marked depolarization shown by reduction of $\Delta V/n$. The latter also takes place at surface pressures of 5 mN/m or below, emphasizing that dipolar interactions are detected at the interface despite this not being reflected by variations of mean molecular areas with film composition, which follow additive behavior in this range of pressures (not shown). The excess free energy of mixing shows that the interactions of c-Fos with PIP_2 are thermodynamically favored (Fig. 5), in contrast to mixtures with PC.

3.2. The contribution of each component to deviation from ideal behavior depends on the composition

The deviations from the ideal behavior of the mean molecular area and average surface potential/molecule at each monolayer composition, at constant surface pressure, may in principle be ascribed to a change of the parameters of only one or of both components in the binary mixture. This can be discerned by analyzing the variation of the partial surface parameter as a function of the mol fraction of one component employing the method of intercepts

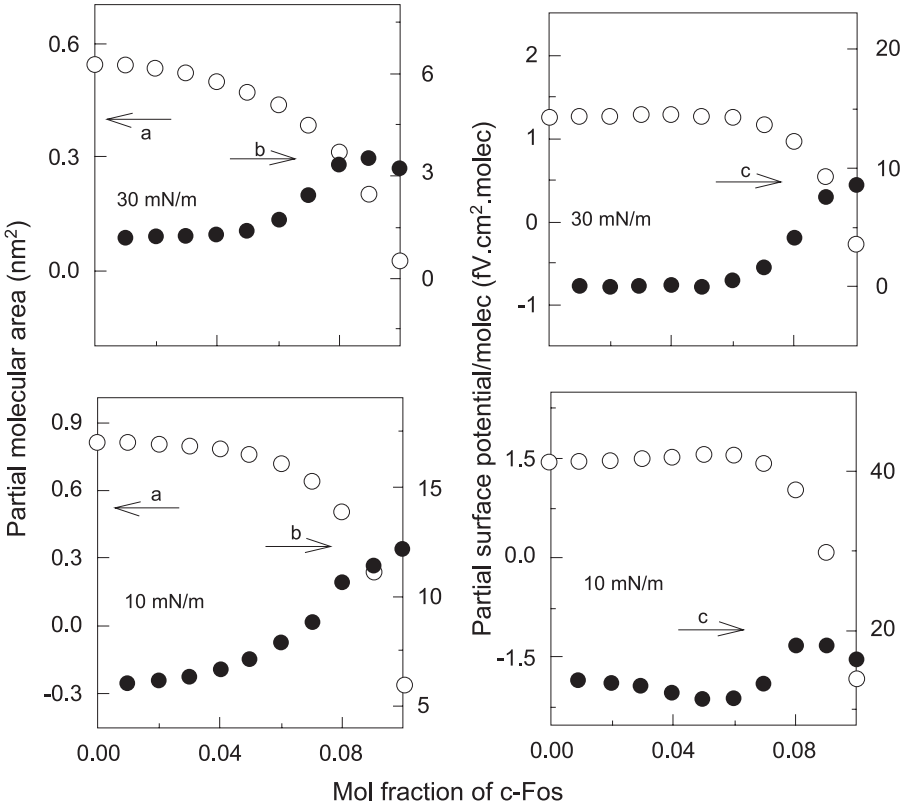

Fig. 6. Partial molecular contributions to surface packing and electrostatics. Partial mean molecular area (left panels) and partial mean surface potential/molecule (right panels) as a function of composition for mixed films of PIP_2 and c-Fos at the surface pressure indicated. The points show the variation of the partial molecular parameter of PIP_2 (○, left scale) or c-Fos (●, right scale) in the mixed monolayers. The minimum possible cross-sectional mean molecular area of PIP_2 is 0.35 nm^2, corresponding to that of two closely packed hydrocarbon chains; the mean molecular area of pure c-Fos is 10.5 nm^2 at 10 mN/m and 3.37 nm^2 at 30 mN/m; and the mean surface potential/molecule of pure c-Fos is 16.5 fV cm^2/molecule at 10 mN/m and 8.2 fV cm^2/molecule at 30 mN/m.

[30,32,33]. Fig. 6 shows that the condensation and depolarization responsible for the negative deviations from the ideal behavior in the mixed monolayers of PIP_2 with c-Fos are due to the marked reduction of the partial molecular area of c-Fos and its depolarization when its mol fraction in the mixture is below ~0.07. On the other hand, the partial molecular area and surface potential/molecule of PIP_2 are mostly unaltered up to that composition, after which the mixture becomes strongly condensed and depolarized. When the mol fraction of c-Fos is above ~0.0 7, the partial molecular area and surface potential/molecule of the protein are within ~10% of the values found in monolayers of pure c-Fos at the same surface pressure. In films containing the highest proportions of c-Fos studied, the partial molecular area of PIP_2 is reduced below its own limiting molecular area at collapse in the pure lipid film (~0.43 nm^2) and may even show negative values, with the partial surface potential/molecule also acquiring negative values. This behavior is typical of "molecular cavity" effects found in a variety of other binary monolayers [30,33–36], and reflects "hiding" of part or all the molecule into lateral packing defects of the surface lattice containing, in this case, more than ~0.07 mol fraction of protein. It should be pointed out that, due

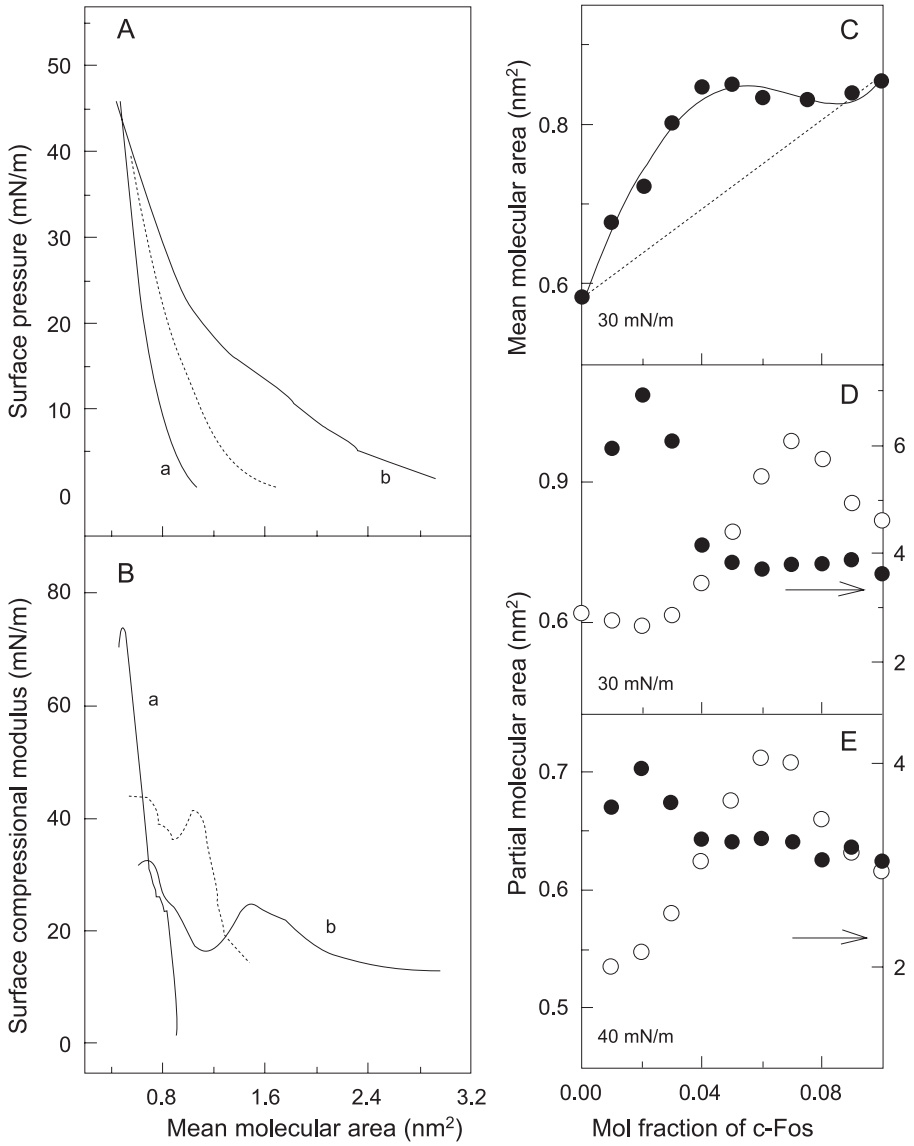

Fig. 7. Interactions of c-Fos with PC. Surface pressure (A) and surface compressional modulus (B) as a function of the mean molecular area are shown for films of pure PC (curve a), a mixed film with a mol fraction of c-Fos of 0.06 (curve b) and the corresponding ideally mixed film (dotted line). The deviations from the ideal behavior (dotted straight line) as a function of composition are shown for the mean molecular area (C) at 30 mN/m. The continuous line indicates the best polynomial fitting to the experimental values (●). Similar positive deviations are found for the variation of the partial surface potential/molecule (not shown). The variations with composition of the partial mean molecular area are shown for PC (○, left scale) or c-Fos (●, right scale) in the mixed monolayers at 30 mN/m (D) and 40 mN/m (E). The minimum possible cross-sectional mean molecular area of PC is 0.35 nm^2, corresponding to that of two closely packed hydrocarbon chains; the mean molecular area of pure c-Fos is 3.37 nm^2 at 30 mN/m and 2.17 nm^2 at 40 mN/m.

to its larger mean molecular area, more than 40% of the film area is occupied by protein in spite of its relatively small mol fraction in the mixture. In these conditions, the surface behavior is mostly controlled by c-Fos, which appears little modified in its surface molecular properties, while PIP_2 is responsible for the film's condensation and depolarization (Fig. 6). A considerable proportion of PIP_2 becomes thus sequestered into packing defects due to irregular lateral interfaces of protein-enriched condensed clusters (see below).

3.3. Mixed interfaces of c-Fos and PC

PC–c-Fos mixtures exhibit thermodynamically unfavorable (Fig. 5) mean molecular area expansion (Fig. 7A curve b, and panel C) and hyperpolarization (not shown). The variation of the partial molecular parameters with the film composition indicates that c-Fos is responsible for the expansion (and hyperpolarization) found in the films containing a small proportion of protein, with the phospholipid remaining mostly unchanged. The contrary occurs when the mol fraction of c-Fos increases beyond ~0.07 (Fig. 7D and E). The resemblance of the behavior of lipid–protein films with that of pure c-Fos as its proportions are increased becomes apparent (Fig. 7A, curve b). Besides, the reorganization that for pure c-Fos occurs at 13 mN/m remains isobaric in the presence of PC, while the change occurring at 32 mN/m for the pure protein is no longer detected in the mixed films, even at the highest proportions of the protein (see Fig. 3B). This is clearly revealed by the invariance with the surface pressure of the partial molecular area of c-Fos, when its mol fraction in the film is above 0.05 (Fig. 7D and E). The partial molecular area of c-Fos in the mixed film remains at ~3.4±0.2 nm^2 either at 30 or 40 mN/m, while the mean area of the pure protein is 3.4 nm^2 at 30 mN/m and 2.2 nm^2 at 40 mN/m. This means that the protein cannot adopt the tightest packing state before reaching the collapse point seen with pure protein or with mixtures with PIP_2, in agreement with the lack of the second protein reorganization point indicated in the phase diagram (Fig. 3B) for mixed films.

3.4. Surface topography

In order to ascertain the phase coexistence indicated by the phase diagrams in Fig. 3, we inspected the surface topography of selected mixed monolayers employing EFM and BAM. Both techniques are helpful for revealing surface inhomogeneity due to phase coexistence [26]; BAM can add information about lipid–protein segregation and/or reorganization without using fluorescent probes [37,38]. Although these techniques bring simultaneous images of the same film, they show different features of the surface. In EFM, the domains are revealed by the different lateral partitioning of the fluorescent lipid probe in the coexisting phases, while BAM reveals domains having different reflectance due to regions of different thickness and/or refraction index caused by molecular tilting [27,37,38].

Pure PC or PIP_2 films show homogeneous liquid-expanded state by EFM and homogeneous reflectance by BAM (not shown). Pure c-Fos (Fig. 8) reveals an inflection of reflectance in the range of surface pressure over which it undergoes molecular reorganization (between 8 and 18 mN/m, centered at ~13 mN/m; see also Fig. 1A, curve e). No gross inhomogeneity is observed by EFM, while BAM shows a mostly homogeneous surface, with occasional small round clusters of greater reflectance above 13 mN/m (not shown).

As an example of the changes in surface topography, we chose PIP_2–protein mixtures with 0.06 mol fraction of c-Fos, in the middle of the composition range in which phase coexistence is indicated by the phase diagrams (see Fig. 3). These films appear by EFM rather homogeneous and in the liquid-expanded state between 3 and 6 mN/m (Fig. 9A) (some occasional dark regions excluding the probe may be found but represent less than ~10% of the field). We find no surface pressure-induced transitions in this region of the isotherm, where there are no deviations of packing from ideality. However, lipid–protein interactions are indicated by slight negative deviations from ideality of $\Delta V/n$ as a function of film composition (Fig. 4). BAM reveals a homogeneous background with some round or elliptic small spots of higher reflectance (Fig. 9B) that will coalesce at higher surface pressure, probably corresponding to relatively small clusters of c-Fos and PIP_2 dispersed in the liquid-expanded lipid phase.

Above 10 mN/m, changes of surface topography mark the entrance to the biphasic region LE2 as indicated by the phase diagram (Fig. 3A). Liquid-expanded and condensed phase domains seen by EFM coexist, with rigid boundary

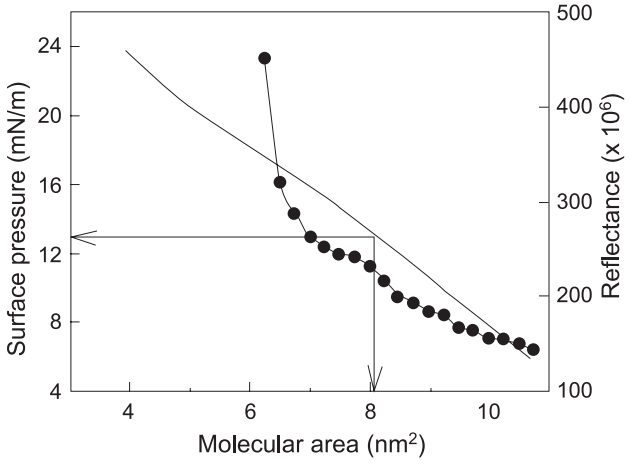

Fig. 8. Surface reflectance of pure c-Fos films. Portion of the surface pressure versus mean molecular area isotherm of pure c-Fos (line, left scale) showing the low pressure-induced reorganization and relative variation of the surface reflectance (circles, proportional to interfacial thickness, right scale) as detected by BAM. The arrows point to the surface pressure on the *Y*-axis and the mean molecular area on the *X*-axis at which c-Fos undergoes molecular reorganization.

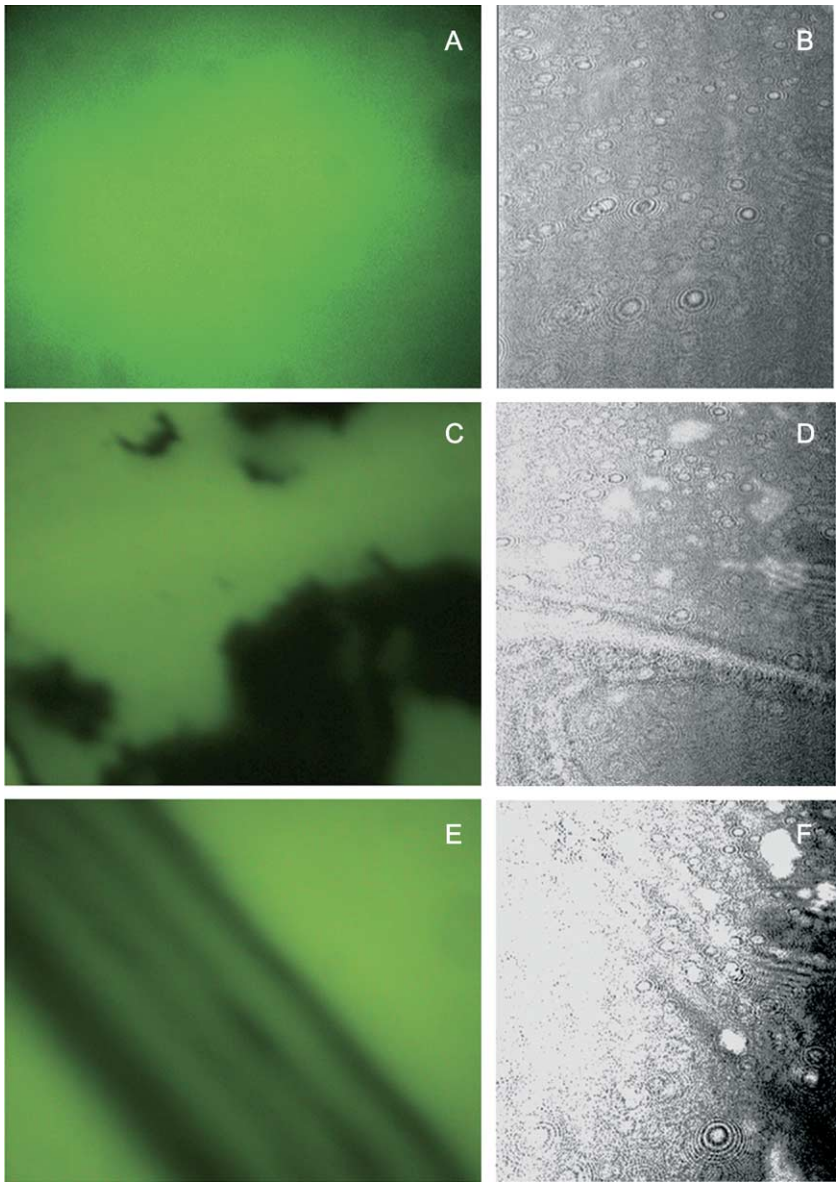

Fig. 9. Surface microscopy of PIP_2/c-fos films. Epifluorescence (A, C and E) and Brewster angle (B, D and F) microscopy images of a PIP_2 with 0.06 mol fraction c-Fos mixture film at 3 (A, B), 15 (C, D) and 27 (E, F) mN/m. The vertical side of the BAM images corresponds to 4.8 mm. The magnification of the epifluorescence images is 10 times that of the BAM images.

lateral interfaces (Fig. 9C). BAM shows that the biphasic system is constituted by highly reflective aggregates dispersed in a phase of lower reflectance (Fig. 9D), and their irregular, non-relaxed boundaries suggest that they correspond to the condensed dark domains seen by EFM. The non-relaxed boundary line tension is in agreement with the reduction of the in-plane elasticity of the mixed lipid–protein film compared to the pure protein.

Above 25 mN/m EFM shows a different topography in the biphasic LE2 region, with a stripe phase structure characteristic of the proximity of a critical point (Fig. 9E, [39]). BAM also shows a defined striped pattern of more reflective bands together with highly reflective irregular clusters (Fig. 9F). Between ~32 and 36 mN/m, the pattern is similar to that shown in Fig. 9E, only with larger dark domains of condensed phase with non-relaxed line tension at the boundary observed by EFM coexisting with a homogeneously fluorescent liquid-expanded phase. BAM shows features similar to Fig. 9F, with larger clusters of very high reflectance, and irregular rigid boundaries (not shown).

For mixed films of PC with 0.06 mol fraction of c-Fos, EFM shows a rather homogeneous surface in general at all surface pressures (not shown). BAM images also reveal less featured surfaces, with some regions of comparatively higher reflectance above 10 mN/m that are similar to the small reflective clusters seen in Fig. 9B. Above 13 mN/m BAM images of films with PC show a similar diffuse reflectance without large aggregates, and EFM coincides in showing a liquid-expanded phase of rather homogeneous fluorescence (not shown). In general, the observations by

EFM and BAM are complementary to and in good agreement with the packing and surface potential changes revealed by the compression analysis. Our results indicate that the thermodynamically favored interactions of c-Fos with PIP_2 (occurring with condensation and marked depolarization) translate to long-range supramolecular structuring, phase state, and domain coexistence at the membrane interface. A full morphometric study of the composition- and surface pressure-dependent film structure is in progress.

We have proposed lateral separation of lipid domains as a mechanism for the modulation of phospholipase activity by c-Fos [3]. We now show that c-Fos indeed mixes with PIP_2 and with PC in a non-ideal manner, inducing marked changes of phospholipid packing. Such changes in the organization of the interface can modulate enzymatic activities that depend on lateral packing [4,6,12]. The PIP_2 modulating proteins MARCKS, GAP43 and CAP23, whose effect on signaling is not direct but exerted through lipid clustering [18], bring more insight into this point. Interestingly, although the three proteins promote PIP_2 clustering, only MARCKS shows increased PLC-driven PIP_2 breakdown. Thus, it is not clustering itself that modulates PLC activity; rather, the nature of the lipid–protein interactions that give rise to clustering probably determines the effect on this and perhaps other membrane activities. The role of MARCKS, GAP43 and CAP23 in neurite outgrowth is linked to the content of basic residues of their effector domain [18], which is defined by a high content of basic residues. The ratio of basic residues over the total number of amino acids in the effector domain is 0.52 (13/25) for MARCKS and 0.35 (8/23) for GAP43. c-Fos has a basic, DNA binding domain, which can be considered the equivalent to the effector domain of MARCKS, GAP43 and CAP23 in that it is presumably responsible for the interaction with phospholipids. Interestingly, the content of lysine and arginine residues in this domain is 12 over 21 residues in total length, giving a ratio (0.57) for c-Fos similar to that for MARCKS. It is noteworthy that c-Fos interacts preferentially not only with PIP_2 but also with other anionic lipids like phosphatidylserine and phosphatidylglycerol in a similar manner [2], further suggesting that the basic DNA domain is responsible for the interaction. With the latter two anionic lipids, similar to PIP_2, c-Fos is able to interact up to cut-off surface pressures as high as 30–35 mN/m [3], a range that corresponds (though with rather large fluctuations [22]) to the average for a bilayer membrane [21]. This is also the range in which the protein undergoes the composition-dependent high surface pressure reorganization (see Fig. 3A). On the other hand, the selectivity of these interactions is clearly pointed out by the fact that the cut-off surface pressure for the occurrence of interactions with other anionic lipids such as PIP and PI is not above 25 mN/m [3]. Although the basic His-tag of the recombinant protein used might be involved in the interactions observed, this should not modify the conclusions reached for a number of reasons. First, the protein preparation induces the selective lipid interactions reported (this work and Ref. [3]). Second, the effects of c-Fos on phospholipid metabolism are induced with the same

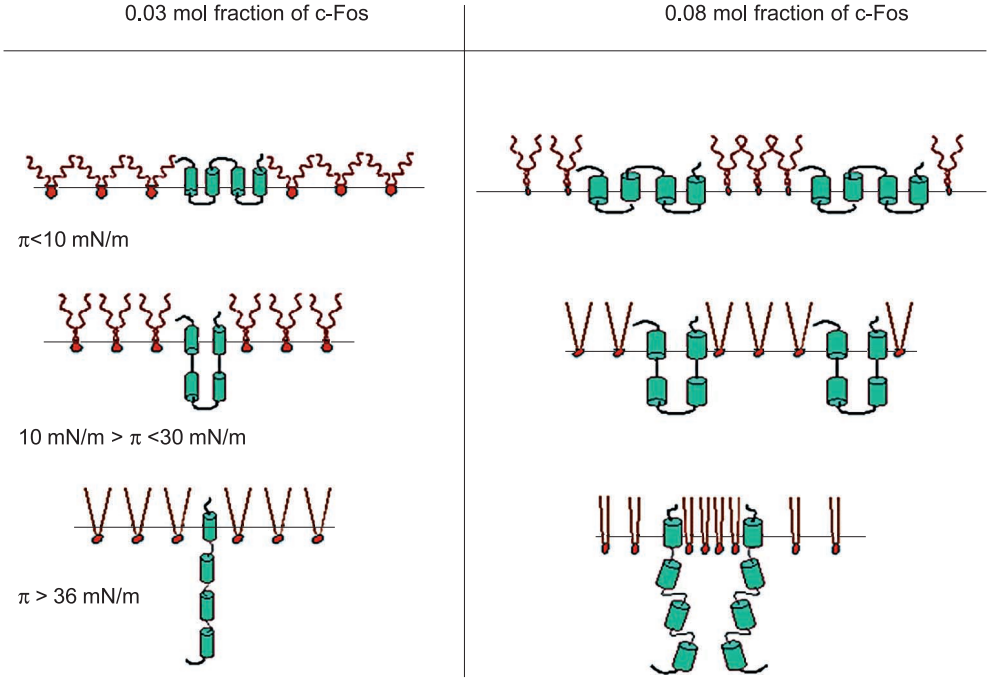

Fig. 10. Schematic representation of interfacial organization of PIP_2–c-Fos monolayers. To the left: mixture with 0.03 mol fraction of c-Fos. To the right: mixture with 0.08 mol fraction of c-Fos. Surface pressures are indicated. The protein is represented as having four α helices (cylinders) [3]. At each stated surface pressure, the molecular area of c-Fos is normal in the right panel and condensed in the left panel, while the molecular area of PIP_2 is normal (wavy lines for hydrocarbon chains) in the left panel and condensed (straight lines for hydrocarbon chains) in the right panel.

recombinant protein while His-tagged c-Jun fails to do so [40]. And third, both recombinant and endogenously induced c-Fos show similar effects on phospholipid metabolism [40]. We found that the effect of c-Fos on lipid reordering depends on the composition of the mixtures. This suggests the possibility that its relative concentration due to expression variation could change its membrane-associated effects. It is known that c-Fos is not constitutive in quiescent cells, but its expression is induced upon stimulation and varies along the cell cycle [41].

Fig. 10 summarizes the surface pressure- and composition-dependent changes of the partial molecular area of c-Fos and PIP_2. It shows a possible organization in mixed films of PIP_2 with c-Fos at 0.03 and 0.08 mol fractions at low (less than 10 mN/m), intermediate (between 10 and 30 mN/m), and high (over 36 mN/m) surface pressure. The phase state of the lipid is not determinant of the effect of c-Fos on packing because, although pure PC and PIP_2 both form liquid expanded films, the former is expanded while the latter is condensed by the protein, indicating that this effect is due to the polar head group. We should also stress that we cannot discern between a looser packing of the helices in the protein and their actual expansion when the mol fraction of protein increases. Thus, the cylinders in Fig. 10, representing the helices in the left and right panels, are shown with the same size but with different spacing, reflecting the mean cross-sectional area. The model does not take into account changes regarding $\Delta V/n$, a parameter that includes complex electrostatic contributions derived from oriented water molecules and possible variations of fundamental group dipoles; these combined influences cannot be directly established with our measurements.

According to Ref. [13], proteins likely have an important role in determining the constituents, structure and dynamics of membranes. c-Fos could share such a role, with a richness that is supported by our preliminary observations of mixed interfaces by microscopy. The ability of c-Fos to undergo reorganization responding to the state and composition of the membrane provides a basis for transduction. Its ability to induce changes in the membrane through lipid segregation and packing adds complexity to its potential as a signaling molecule.

4. Conclusions

As a summary of our findings, we conclude that thermodynamically favorable interactions of c-Fos with PIP_2 occur at all proportions, with an optimum at the 0.08 mol fraction of protein mixture. On the contrary, interactions with PC are unfavorable (Fig. 5).

Condensation of the mean molecular area and concomitant reduction of the overall interfacial polarization occur in the mixed films of c-Fos with PIP_2 (Fig. 4). By contrast, large molecular area expansions are found in mixed monolayers of the protein with PC (Fig. 7). The contribution of each component to these deviations of ideality depends on the amount of protein in the mixtures.

The thermodynamically favored phase coexistence in mixtures of c-Fos with PIP_2 translates to a varied surface structuring over the long range, with domain segregation of protein-induced condensed, optically thicker phases (Fig. 9). Conversely, the surface topography in mixed films of c-Fos with PC (not shown) is in keeping with lipid–protein unfavorable associations, with tendency of both components to remain dispersed.

Acknowledgement

We wish to thank Dr. T. Curran for providing the plasmid to purify c-Fos, and C. Rosetti for help with BAM microscopy. FONCyT, CONICET, Fundación Antorchas, SeCyT-UNC and James S. McDonnell Foundation supported this work.

References

[1] D.F. Bussolino, M.E. Guido, G.A. Gil, G.A. Borioli, M.L. Renner, V.R. Grabois, C.B. Conde, B.L. Caputto, c-Fos associates with the endoplasmic reticulum and activates phospholipid metabolism, FASEB J. 15 (2001) 556–558.
[2] M.E. Guido, G.A. de Arriba Zerpa, D.F. Bussolino, B.L. Caputto, Immediate early gene c-*fos* regulates the synthesis of phospholipids but not of gangliosides, J. Neurosci. Res. 43 (1996) 93–98.
[3] G.A. Borioli, B.L. Caputto, B. Maggio, c-Fos is surface active and interacts differentially with phospholipid monolayers, Biochem. Biophys. Res. Commun. 280 (2001) 9–13.
[4] G.A. Borioli, L. Fanani, B. Caputto, B. Maggio, c-Fos is a surface pressure-dependent diverter of phospholipase activity, Biochem. Biophys. Res. Commun. 295 (2002) 964–969.
[5] J.H. Exton, Phosphatidylcholine breakdown and signal transduction, Biochim. Biophys. Acta 1212 (1994) 26–42.
[6] G.A. Borioli, B.L. Caputto, B. Maggio, Phospholipase activity is modulated by c-Fos through substrate expansion and hyperpolarization, FEBS Lett. 570 (2004) 82–86.
[7] D.W. Grainger, H. Rechert, H. Ringsdorf, C. Salesse, Hydrolytic action of phospholipase A_2 in monolayers in the plane transition region: direct observation of enzyme domain formation using fluorescence microscopy, Biochim. Biophys. Acta 1023 (1990) 365–379.
[8] J.M. Muderhwa, H.L. Brockman, Lateral lipid distribution is a major regulator of lipase activity. Implications for lipid-mediated signal transduction, J. Biol. Chem. 267 (1992) 24184–24192.
[9] R. Burack, R.L. Biltonen, Lipid bilayer heterogeneities and modulation of phospholipase A_2 activity, Chem. Phys. Lipids 73 (1994) 209–222.
[10] B. Maggio, Control by ganglioside GD1a of PLA_2 activity through modulation of the lamellar-hexagonal (HII) phase transition, Mol. Membr. Biol. 13 (1996) 109–112.
[11] G. Basañez, G.D. Fidelio, F.M. Goñi, B. Maggio, A. Alonso, A twofold inhibitory effect of glycosphingolipids on phospholipase C-promoted fusion of lipidic vesicles, Biochemistry 35 (1996) 7506–7513.
[12] M.L. Fanani, S. Härtel, R.G. Oliveira, B. Maggio, Bi-directional control of sphingomyelinase activity and surface topography in lipid monolayers, Biophys. J. 83 (2002) 3416–3424.
[13] G. Vereb, J. Szollosi, J. Matko, P. Nagy, T. Farkas, L. Vigh, L. Matyus, T.A. Waldmann, S. Damjanovich, Dynamic, yet structured:

the cell membrane three decades after the Singer–Nicolson model, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 8053–8058.
[14] B. Maggio, J.M. Sturtevant, R.K. Yu, Effect of myelin basic protein on the thermotropic behavior of aqueous dispersions of neutral and anionic glycosphingolipids and their mixtures with dipalmitoylphosphatidylcholine, J. Biol. Chem. 25 (1987) 2652–2659.
[15] E. Sackmann, Physical basis of self-organization and function of membranes: Physics of vesicles, in: R. Lipowsky, E. Sackmann (Eds.), Structure and Dynamics of Membranes, vol. 1A. From Cells to Vesicles, Elsevier Science, Amsterdam, 1995, pp. 213–304.
[16] I.D. Bianco, G.D. Fidelio, R.K. Yu, B. Maggio, Concerted modulation by myelin basic protein and sulfatide of the activity of phospholipase A_2 against phospholipid monolayers, Biochemistry 31 (1992) 2636–2642.
[17] B. Maggio, R. Yu, Modulation by glycosphingolipids of membrane–membrane interactions induced by myelin basic protein and melittin, Biochim. Biophys. Acta 1112 (1992) 105–114.
[18] T. Laux, K. Fukami, M. Thelen, T. Golub, D. Frey, P. Caroni, GAP43, MARCKS, and CAP23 modulate PI(4,5)P2 at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism, J. Cell Biol. 149 (2000) 1455–1471.
[19] T. Magee, N. Pirinen, J. Adler, S.N. Pagakis, I. Parmryd, Lipid rafts: cell surface platforms for T cell signaling, Biol. Res. 35 (2002) 127–131.
[20] H. Brockman, Lipid monolayers: why use half a membrane to characterize protein–membrane interactions? Curr. Opin. Struck. Biol. 9 (1999) 438–443.
[21] D. Marsh, Lateral pressure in membranes, Biochim. Biophys. Acta 1286 (1996) 183–223.
[22] M.C. Phillips, D.E. Graham, J. Hauser, Lateral compressibility and penetration into phospholipid monolayers and bilayer membranes, Nature 254 (1975) 154–155.
[23] M. Edidin, Lipids on the frontier: a century of cell–membrane bilayers, Nat. Rev., Mol. Cell Biol. 4 (2003) 414–418.
[24] I.D. Bianco, G.D. Fidelio, B. Maggio, Effect of sulfatide and gangliosides on phospholipase C and phospholipase A_2 activity. A monolayer study, Biochim. Biophys. Acta 1026 (1990) 179–185.
[25] D.C. Carrer, B. Maggio, Phase behavior and molecular interactions in mixtures of ceramide with dipalmitoylphosphatidylcholine, J. Lipid Res. 40 (1999) 1978–1989.
[26] R.G. Oliveira, B. Maggio, Compositional domain immiscibility in whole myelin monolayers at the air–water interface and Langmuir–Blodgett films, Biochim. Biophys. Acta 1501 (2002) 238–250.
[27] C.M. Rosetti, R.G. Oliveira, B. Maggio, Reflectance and topography of glycosphingolipid monolayers at the air–water interface, Langmuir 19 (2003) 377–384.
[28] G.L. Gaines, Insoluble monolayers at liquid–gas interfaces, in: I. Prigogine (Ed.), Interscience Monographs on Physical Chemistry, Wiley-Interscience, New York, 1966, pp. 136–207.
[29] S. Alí, H.L. Brockman, R.L. Brown, Structural determinants of miscibility in surface films of galactosylceramide and phosphatidylcholine: effect of unsaturation in the galactosylceramide acyl chain, Biochemistry 30 (1991) 11198–11205.
[30] B. Maggio, T. Ariga, R.O. Calderón, R.K. Yu, Ganglioside GD3 and GD3-lactone mediated regulation of the intermolecular organization in mixed monolayers with dipalmitoylphosphatidylcholine, Chem. Phys. Lipids 90 (1997) 1–10.
[31] X.-M. Li, J. Smaby, M.M. Momsen, H.L. Brockman, R.E. Brown, Sphingomyelin interfacial behavior: the impact of changing acyl chain composition, Biophys. J. 78 (2000) 1921–1931.
[32] P.W. Atkins, Physical Chemistry, Fourth ed., W.H. Freeman and Company Publishers, New York, 1990, p. 182.
[33] D.C. Carrer, B. Maggio, Transduction to self-assembly of molecular geometry and local interactions in mixtures of ceramides and ganglioside GM1, Biochim. Biophys. Acta 1514 (2001) 87–89.
[34] D.O. Shah, Surface chemistry of lipids, Adv. Lipid Res. 8 (1970) 347–431.
[35] B. Maggio, J.A. Lucy, Studies on mixed monolayers of phospholipids and fusogenic lipids, Biochem. J. 149 (1975) 597–608.
[36] B. Maggio, J.A. Lucy, Polar group behaviour in mixed monolayers of phospholipids and fusogenic lipids, Biochem. J. 155 (1976) 353–364.
[37] J. Miñones Jr., C. Carreras, P. Dynarowicz-Latka, J. Miñones, O. Conde, R. Seoane, J.M. Rodriguez Patiño, Orientational changes of amphotericin B in Langmuir monolayers observed by Brewster Angle Microscopy, Langmuir 17 (2001) 1477–1482.
[38] P. Wu, B. Corsico, C.R. Flach, D.P. Cistola, J. Storch, R. Mendelsohn, Deletion of the helical motif in the intestinal fatty acid-binding protein reduces its interactions with membrane monolayers: Brewster Angle Microscopy, IR reflection-absorption spectroscopy, and surface pressure studies, Biochemistry 40 (2001) 1976–1983.
[39] H.M. McConnell, Structure and transitions in lipid monolayers at the air–water interface, Annu. Rev. Phys. Chem. 42 (1991) 171–195.
[40] G.A. Gil, D.F. Bussolino, M.M. Portal, A.A. Pecchio, M.L. Renner, G.A. Borioli, M.E. Guido, B.L. Caputto, c-Fos activated phospholipid synthesis is required for neurite elongation in differentiating PC12 cells, Mol. Biol. Cell 15 (2004) 1881–1894.
[41] D. Lallemand, G. Spyrou, M. Yaniv, C.M. Pfarr, Variations in Jun and Fos protein expression and the AP-1 activity in cycling, resting and stimulated fibroblasts, Oncogene 14 (1997) 819–830.