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Protein-mediated surface structuring in biomembranes

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Abstract

The lipids and proteins of biomembranes exhibit highly dissimilar conformations, geometrical shapes, amphipathicity, and thermodynamic properties which constrain their two-dimensional molecular packing, electrostatics, and interaction preferences. This causes inevitable development of large local tensions that frequently relax into phase or compositional immiscibility along lateral and transverse planes of the membrane. On the other hand, these effects constitute the very codes that mediate molecular and structural changes determining and controlling the possibilities for enzymatic activity, apposition and recombination in biomembranes. The presence of proteins constitutes a major perturbing factor for the membrane sculpturing both in terms of its surface topography and dynamics. We will focus on some results from our group within this context and summarize some recent evidence for the active involvement of extrinsic (myelin basic protein), integral (Folch-Lees proteolipid protein) and amphitropic (c-Fos and c-Jun) proteins, as well as a membrane-active amphitropic phosphohydrolytic enzyme (neutral sphingomyelinase), in the process of lateral segregation and dynamics of phase domains, sculpturing of the surface topography, and the bi-directional modulation of the membrane biochemical reactivity.

Key words
- Lipid monolayers
- Sphingomyelinase
- c-Fos
- Segregated lipid domains
- Lipid-protein interaction
- Amphitropic proteins

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Introduction

The plasma membrane of eukaryotic cells has a formidable compositional heterogeneity in that it contains a wide variety of lipid and protein species. These are thermodynamically constrained to coexist within a lateral and transverse narrowly restricted anisotropic (vectorial) structure whose fundamental topology was conceived on the basis of the fluid lipid bilayer paradigm (1). However, the lipids and proteins forming the membrane exhibit highly dissimilar conformations, geometrical shapes, amphipathicity, and thermodynamic properties regarding their two-dimensional molecular packing, electrostatics, and interaction preferences. This causes inevitable development of large local tensions along the lateral and transverse planes of the membrane. Their tendency to relaxation introduces considerable structural dynamics and metastability that transduce those tensions from the local to the supramolecular and further to the topological level, even beyond the bilayer membrane itself through hydration structuring and double layer electrostatic potential (2). Actually, these ef-

fects constitute the molecular and structural codes that mediate changes of phase state, domain segregation determining lateral and transverse topography, surface electrostatics, membrane curvature and non-bilayer phases, as well as membrane-membrane recognition and/or recombination (2-6).

The above phenomena can also occur in protein-free lipid bilayers due to lipid phase segregation brought about by polar head group, hydrocarbon chain or packing geometry incompatibilities leading to immiscibility (2,7). On the other hand, the presence of proteins constitutes a major perturbing factor for the membrane sculpturing both in terms of its surface topography and dynamics (8,9). In turn, the compensating structural features generated by their presence and activity concomitantly affect and regulate protein function (10). Moreover, some membrane active proteins and enzymes do not remain permanently integrated to the membrane but are extrinsically adsorbed or exhibit amphitropic behavior whereby they can become associated or not with the biointerface depending on composition, interactions, or dynamic changes of membrane topology (11). Although the latter properties have been less popularized for lipids, several species can be readily incorporated, released or rapidly exchanged between membranes depending on the membrane composition and structural dynamics (12).

As a general overall concept, it is thermodynamically inevitable that at least one way of relieving lateral and transverse tensions is by segregation of immiscible components into separate domains of different composition and/or phase state. However, it has been much more difficult to describe the local molecular properties and defined interactions representing the critical thresholds driving the intermolecular immiscibility processes leading to domain formation and surface microheterogeneity at the mesoscopic level (3,13). Although most details still remain obscure, it has long been known to membrane biophysicists that immiscible domains exist even in very simple binary and ternary systems (14,15), let alone in the compositional complexity of whole cell membranes (3), and that both the lipid and protein components are likely to participate in establishing the phenomena of membrane phase separation. The importance of this fact was rediscovered relatively recently in the fields of membrane biology and biochemistry (16, 17). Research in this area gained a long postponed and deserved impetus raising some hopes of recognizing "lipidomics" or "membranomics" as a formidable problem to be tackled to allow the understanding of one of the more complex condensed states of matter, whose surface, metaphorically, we are only beginning "to scratch".

In this paper we will focus on some recent results from our group within this context and summarize some evidence for the involvement of some extrinsic, integral or amphitropic proteins and a membrane-active phosphohydrolytic enzyme in the process of lateral segregation of phase domains, sculpturing of the surface topography, and the bi-directional modulation of its biochemical and structural reactivity. We do not attempt to cover all the literature and our description will be restricted to some surface effects of myelin basic protein (MBP), a neutral sphingomyelinase, the amphitropic transcription factors c-Fos and c-Jun, and the Folch-Lees proteolipid protein (PLP).

Thermodynamic domains induced by myelin basic protein in lipid mixtures of myelin glycosphingolipids

In pure binary lipid systems with fully compatible hydrocarbon moieties, polar headgroup-driven favorable and unfavorable interactions can induce lateral immiscibility (2-4,18). Among these, we recently described the surface miscibility preferences of ceramide and glycosphingolipids in close correlation with the metabolic segregation of their

biosynthetic pathways (7). In more complex systems other factors may override the intrinsic thermodynamic tendencies of glycosphingolipids to undergo mixing or demixing processes. Among several physical factors, long-range tensions related to curvature and topological stress cause lateral and transverse reorganization through thermodynamic-geometric compensation (4,19-21) which may translate to alterations of the activity of membrane-related enzymes (22). Regarding composition, a major factor is the existence of differential interactions of particular glycosphingolipids with some membrane proteins such as MBP (23,24). As a result, defined glycosphingolipids may be laterally sequestered or segregated into different compositional domains as recently described in whole myelin monolayers (25).

Apart from preferential interactions with negatively charged interfaces containing sulfatides and gangliosides, MBP causes lateral condensation (23) and affects differentially the thermotropic behavior of single glycosphingolipids (24). In ternary systems of MBP in bilayers of defined composition constituted by dipalmitoyl phosphatidylcholine (dpPC) and different glycosphingolipids, the protein causes phase separation and can induce membrane-membrane interactions and recombination depending on the oligosaccharide chain of the glycosphingolipid (4). The protein affects the thermotropic behavior. Figure 1A shows that in mixtures of dpPC with the neutral galactosylceramide the protein causes an asymmetric distortion of the phase transition; this indicates that MBP preferably partitions into the liquid-crystalline lipid phase in which the high temperature asymmetry can be resolved into a broad low-cooperativity peak. Statistical thermodynamic calculations (26) of the cluster size distribution functions reveal that the most probable size (number of thermodynamically correlated molecules undergoing the transition at $T_{1/2}$) of the high temperature clusters induced by MBP is below 40 and is narrowly distributed. On the other hand, the cluster size and distribution of the segregated domains undergoing the phase transition at the $T_{1/2}$ corresponding to the protein-free mixture remain practically unchanged (Figure 2A).

In binary lipid mixtures of dpPC with sulfatide or ganglioside GM1 (two major anionic sphingolipids of myelin) MBP induces major changes of thermotropic behavior. Pre-

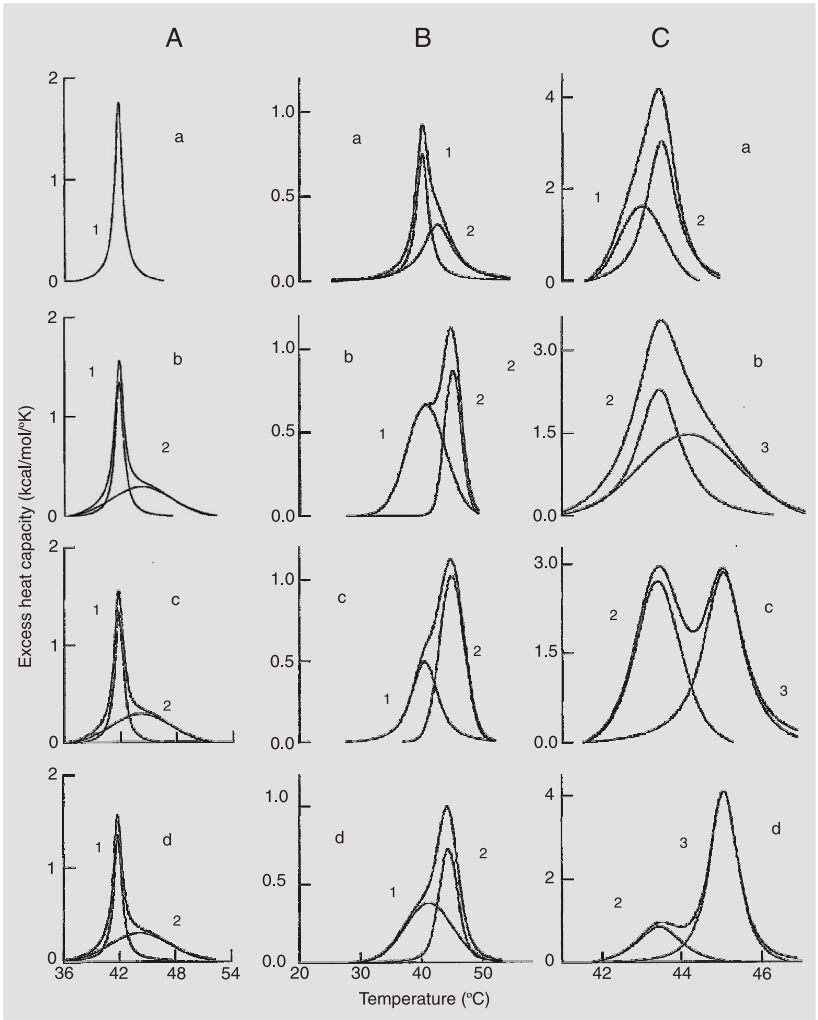

Figure 1. Myelin basic protein induces phase separation in mixtures of dipalmitoyl phosphatidylcholine (dpPC) and glycosphingolipids. Excess heat capacity-temperature scans (0.5°C/min) of mixtures of: *Column A*, galactosylceramide-dpPC (1:3) with a proportion of myelin basic protein (MBP) of 0 (a), 0.6 (b), 2.3 (c), and 4.3 (d) mol%; *Column B*, mixtures of sulfatide-dpPC (1:3) with a proportion of MBP of 0 (a), 0.3 (b), 1.1 (c), and 4.4 (d) mol%; *Column C*, mixtures of ganglioside GM1-dpPC (1:5) with a proportion of MBP of 0 (a), 0.1 (b), 1.2 (c), and 4.5 (d) mol%. Coexisting phase transitions are identified by numbers on the corresponding peaks.

vious publications have reported the complete temperature-composition phase diagrams for binary mixtures of several glycosphingolipids with dpPC. These studies revealed that for protein-free mixtures containing sulfatide and gangliosides the phase diagrams were quite broad and, depending on composition, phase coexistence of phospholipid domains excluding glycosphingolipids into enriched clusters was present (18). This can be seen in Figure 1Ba and 1Ca where two overlapping calorimetric transitions are found in the protein-free mixtures. The presence of MBP in increasing amounts facilitates formation of high-temperature segregated glycosphingolipid-enriched domains (Figure 1B, peak 2, Figure 1C, peak 3). The statistical thermodynamic analysis (Figure 1B,C) reveals that the most probable cluster size of the high-temperature transition component (glycosphingolipid-enriched domains) becomes larger and more broadly distributed with increasing proportions of MBP (Figure 2B, peak 2, Figure 2C, peak 3; see also Figure 1B,C). On the other hand, the most probable clusters of the segregated low-temperature component whose transition temperature mostly remains unaltered in the presence of MBP become very small and more narrowly distributed (mixtures with sulfatide; Figure 2B, peak 1) while the opposite occurs in mixtures with GM1 (Figure 2C, peak 2). This points to the existence of a long-range structural influence and intercommunication among the coexisting phase domains on the thermodynamic level whereby the transition features of one type of segregated cluster become influenced by the presence of another type.

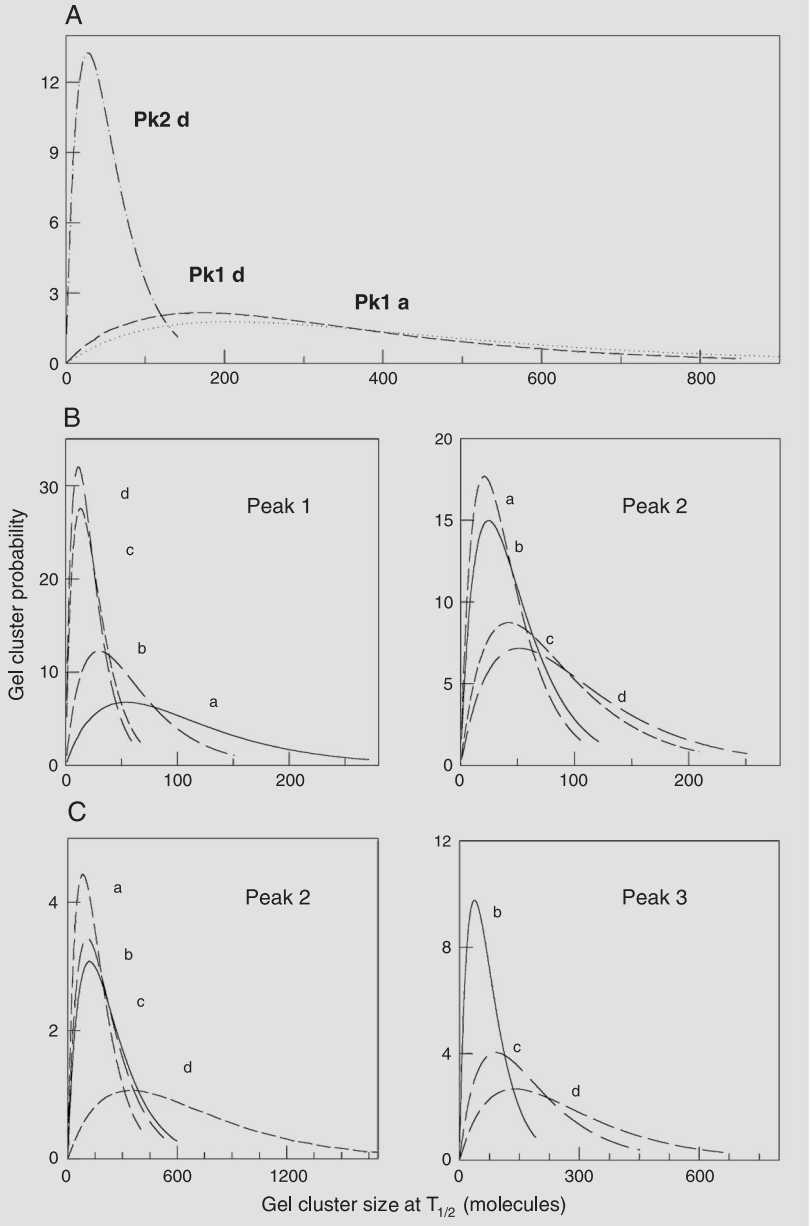

Figure 2. Statistical thermodynamic cluster functions of phase-segregated domains induced by myelin basic protein (MBP) in mixtures of dipalmitoyl phosphatidylcholine (dpPC) and glycosphingolipids at $T_{1/2}$. Most probable cluster size (peak maxima) of the different phase components of the transition shown in Figure 1 and gel cluster probability as a function of cluster size. *Upper panel* (A): mixtures of galactosylceramide-dpPC (1:3) with a proportion of MBP of 0 (a) and 4.3 (d) mol%. *Middle panels* (B): mixtures of sulfatide-dpPC (1:3) with a proportion of MBP of 0 (a), 0.3 (b), 1.1 (c), and 4.4 (d) mol%. *Lower panels* (C): mixtures of ganglioside GM1-dpPC (1:5) with a proportion of MBP of 0 (a), 0.1 (b), 1.2 (c), and 4.5 (d) mol%.

Lipid-protein surface reorganization induced by the transcription factors c-Fos and c-Jun

Two of the most studied transcription factors are the immediate early gene protein products c-Fos and c-Jun. Their amounts in the cell are very rapidly and transiently increased in response to stimuli, culminating with the activation of an array of target genes involved in normal cellular processes such as growth, differentiation and prolif-

eration (27). Imbalance of their highly regulated expression and or activity leads to oncogenic or apoptotic processes (28,29). The transcriptional activity of c-Fos and c-Jun is conditioned to their entering the nucleus as a heterodimeric complex, the activator-protein 1 (AP-1), whose formation depends on the relative amounts of both proteins, their presence in a same cellular compartment, and their post-translational state (30,31). These in turn depend on the cell type and its microenvironment. Phosphorylation, among the modifications that modulate AP-1 formation (32), may be seen as a mechanism involving competition between the association of its components with membranes and their dimerization. c-Fos association with membranes was first suggested by its recently reported capacity to specifically regulate phospholipid metabolism (33). This is one of the normal c-Fos functions, occurring when the protein is expressed very rapidly after a cell or tissue receives stimuli of various kinds and associates with the endoplasmic reticulum (34). The other normal, more studied, function of the protein is to transcriptionally regulate target genes of AP-1 at longer times (usually about one hour) after the stimulus, when located in the nucleus.

A few but important lines of evidence have opened new insights into the molecular function of some gene-regulatory proteins; among the most unexpected was the impressive membrane activity of some of them, like the DnaA protein (35). Within this context, the finding that c-Fos is strongly amphitropic and that it interacts differentially with phospholipids (Ref. 36 and Figure 3) was a first step in the way to understanding the nature of its association with membranes. This was followed by demonstration of its capacity to finely modulate phospholipase activity against organized biointerfaces (37). On the other hand, c-Jun, the partner of c-Fos in the AP-1 complex, is also amphitropic but its interaction with phospholipids is not specific (Ref. 38 and Figure 3). A further step in the characterization of the c-Fos association with membranes indicated more clearly the differential quality of the protein/lipid interaction: interestingly, c-Fos elicits opposite lipid packing and electrostatic effects in dilauroyl phosphatidylcholine (dlPC) and phosphatidylinositol diphosphate (PIP_2), inducing expansion and hyperpolarization of the former and condensation and depolarization of the latter (Fig-

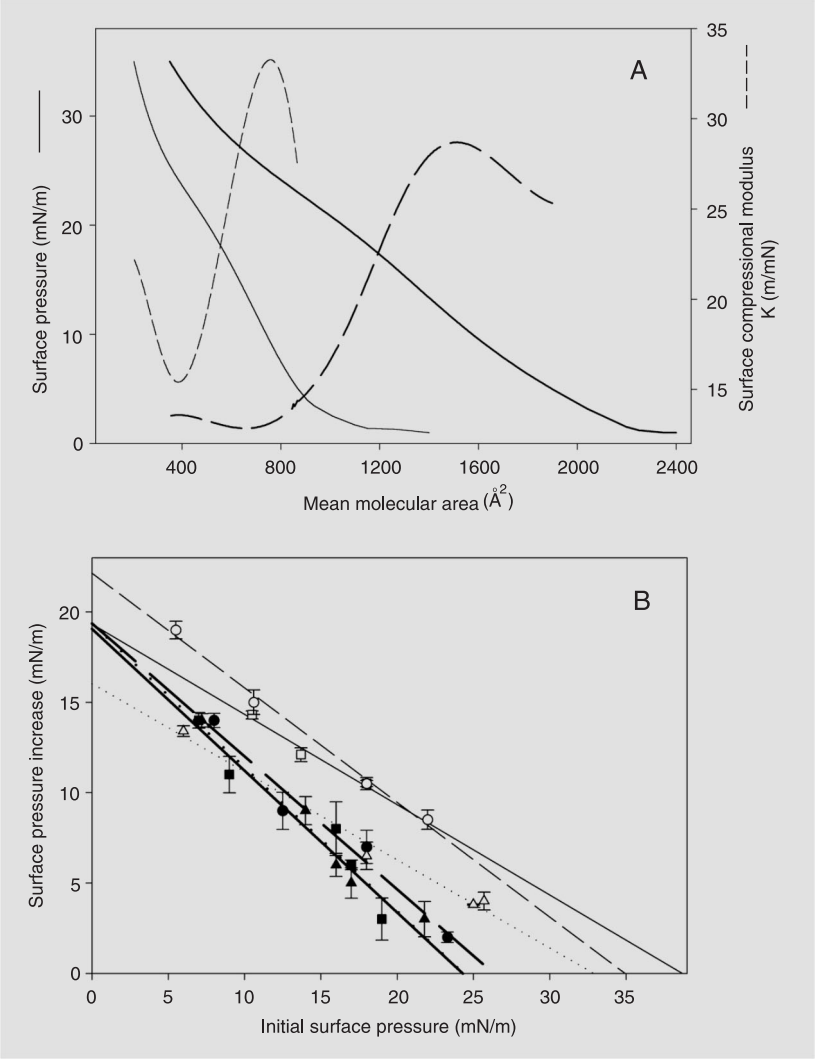

Figure 3. Interfacial behavior of c-Fos and c-Jun and their interaction with phospholipids. *A*, Isotherms of the pure proteins (full lines), c-Fos (thin lines), c-Jun (thick lines) and their corresponding compressional moduli (dashed lines). *B*, Penetration curves of each protein (c-Fos, open symbols; c-Jun, filled symbols) in phosphatidylserine (circles, dashed lines), phosphatidylcholine (triangles, dotted lines), and phosphatidylinositol 4,5 diphosphate (squares, full lines). Data are reported as means ± SD.

ure 4). Moreover, its effects on the organization of dlPC explain its capacity to modulate phospholipase A_2 (PLA_2) and PLC activity on this phospholipid (37,39). The contrasting effects of the intermolecular interactions of c-Fos with dlPC and PIP_2 are supported by the thermodynamic drive, as assessed by the excess free energy of mixing, regulating the composition-dependent specific lipid-protein interactions (Figure 4C). The two-dimensional phase diagram for the mixture of c-Fos with PIP_2 also shows regions of protein-induced surface immiscibility (40). This is illustrated by epifluorescence and Brewster angle microscopy images of the mixtures (Figure 5).

The phenomena underlying the interaction of c-Fos and c-Jun with phospholipids may eventually affect their dimerization tendency, which leads to nuclear translocations and further transcriptional regulation. With respect to AP-1 heterodimers, their operational formation and involvement in cellular responses have been ascertained (28). However, although association of c-Fos and c-Jun has been described (27-30), we have not found publications describing the actual isolation of this complex as a stable chemical entity and we were not successful in achieving its formation in solution under a variety of conditions (38). On the other hand, we and others (27,38) obtained direct evidence indicating that interface interactions are essential to drive AP-1 formation. In this regard, not only both proteins can interact with each other at the interface, as they do separately with phospholipids, but their equimolecular complex is also strongly stabilized by the favorable thermodynamics of their association (Ref. 38 and Figure 6). The interplay and balance between the two sets of interaction forces are likely to determine the fate and possible reversible location of these transcription factors in subcellular compartments, thus accomplishing the fine regulation of their function in the cell.

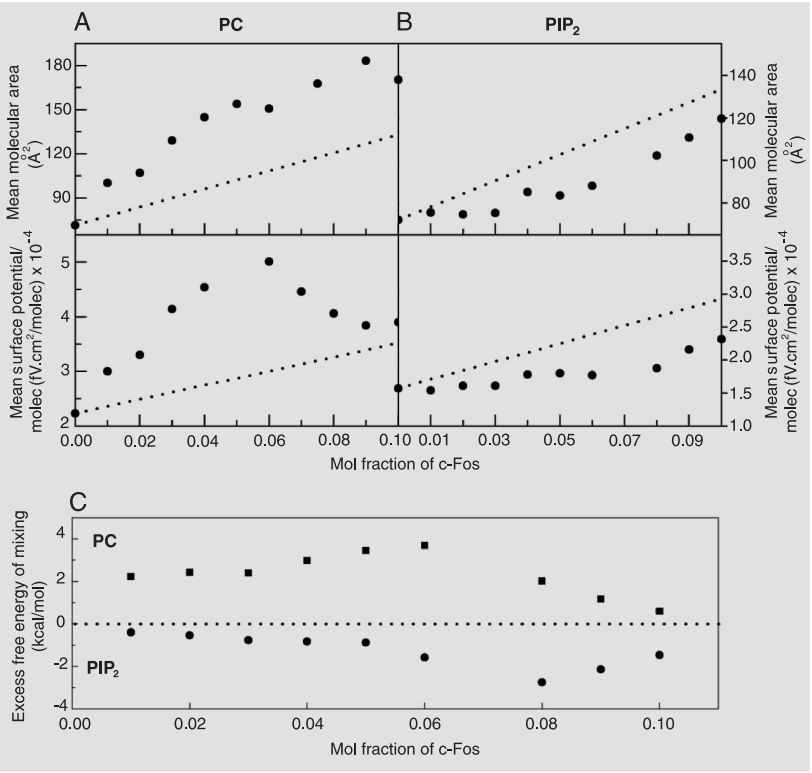

Figure 4. c-Fos-induced lipid reorganization. *A*, Mixtures of c-Fos with phosphatidylcholine (PC) showing expansion (upper panel) and hyperpolarization (lower panel) with respect to ideal behavior (dashed lines). *B*, Mixtures of c-Fos with phosphatidylinositol biphosphate (PIP_2) showing condensation (upper panel) and depolarization (lower panel) with respect to ideal behavior (dashed lines). *C*, Excess free energy of mixing. The excess compression free energy as a function of composition is given for mixed films of c-Fos with PIP_2 (circles) or with PC (squares). molec = molecule.

Bi-directional information transduction between surface domain structuring and membrane biocatalysis

A "dynamic" type of protein-induced surface structuring is mediated by the action of phospholipases (10,41). All phosphohydrolytic enzymes, usually a kind of amphitropic proteins producing lipid second messengers, are currently thought to be involved in some sort of membrane signal transduction process (42,43). With respect to sphingolipid signaling, ceramide is a pivotal compound that links the metabolism of phospho, sphingo-, and glycosphingolipids, all of which are important biomodulators that control membrane topology and phospholipase activity. Besides the concept that ceramide

is an important second messenger derived from the sphingomyelin cycle (43), there are direct metabolic, structural and functional consequences of the sphingomyelinase-driven conversion of sphingomyelin to ceramide in biomembranes (43,44).

Studies on the molecular and structural codes that underlay the enzyme specificities and the kinetics of the complex surface reactions are beginning to redefine the concept of membrane signaling by including processes taking place over length and time domains spanning several orders of magnitude (21). This involves bi-directional transduction through a "bottom-up" flow of information from the molecular level to the mesoscopic level, thus redefining local interactions on a wider range topological scale; conversely, the membrane topology and derived tensions at that level generate a "top-down" transduction to the local properties of the individual membrane molecules with a regulatory power on their function (10,45).

It is well documented that the activity and kinetics of lipases and phospholipases (mostly those of the A_2 and C types) are correlated with the existence of lateral membrane defects and structural domain microheterogeneity (41,46,47), coexistence of bilayer and non-bilayer phases (48,49) and with the presence of non-substrate lipids or proteins in the interface (21,39). Lipid mixing-demixing processes and concomitant structuring of segregated domains with different lipid composition or phase state profoundly influence the pre-catalytic and catalytic steps of the enzymatic reactions (10,41,

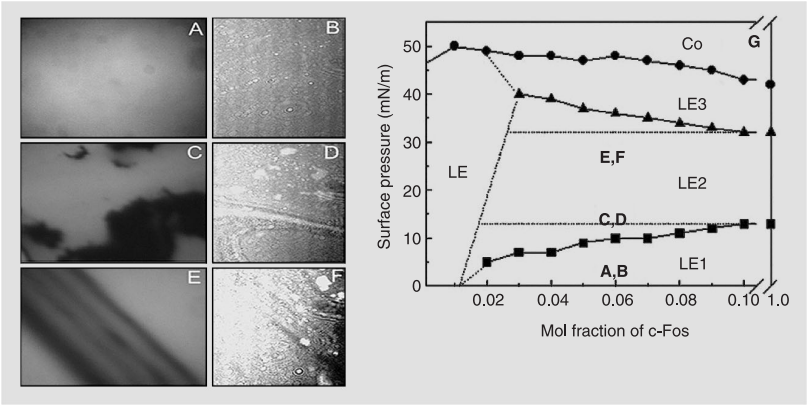

Figure 5. Surface pressure- and composition-induced phase transitions by c-Fos of mixed films with phosphatidylinositol biphosphate (PIP_2). The domain segregation is shown in the left panels and the respective phase diagrams in the right panels. Epifluorescence (panels A, C and E) and Brewster angle microscopy (BAM, panels B, D, and F). Microscopy images of a film with a 0.06-mol fraction of c-Fos 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 ten times that of the BAM images. Two-dimensional partial phase diagram of mixtures of PIP_2 (G) 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. *AB*, *CD*, and *EF* indicate the region of the phase diagram illustrated by the corresponding surface patterns on the left panels.

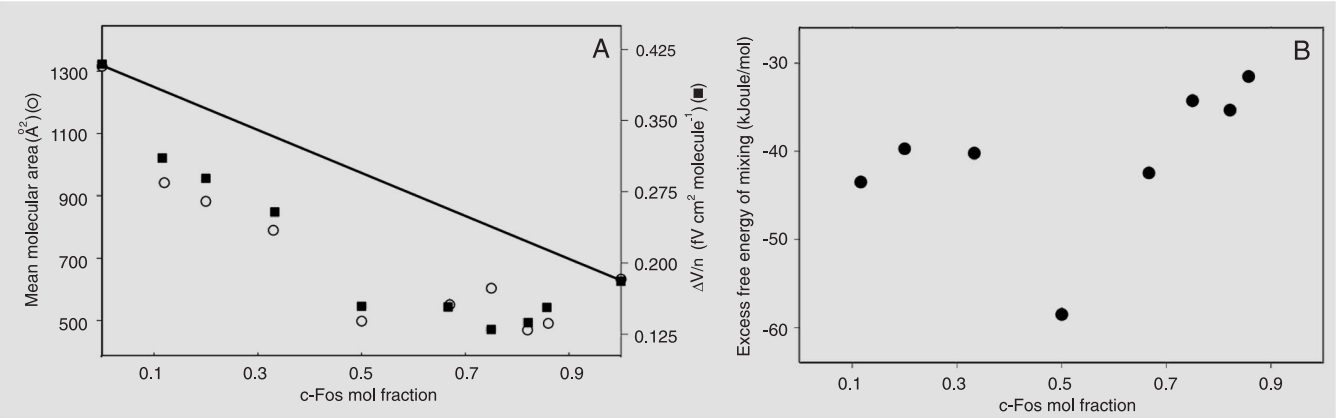

Figure 6. Interactions between c-Fos and c-Jun. *A*, Mean molecular area (open circles, left scale) and dipole potential density (filled squares, right scale) as a function of composition for Jun-Fos mixtures; the straight line corresponds to ideally mixed films. *B*, Excess free energy of mixing as a function of film composition.

45,49). A common structural element in all of these factors is the existence of lateral and/or transverse packing defects and interface tensions introduced by changes of lipid composition, anisotropic interactions, phase coexistence and connectivity of the domain lattice (10,21,41,45,49). Within this context, critical amounts of non-substrate lipids or proteins that may induce or disrupt specific surface super-structuring (10,45,47) over ranges that exceed the local intermolecular interactions become important membrane-regulating factors, and some of the topological variations may be induced by the phosphohydrolytic enzymes themselves.

The formation by a membrane-associated enzyme of a lipid product with markedly different surface properties and interactions with the parent substrate generates a major thermodynamic problem in a local micro-region (7,10, 21). This is because of the emergence of local tensions due to variations of the relative composition in lipid species having different miscibility properties. These tensions can only be resolved through long-range variations of the membrane topography involving compositional and/or phase domain segregation, super-lattice structuring and curvature alterations (3,4). For sphingomyelinase, we could demonstrate that the enzyme not only actively modifies the surface topography but also that the dynamic features and shape variations of the latter finely regulate the time-course and extent of the enzymatic reaction (10,45).

Sphingomyelinase preferably degrades sphingomyelin in the liquid-expanded state, both in monolayers and bilayer vesicles (21, 49). The steady-state reaction is preceded by a pre-catalytic latency period that involves enzyme adsorption and a rate-limiting interface activation step (21). We were able to show the real-time dynamic sculpturing of the surface topography by sphingomyelinase through the organized formation, morphological evolution, and super-structuring of ceramide-enriched domains under conditions of controlled and known intermolecular organization of the substrate (45). A succession of discrete shape transitions and lateral distribution of domains at defined times during the reaction underlay the topography generated by the activity of the enzyme, that is not found in enzyme-free mixtures of sphingomyelin and ceramide of the same composition (10,45). Advanced image processing routines in combination with time-resolved epifluorescence microscopy (45) on Langmuir monolayers revealed: i) spontaneous nucleation and circular growth of ceramide-enriched domains after injection of sphingomyelinase into the subphase of the sphingomyelin monolayer, ii) domain-intrinsic discrete shape transitions from circular to periodically undulating shapes followed by a second transition towards increasingly branched morphologies, iii) lateral superstructure organization into predominantly hexagonal domain lattices, iv) formation of super-superstructures by the hexagonal lattices, and v) rotationally and laterally coupled domain movement prior to domain border contact (Figure 7).

The cross-talk between lateral domain structures and dipolar electrostatic fields added new perspectives to the study of the mechanisms of phospholipase-mediated signal transduction in biological membranes. The dynamic structural rearrangements couple to generate two-dimensional electrostatic fields originating from the specific orientation and magnitude of the lipid dipole moments in the ceramide-enriched domains. The unfavorable increase of the dipole moment density due to the accumulation of ceramide in segregated domains because of its immiscibility with the substrate sphingomyelin, in competition with the tendency to relaxation of the domain line tension at the boundary, induces shape ondulations (45). On the other hand, once the domains exceed a threshold dimension, morphology, and proximity, the electrostatic field due to the domain resultant dipole moments causes self-organization by lateral repulsion and determines the dynamic formation of hex-

agonal lattices and super-structuring over the long range (Figure 8). Lateral enzyme-specific out-of-equilibrium organization of lipid domains represents a new level of signal transduction (45) from local (nm) to long-range (μm) scales, which controls and regulates information exchange among various hierarchical levels of the membrane function. This includes the variations in composition due to insertion of regulatory lipids and proteins that inherently modify the structure (4,21,39). This can be correlated with alterations of the membrane surface in terms of domain segregation and topological restructuring (10,41,45). In this manner, local and/or supramolecular information generated by one phosphohydrolytic pathway may be transduced to a non-biochemical regulation of other enzymatic pathways that do not even share common substrates or products. This was specifically shown for the cross-talk between the PLA_2- and sphingomyelinase-driven reactions whereby they can become mutually amplified or dampened by fluctuations of the lateral surface pressure controlling the lateral lipid packing and dipolar organization (21). Recently, it was shown that the lateral packing and in-plane elasticity of the lipid-protein interface can also regulate the activity of the GPI-anchored protein alkaline phosphatase and modulate information exchange between the interface and the aqueous environment (50).

Surface topography of whole myelin monolayers and influence of Folch-Lees proteolipid protein on domain structuring and dynamics

In contrast to the commonly used binary or ternary lipid and lipid-protein model membrane systems, multicomponent systems such as natural membranes can establish a wide diversity of interactions among their lipid and protein components giving rise to an increasing number of ways to reduce local tensions derived from molecular asymme-

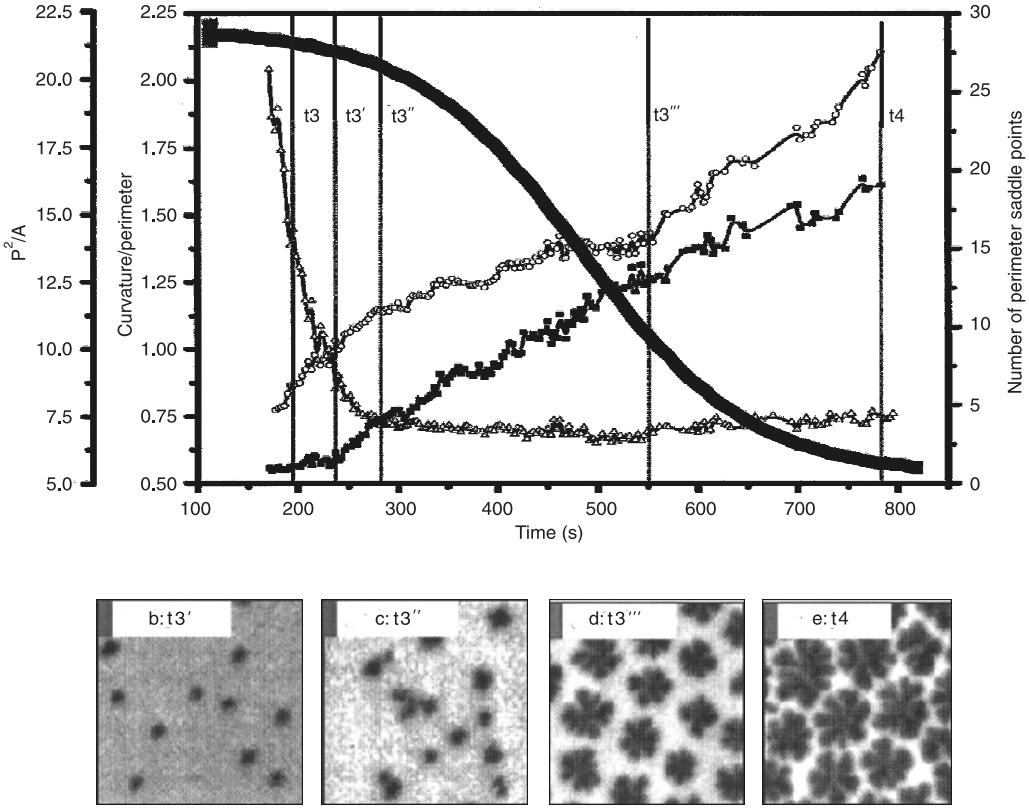

Figure 7. Morphological changes induced by sphingomyelinase. Shape transitions of ceramide-enriched domains during the time course of the reaction against sphingomyelin monolayers at 10 mN/m. The shape-sensitive parameter of circularity P^2/A (open circles), the perimeter-normalized curvature of the border trajectory (open triangles) and the number of saddle points (full squares) are shown as mean values (taken over 725 ceramide-enriched domains; see Ref. 45 as a function of time of the enzymatic reaction). The thick line represents the time course of sphingomyelin degradation as a function of time from 100 s after enzyme addition in the subphase (0% degradation) to 800 s (about 70% degradation). The lower panels show representative images indicating discrete stages of the surface morphology at the stated times.

tries. The resultant loss of cooperativity, as the system becomes more complex, induces an apparent gradual (diffuse) response to changes in temperature, pressure and compositional changes (4,25,51). Nevertheless, although some responses become buffered in multicomponent systems, marked rearrangements transducing local stress over wider range scales occur under the effects of some perturbing factors that introduce large tensions in membranes. Examples of the latter are HII phase-forming molecules (19-22), cholesterol (52,53), and proteins (54). Cholesterol is known to induce, above a certain threshold, the coexistence of immiscible liquid phases (52). This effect has been recognized as the basis for the surface heterogeneity of monolayers prepared from some natural lipid membranes (55), and is postulated as an important factor in the origin of the generically called "raft domains" (17). Membrane proteins embedded in membranes can behave as strong perturbing agents acting at different levels of organization.

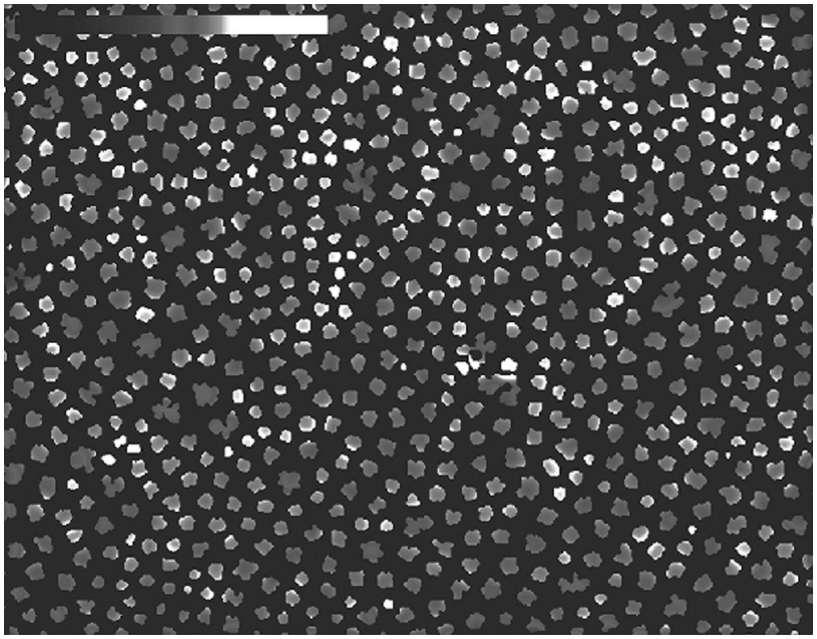

Figure 8. Repulsive electrostatic energy between enzymatically generated domains. Intensity-coded representation of repulsive electrostatic energy between ceramide-enriched domains generated by sphingomyelinase at t3" < t < t4 (see also Figure 7). For each domain the energy was calculated pixelwise with respect to the six nearest neighbor domains (45). The bar code at the top left represents a domain energy W of 10E-21-6E-20 J.

They not only affect the immediate lipid environment but also influence the long-range thermotropic behavior of lipids according to their tendency to undergo aggregation or partitioning into particular lipid phases. Furthermore, peripheral and integral proteins have been shown to induce phase domain segregation in model lipid membranes consisting of few components (4,8, 9,24). Solvent-solubilized whole myelin membranes can form monomolecular layers at the air-water interface (23,56). We first reported (57) that the surface topography of a compositionally complex lipid-protein natural membrane, under precisely controlled conditions of intermolecular organization, is characterized by the coexistence of at least two liquid phases at low- and high-lateral surface pressures with a transition from round-border domains to fractal domains occurring during compression (Figure 9a-d). Prior to our studies, the presence of liquid-liquid surface immiscibility was described in complex monolayers containing cholesterol prepared with lipids from red blood cell membranes (55). In agreement with our observations on the complex natural myelin interface (57), fluid-phase coexistence was subsequently found in bilayer vesicles made from natural kidney brush border membranes (58) and was also recently reported in bilayer vesicles prepared with a natural mixture of lipids and pulmonary surfactant protein (59). In whole myelin monolayers we identified the contribution of some protein and lipid components to the topographic organization. A segregation of the protein components excluded from domains containing condensed lipids such as cholesterol and galactosylceramide at all surface pressures pointed to compositional immiscibility as an important factor for the domain phase coexistence (25).

The protein-depleted mixture of myelin lipids at low-surface pressures shows coexistence of cholesterol-enriched and cholesterol-depleted liquid phases organized as

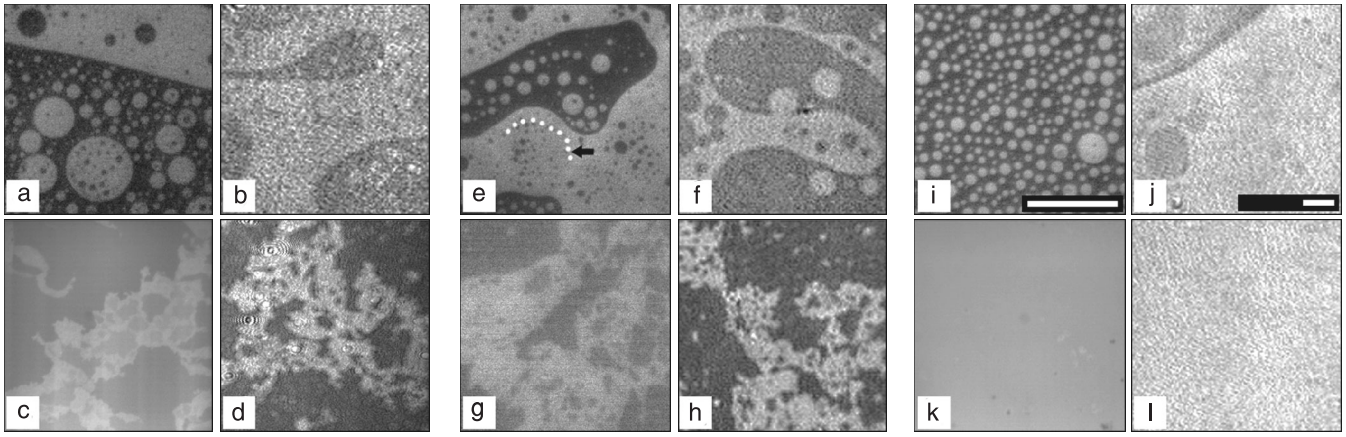

Figure 9. Surface topography of whole myelin and myelin lipid monolayers. Representative images of whole myelin extract monolayers (a, b, c, and d), lower phase extract monolayers (e, f, g, and h) and myelin lipid monolayers, except gangliosides (i, j, k, and l) containing 0.5 mol% of egg-Rho-PE as seen by epifluorescence microscopy (a, c, e, g, i, and k) and Brewster angle microscopy (b, d, f, h, j, and l). The lower phase extract contains only proteolipid protein as the protein component. The upper row of images corresponds to surface pressures between 1 and 1.5 mN/m during the second compression. The arrow in image "e" is pointing to the border of the gray phase (outlined with a dotted line) that is surrounded by a rim of bright phase. The images in the bottom row were taken between 35 and 37 mN/m. The reference bars in epifluorescence and Brewster angle microscopy images represent 150 µm.

rounded domains of homogenous size distributed in rather regular lattices (Figure 9i,j) but fails to undergo the topographic changes involving the formation of fractal domains under increasing compression that is characteristic of whole myelin monolayers (compare Figure 9k,l with Figure 9c,d). On the other hand, one of the major protein components of myelin membranes, the Folch-Lees PLP (about 50% by weight of the protein fraction), when mixed with myelin lipids in the absence of all the other proteins (51) is capable of reproducing the topographic organization of the whole myelin monolayer in a concentration-dependent manner (Figure 9e-g).

The addition of PLP to myelin lipids preserves the liquid character of the coexisting phases at low-surface pressures but modifies the size and shape distribution of domains (compare Figure 9i,j with Figure 9e,f). At high-surface pressures the PLP overrides the tendency of the lipids to merge, and the surface aggregation of PLP-enriched fractal domains provides a topographic explanation for the surface heterogeneity of the monolayer. The fractal nature of the structure indicates that it originates from a non-equilibrium process (possibly an out-of-equilibrium aggregation of PLP). In monolayers where the PLP has been diluted, a pattern of disconnected domains induced at high-surface pressures becomes progressively indistinguishable from that of monolayers with a larger content of PLP. This is due to aggregation with time of the individual domains, thus forming large fractal clusters (Figure 10). Interestingly, the character of the pattern is fully determined by the presence of PLP independent of its concentration, although the latter determines the rate for its acquisition (51). Thus, the dynamics of the out-of-equilibrium protein-induced surface topography depends on the interplay of time and the concentration of PLP in the monolayer. Moreover, the aggregation process of fractal clusters is vectorially directed in time since the large clusters do not reversibly disperse and reform under successive expansion and recompression cycles (51), thus storing information content regarding both time-dependence and structural morphology.

In summary, the Folch-Lees PLP, as a single protein component, constitutes at least

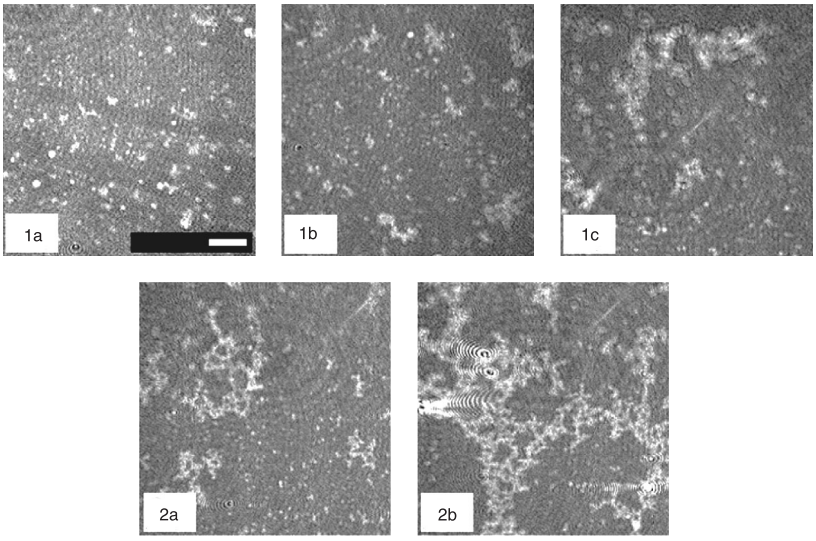

Figure 10. Dynamic structuring of the fractal pattern in mixtures with proteolipid protein (PLP). Representative Brewster angle microscopy images of a film of myelin lipids (except gangliosides) containing 5.3 x 10^{-4} mol fraction of PLP. The monolayer was compressed up to 37 mN/m and was left at the same surface pressure for 100 min. Images 1a, 1b and 1c are representative of the topography of the films at 2, 50, and 100 min, respectively, after the target surface pressure was reached during the first compression. Images 2a and 2b show the relaxation pattern after 10 and 70 min elapsed from the time when the film was brought to 37 mN/m during the second compression. The scale bar represents 300 µm.

one of the fundamental structuring factors capable of determining the pattern and dynamics of the surface topography in a manner indistinguishable from that observed in the whole myelin membrane monolayers that contain all the protein and lipid components (57).

Differing from the view of proteins as passively partitioning according to the features of pre-assembled lipid domains in cell membranes (8), our results directly demonstrate that some proteins such as PLP can have an active role of paramount importance and should be considered as key sculpturing factors determining membrane structuring over the long range. In addition, the presence of the protein brings about features of microheterogeneity in the pattern and dynamics of the surface topography, thus conveying molecular to supramolecular differential responses over to variations of the molecular packing (and surface pressure). Simultaneously with the directionality of out-of-equilibrium processes, the protein-induced effects described indicate the capacity of membrane-associated proteins to store and vectorially convey information with respect to membrane perturbations and dissipation of surface tensions. This cannot be overlooked within the context of the extraordinary capability of membranes to act as sensing, transducing, and modulatory manifold elements for cell function as bio-electro-mechano-chemical devices.

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