FEBS Letters 584 (2010) 1653–1658

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Stability of lipid domains

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ARTICLE INFO

Article history:
Received 2 November 2009
Accepted 18 December 2009
Available online 27 December 2009

Edited by Wilhelm Just

Keywords:
Lipid–lipid interaction
Cholesterol
Sphingolipid
Liquid ordered phase
Line tension

ABSTRACT

Membranes with simple lipid composition exhibit complex phase behavior. Ordered and disordered liquid phases can coexist in cholesterol-containing membranes with lipid compositions resembling biological membranes and at physiological temperatures. Research during the last years suggests that these lipid domains play a role in the organization of biological membranes. Understanding the principles that govern the formation and stability of lipid domains is of great importance to build a model that properly describes membrane structure and function. In this review, we describe the current knowledge of the chemical and physical basis of lipid domains and its application to biological membranes.

1. Introduction

Cellular membranes are liquid films that form closed volumes and serve as semi-permeable barriers, thus, defining the cell itself and its organelles. Membranes fulfill additional functions that are necessary for life, like the directional transport of materials, energy and information. As a consequence, a number of reactions happen within cellular membranes, including energy production, the biosynthesis of its components or the formation of complexes between biomolecules. The two-dimensional nature of membranes provides a platform in which these reactions are compartmentalized and biochemical efficiency is optimized. Another important property of membranes is their ability to deform, which is necessary for the adoption of specialized shapes and for budding, fission and fusion. These processes are essential for membrane trafficking, fertilization, and cell division and differentiation.

Scientists have long been trying to understand how cellular membranes are organized and the molecular mechanisms associated with their functions. The analysis of their constituents, which is becoming increasingly precise, has revealed an extremely complex composition. Cellular membranes are mainly formed by proteins and lipids. There is a huge variety of membrane-associated proteins, which account for around 30% of genomes [1]. Lipids form the membrane matrix thanks to their amphipathic structure: they contain a hydrophilic headgroup and a hydrophobic tail that drive spontaneous organization into a bilayer. The combination of multiple headgroups and hydrophobic tails gives rise to thousands of different species. And although some lipids have been involved in specific interactions, the reasons for the evolutionary selection of such an intricate lipidome are not well understood yet.

The relationship between lipid composition and function is reflected in the heterogeneity found in biological membranes, which can be found at three levels. First, the composition of membranes varies not only with cell type, but it also differs between the organelles within the same cell. Second, the composition of each of the leaflets that forms the bilayer is different, and this transversal asymmetry is actively maintained with specific mechanisms. Third, there are also lateral heterogeneities within the membrane plane that spatially compartmentalize certain cellular processes. It is generally assumed that the composition of each cellular membrane has evolved to optimize the functions associated with it. For a review on the differential localization of lipids, see [2].

Since the fluid mosaic model proposed by Singer and Nicholson, in which the membrane is seen as a homogeneous fluid, several theories considered the presence of lateral heterogeneities in membranes [3]. The controversy generated by the "raft" hypothesis in the late 90's has brought much attention to the issue of lateral organization of membranes. This theory predicts the existence of lipid and protein microdomains that are enriched in cholesterol and sphingolipids and have a functional role in processes like signaling, membrane trafficking or viral infection. Despite intense research, the actual nature and functioning of lipid rafts remains poorly understood. However, the last years have seen enormous progress in our understanding of the lateral organization of membranes. In this review, we describe the current knowledge of the stability of lipid domains, meaning the physical forces that

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.12.036

determine their existence and behavior, and its connection to the organization of cellular membranes.

2. Lipid–lipid interactions

Much of our knowledge about membrane organization comes from model systems. The chemical composition of lipids determines the interactions between adjacent molecules of the same and different species within the membrane. Preferential interactions between certain lipid molecules in a lipid mixture are involved in the clustering and segregation of lipids within the membrane matrix leading to the formation of domains.

In model systems, lipids exhibit a rich phase behavior with multiple phase transitions that depend on their chemical composition and on temperature. A phase is a portion of the system with uniform chemical and physical properties. Lipids dispersed in water can organize into lamellar and non-lamellar phases. The former is the most commonly found in biological systems. Lamellar, or bilayer, phases can be liquid or solid, depending on the translational and conformational order of the lipid chains [4]. In the solid ordered (s_o) phase, the acyl chains of lipids are in an extended conformation and the lateral diffusion coefficient in the plane of the membrane is very low. The liquid disordered (l_d) phase is characterized by conformational freedom and a higher diffusion coefficient. Cholesterol, with its flat and rigid structure, can associate with lipids and form a liquid ordered (l_o) phase in which the conformational order is high, but the diffusion coefficient is only slightly lower than in the l_d phase. Interestingly, the l_d and l_o phases can coexist in a situation of liquid–liquid immiscibility. Understanding the physical and chemical properties of membranes exhibiting lipid domain coexistence and their possible relationship with the structure of biological membranes is a very active area of research.

The energy differences between distinct lipid/lipid interactions govern their preferential association and, subsequently, their spatial distribution in equilibrium. For example, in a binary mixture of species A and B, there are three types of lipid–lipid interactions AA, BB and AB. These interactions can be described thermodynamically with the unlike nearest-neighbor interaction parameter, ω_{AB}, which corresponds to the difference between the AB interaction and the average of AA and BB interactions [5]. If $\omega_{AB} < 0$, the unlike interactions AB are favored over the like interactions, and vice versa in the case of $\omega_{AB} > 0$. The physical forces that contribute to ω_{AB} are not defined, but contain all effects that are significant for a nearest-neighbor interaction, such as the conformational entropy of acyl chains, the London dispersion forces, hydrogen bonds, hydrophobic interactions and electrostatics repulsions in the case of charged membranes. The values for ω_{AB} can be obtained from DSC (differential scanning calorimetry) and Monte Carlo simulations, NNR (nearest neighbor recognition), ITC (isothermal titration calorimetry) or from the analysis of lipid phase diagrams [5]. Assuming a simple lattice model for the bilayer, small values that vary between −300 and +300 cal/mol are typically obtained for most unlike lipid–lipid interactions. Usually they are repulsive, indicating that lipids prefer to interact with neighbors of the same type, but in some cases, for example the interaction with cholesterol with sphingomyelin (SM) or with saturated phosphatidylcholine (PC), $\omega_{AB} < 0$ and the interaction is attractive [5].

In binary mixtures of a phospholipid and cholesterol, the preferential interaction of the latter with the ordered acyl chain state of the former leads to phase separation depending on the nature of the phospholipids [5]. In the l_d phase, ω_{AB} between cholesterol and saturated PC is close to zero. However, cholesterol has slightly repulsive interactions with unsaturated PC [6] and very unfavorable interactions with polyunsaturated PC [7], due to the flexibility that the double C–C bonds promote in the acyl chain [8]. This ordering that cholesterol induces in the unsaturated PC has a positive entropic cost. Estimation of the interactions between SM and cholesterol in the l_d phase give a value of ω_{AB} around −200 cal/mol, which is more favorable than the cholesterol/PC interactions in the l_d phase, but less attractive that cholesterol/SM in the l_o phase ($\omega_{AB} \sim -350$ cal/mol) [9]. Indeed, the interaction between cholesterol and saturated phospholipids is especially important in the l_o phase, where the content of cholesterol is high. For example, the interaction between saturated PC and cholesterol is one of the most favorable found between lipids, and the interaction of cholesterol with SM is similar or even more attractive [5]. In the l_d phase, the interactions between unsaturated PC and cholesterol are small, but it becomes repulsive in the l_o phase [10].

In ternary mixtures of di-oleoyl-phosphatidyl-choline/di-palmitoyl-phosphatidyl-choline/cholesterol or palmitoyl-oleoyl-phosphatidyl-choline/SM/cholesterol, which clearly show phase separation, the thermodynamics of lipid–lipid interactions are slightly different [5]. Here, cholesterol interacts more favorably with the saturated PC or SM, which are in the ordered state, and the interaction is repulsive with the disordered, unsaturated PC. Interestingly, several studies have concluded that the phase behavior of these ternary mixtures can be explained with three pairs of binary interactions: the unfavorable interactions between the two phospholipids, the favorable interactions between cholesterol and the ordered phospholipid and the unfavorable interaction of cholesterol with the disordered lipid are sufficient to create a closed loop of phase coexistence in the phase diagram [10,11]. An especially relevant observation is that very simple lipid mixtures show a surprisingly complex behavior.

3. Effect of line tension and membrane deformations

As observed by AFM and X-ray scattering measurements [12,13], the bilayer in the l_o phase is thicker than in the l_d phase. This is due to the extended conformation of the lipid acyl chains in the l_o phase and leads to a height mismatch at the domain edge in phase coexisting membranes. To minimize the unfavorable effect of the exposure of the hydrophobic tails of lipids to the water solvent, the membrane elastically deforms at the domain interface [14]. The height mismatch, together with steric interactions at the phase interface, has an energetic cost that depends on the length of the phase boundary. This effect is called line tension and is applicable when the membrane is approximated to a continuum solvent, instead of considering the individual lipid molecules. To minimize line tension, and hence, the energy of the system, the membrane tries to optimize the perimeter of the phase boundary. As a consequence, the lipid domains tend to adopt a circular shape and, in some cases, bud out of the membrane plane.

Line tension has been calculated from experiments in giant unilamellar vesicles (GUVs) and in supported bilayers with phase separation [15,16]. By systematically changing the height mismatch, we showed that the line tension has a great effect on the lateral organization of the membrane (Fig. 1) [17]. The domain melting temperature, growth rate and size in equilibrium exhibited a strong correlation with the line tension.

If line tension were the only parameter governing phase distribution, all domains in a membrane would merge with time into one big domain. However, a stable distribution of domain sizes can be found both in model and biological membranes. This indicates that other processes, in addition to line tension, affect the coalescence of lipid domains. Beside entropic effects and lipid recycling due to membrane trafficking in living cells, there are membrane mediated forces that inhibit domain merging [18,19]. Depending on bending stiffness, line tension and membrane

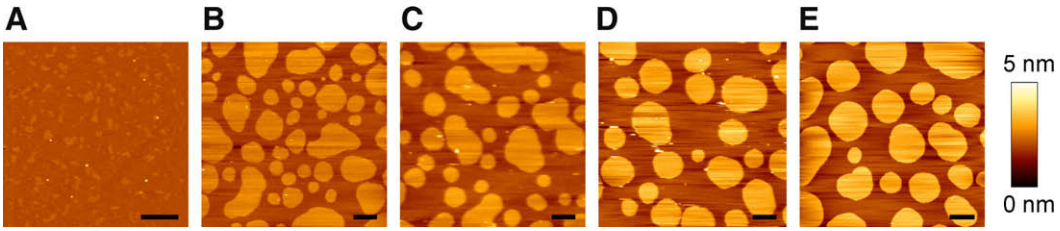

Fig. 1. Effect of line tension on lipid domains. AFM images of domain-coexisting membranes with different phase height mismatch. The lipid composition of the membranes was: (A) DEruPC:SM:Chol (2:2:1), (B) DEiPC:SM:Chol (2:2:1), (C) DOPC:SM:Chol (2:2:1), (D) DPoPC:SM:Chol (2:2:1) and (E) DMoPC:SM:Chol (2:2:1). l_o domains correspond to the thicker regions of the membrane and a color coding has been used so that bright and dark regions represent l_o and l_d phases, respectively. Scale bar 20 μm.

tension, domains merge up to a critical size at which they switch from a flat to a dimple shape. This causes a deformation of the membrane in the area surrounding the domains gives rise to elastic interactions between domains that inhibit their coalescence.

Membrane proteins and other membrane-embedded molecules can affect the lateral organization of lipid domains. We combined AFM and confocal microscopy to show that binding of a membrane-active peptide derived from the apoptotic protein Bax reorganized phase-separated membranes into irregular domains (Fig. 2). This was linked to a reduction of the line tension at the domain interface [20]. Moreover, glycolipid clustering induced by Cholera toxin subunit B or Shiga toxin induced phase segregation in GUVs and in giant plasma membrane vesicles [21,22].

Recently, considerable attention has been brought to critical fluctuations in lipid membranes. Veatch et al. showed that composition fluctuations of a few tens of nanometers were present in model membranes containing cholesterol near critical points. In such membranes, the line tension decreased to zero when the critical point was approached from low temperatures and the system evolved in agreement with the two-dimensional Ising model for critical phase transitions [23]. A similar critical behavior was also found in giant plasma membrane vesicles derived from cells [24]. This is of special interest because this type of vesicles has a complex composition closer to that found in cell membranes (see below). Because of this, the authors hypothesized that the plasma membrane composition of mammalian cells is close to a miscibility critical point, so that composition fluctuations may exist and be related to rafts.

The interactions between lipids across the two leaflets that form the bilayer had received little attention until the recent interest in domain coupling within phase separated membranes containing cholesterol. Using asymmetric bilayers, Tamm and coworkers showed that ordered domains can be induced in the inner leaflet of supported bilayers by domains in the outer leaflet [25]. Interestingly, a recent report shows that tuning the lipid composition can induce or suppress domain formation across leaflets of pure-lipid asymmetric bilayers [26]. In the case of gel phases, asymmetric membranes were shown to evolve differently, depending on the relative area fraction of the gel phase between the two leaflets [27]. However, the magnitude of domain coupling across bilayers and the mechanisms involved remain poorly understood. Though cholesterol probably plays a role, it is likely that the monolayers interact at the membrane midplane in a similar way that the l_o and l_d phases interact at their interfaces [28].

Membrane curvature has been shown to have an effect on the lateral organization of membranes. For example, the lipid distribution in thin tubes pulled from GUVs with raft-like composition is different from that of the rest of the vesicle, indicating curvature-induced lipid sorting [29]. This depends on the collective behavior of lipids and can be affected by lipid-clustering proteins [30,31]. Moreover, it was shown that beyond a certain threshold, the membrane curvature governs the spatial distribution of the l_o and l_d domains due to differences in the bending rigidity between the phases [32].

4. Domains in biological membranes

There are very important differences between model and cellular membranes that limit the applicability of the results obtained with the first to the understanding of the second. One of the most important ones is that most experiments with model membranes are carried out under equilibrium conditions, while cellular membranes in living cells are in a constant turnover. And although model membranes have proven extremely useful for the understanding of the general principles that govern membrane organization, one should be cautious when extrapolating conclusions.

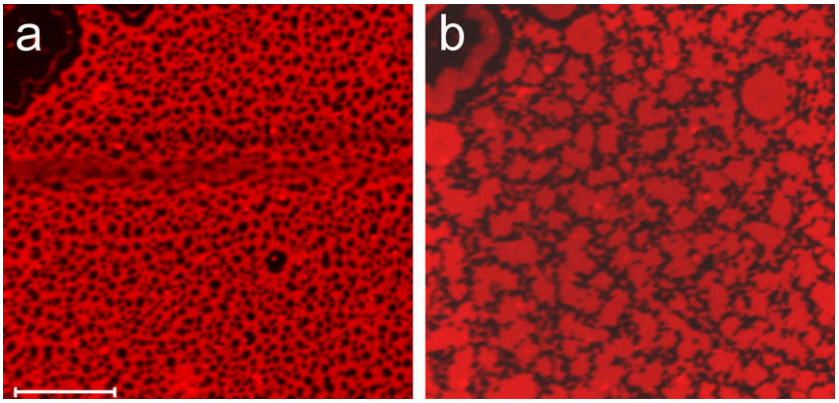

Fig. 2. A membrane-active peptide derived from Bax induces lipid domain reorganization. (A) Confocal image of the membrane before peptide addition. (B) 10 min after incubation with the peptide at a lipid to protein ratio of 109. Membrane composition: DOPC:SM:cholesterol (1:1:0.67). The dark domains correspond to the l_o phase, while the ld phase has been stained with 0.05% DiD (shown in red). Scale bar 20 μm.

Some attempts have been made to characterize the behavior of model membranes out of equilibrium, mainly in following the evolution of lipid domains upon changes in temperature or mechanical stress [17,33]. In those examples, the perturbation of the systems provokes directional evolution towards equilibrium, which is still very different from the steady state of the membrane found in non-stimulated cells. However, it gets closer to the situation in some cellular processes, like the formation of signaling synapses upon ligand binding, which is also directional.

Another big and evident difference is the complexity of composition. Membranes in cells contain far more than three or four components with features that can vary gradually, as the number of unsaturations or the length of the acyl chains in the case of lipids. This will not only affect lipid–lipid packing in membrane areas that resemble both l_o and l_d phases, but also the nature of the boundaries between lipid domains and the membrane matrix. For example, the hydrophobic mismatch found between l_o and l_d phases in ternary lipid mixtures would very likely be compensated in cell membranes by lipids with intermediate length, thus decreasing the abruptness of the boundary and, thereby, the line tension. In addition, certain membrane proteins tend to concentrate at the packing defects of the domain edges, hence also contributing to the reduction of the line tension [20]. And one should keep in mind that the high protein concentration in cellular membranes creates a crowded environment in which lipid–lipid packing is affected by proteins, probably with a stronger effect inside the more ordered raft-like domains.

The heterogeneity of rafts is also related to the complex composition of cellular membranes. For example, lipidomics analyses have shown that isolated rafts, enriched in different GPI-anchored proteins, have significantly different levels of several lipids, including cholesterol [34]. This led to the idea that cellular rafts are heterogeneous in protein and lipid composition and, as a consequence, also most probably in stability. Other interesting concepts have emerged from lipidomics approaches. The fact that the lipidome of the HIV virus resembled the composition of the lipid rafts of the cells from which it budded constituted a strong evidence for the existence of rafts in intact cells [35]. Another intriguing result from lipidomics studies is the observation that raft-associated glycerol–phospholipids contain high amounts of mono-unsaturated acyl chains, in contradiction with the more or less established idea that rafts are preferentially formed by saturated phospholipids [3].

Although lipid–lipid interactions can drive domain formation in model membranes, the situation in cells is much more complicated, with many more lipid and protein components that will affect the process. In fact, the current definition agreed on at the 2006 Keystone symposium of Lipid Rafts and Cell Function is as follows: "Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" [36]. According to this description, proteins play an essential role in the clustering of rafts and most likely they also have an effect on the chemical and physical parameters that govern their stability. However the underlying mechanisms are not properly characterized yet. It could be that certain raft proteins tend to interact with raft lipids, so that when these proteins cluster they bring the lipids together and form a raft. For example, MAL is a membrane protein implicated in lipid-raft-mediated apical sorting that forms oligomers via transmembrane protein–protein binding domains. Magal et al. have recently proposed that hydrophobic mismatch is implied in the association of MAL with lipid rafts and that the combination of these two effects could have a role in raft formation [37].

A model membrane system that closely resembles the complex composition of the plasma membrane of cells is the so-called giant plasma membrane vesicles (GPMVs). However, they are less controlled systems and the methods reported so far for their preparation consist of relatively harsh chemical treatments that probably affect the membrane composition [21,38]. Nevertheless, these systems have already provided insight into the organization of biological membranes. For example, they exhibited phase separation when cooled down below 25 °C [38], or upon cholera toxin-mediated cross-linking at 37 °C [21]. The segregated domains resembled in fluidity the l_o and l_d phases of ternary model systems, and several membrane proteins were shown to redistribute between the two phases. Recently, composition fluctuations associated with the existence of a miscibility critical point and the effect of cholesterol on the phase separation have also been demonstrated in these complex membrane systems [24,39]. However, the membrane alterations associated with the preparation protocols have been shown to play a role in the partitioning of raft-associated proteins. Giant vesicles prepared as in Baumgart et al. [38] exhibited an ordered phase with much higher lipid packing when compared to the ordered phase prepared as in Lingwood et al. [21] and, interestingly, the differences in lipid packing accounted for the differential partitioning of raft proteins in both systems [40].

All this said, the fact is that lipid domains cannot be directly observed with normal optical methods in intact cells. If they exist, they must be very small and transient. During the last years, powerful techniques have been developed or improved to allow the study of membrane structure on the nanometer scale. For example, the role of the cytoskeleton on membrane organization has been investigated with single particle tracking (SPT), which follows the motion of individual membrane molecules. Analysis of the single trajectories of several types of membrane proteins and lipid analogs showed that all membrane molecules, including raft and non-raft markers, exhibit hop diffusion and transient confinement in nanometer-sized zones in a cytoskeleton dependent manner [41–43]. Kusumi and coworkers proposed the "picket and fence" model for membrane organization: the membrane is compartmentalized by the actin-based membrane cytoskeleton that acts like "fences", and anchored by transmembrane proteins that would be the "pickets" [44]. In an alternative approach, Lenne and coworkers varied the size of the focal volume to measure fluorescence correlation spectroscopy (FCS) at different spatial scales [45]. Interestingly, when they calculated the so-called FCS diffusion laws with sub-diffraction apertures, they could access the nanostructural organization of cell membranes and estimate the size of lateral heterogeneities [46]. With a similar strategy of reducing the measured membrane area under the diffraction limit, Eggeling et al. combined FCS with STED illumination to detect and characterize the size and dynamic properties of raft-like nanodomains [47]. HomoFRET (Förster resonance energy transfer between equal fluorophores), has also proven very useful to investigate the organization of the plasma membrane in living cells. Mayor and colleagues found that GPI-anchored proteins form cholesterol-dependent nanoclusters, unlike other transmembrane proteins with random distribution [48]. The distribution and endocytosis of protein domains could be affected by cross-linking of GPI-anchored proteins, which indicates a functional, lipid-dependent clustering [49]. A high temporal resolution analysis of the cluster dynamics showed that they are immobile and that the redistribution dynamics between monomers and clusters is heterogeneous. Interestingly, depletion of cortical actin affects the dynamics and organization of the nanoclusters [50]. All these examples support the existence of a heterogeneous organization of the membrane at the nanometer scale. However, if the basic phenomena observed in model membranes apply, it is not understood yet why the lipid domains do not grow bigger. To explain this paradox, some cellular

processes have been proposed to control domain size, like the constant membrane recycling via the exo- and endocytic pathways, or the interaction of the cytoskeleton with the membrane.

5. Conclusions

In this review, we have described the current knowledge regarding the formation and stability of lipid domains. In model membranes containing cholesterol, favorable interactions between cholesterol and a saturated lipid, together with repulsive interactions between saturated and disordered phospholipids, and between cholesterol and disordered phospholipids, are enough to yield a closed loop of phase coexistence in the phase diagram. Under conditions of phase coexistence, the difference in thickness between the two phases is a major contributor to the line tension at the domain boundary that promotes domain coalescence and budding. However, a stable distribution of domain sizes is usually found at equilibrium due to the existence of elastic membrane-mediated interactions that inhibit domain merging. Interestingly, membrane-associated proteins and peptides have been shown to affect the lateral organization of membranes. With caution, some of the results obtained in model membranes can be extended to biological membranes. However, the latter have a much more complex composition and are in constant turnover. Better model systems are needed that overcome these issues, at least partially. Recently developed techniques that sample the membrane on the nanometer scale have proven the existence of lateral heterogeneities in the plasma membrane of living cells. In spite of these advances, much work still needs to be done until we form an accurate picture of the mechanisms governing the formation and stability of lipid domains in living cells.

Acknowledgements

We thank Jakob Suckale for the careful reading of the manuscript. This work was supported by the Max Planck Society (A.J.G.S.).

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