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Review

Protein-promoted membrane domains

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Abstract

The current notion of biological membranes encompasses a very complex structure, made of dynamically changing compartments or domains where different membrane components partition. These domains have been related to important cellular functions such as membrane sorting, signal transduction, membrane fusion, neuronal maturation, and protein activation. Many reviews have dealt with membrane domains where lipid–lipid interactions direct their formation, especially in the case of raft domains, so in this review we considered domains induced by integral membrane proteins. The nature of the interactions involved and the different mechanisms through which membrane proteins segregate lipid domains are presented, in particular with regard to those induced by the nAChR. It may be concluded that coupling of favourable lipid–lipid and lipid–protein interactions is a general condition for this phenomenon to occur.

Keywords: Lipid sorting; Membrane protein; Lipid–protein interaction; Lateral segregation; Membrane organization

Contents

1. Introduction

The notion of biological membranes has radically changed over the last decades. From the fluid mosaic model [1], where the membrane was considered a homogeneous lipid media in which proteins floated, different experimental evidences have led to the current model which encompasses a very complex structure, made of dynamically changing compartments or domains where different membrane components partition. These domains are formed in a wide temporal and spatial scale, ranging from the nanometer [2,3] to the micrometer [4,5] and from ~10 ns [6] up to hours for some cell structures [7]. In all cases, regardless of size or duration, the molecules in such domains are continuously exchanged with those in surrounding membrane regions [5,8]. Although there is not a clear picture about the origin and structure of these domains, there are increasing evidences about its biological relevance. For instance, they have been related to important cellular functions such as membrane sorting [8–10], signal transduction [11–13], membrane fusion [4,14,15], neuronal maturation [16], and protein activation [17,18].

Abbreviations: DMPA, dimyristoyl phosphatidic acid; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; nAChR, nicotinic acetylcholine receptor; PA, egg phosphatidic acid; PC, egg phosphatidylcholine

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The first hints that biological membranes may not be as simple structures as predicted from the fluid mosaic model came from its complex lipid composition, with up to 500 different lipid species. It has been proposed that this vast number of structurally different elements would facilitate the "solvation" of the large variety of proteins that compound the membrane. It is not unreasonable to expect lateral and transverse tensions in this multicomponent chemical milieu which might be relieved by the segregation of immiscible components into separate domains. In accordance to these ideas experimental and theoretical evidences point out to the existence of lipid domains in both artificial and biological membranes [8,19–24]. From the very beginning and mainly due to the different model systems used for the experiments, two types of lipid domains have been considered: those caused solely by lipid–lipid interactions and those induced by proteins. This has led to a dual picture of biological membranes: that where lipid–lipid interactions direct a compartmentalization of the membrane, the proteins being sorted depending on its affinity for these lipid domains, or that where lipid–protein interactions direct the formation of lipid domains around membrane proteins. The more real picture should probably come from considering both, lipid–lipid and lipid–protein interactions. Thus, while lipid–lipid interactions *per se* may cause the formation of lipid domains, such domains may be modified by the presence of membrane proteins, especially if they have a high affinity for a particular lipid component. The reverse is also true, for a membrane protein to segregate a lipid domain, there should be adequate lipid–lipid interactions in addition to those between the protein and the lipids. In this sense, it is considered unlikely that pure lipid domains are self-sufficient to concentrate molecules to form functional platforms for biological processes in live cells. Therefore, the interplay of lipid-based domains and protein-mediated assemblies would generate stable functional domains in cell membranes [25–27]. This is supported by experimental results. For example, raft domains in the plasma membrane of non-polarized cells are normally small and highly dispersed, but their size can be modulated by oligomerization of raft protein components [25,26]. Also, the cytoskeleton possibly contributes to the final picture by providing barriers to long-range diffusion of lipid domain components and enabling those at the inner leaflet to segregate independently from those at the outer leaflet in live cells [13].

Although many reviews have dealt with membrane domains where lipid–lipid interactions direct their formation, especially the so-called raft domains, there are few reviews on protein-promoted lipid domains. This chapter deals with this latter issue, specifically with those cases where integral membrane proteins are involved.

2. Features of protein-promoted lipid domains

The initial evidences that membrane proteins modify its surrounding lipids came from electron spin resonance (ESR) experiments [28], where a population of motionally restricted lipids were detected, leading to the "lipid annulus" concept. The lipid annulus would be formed by the first shell of lipids around the transmembrane portion of the protein, with a residence time in such boundary region 10 fold higher than in the surrounding bulk bilayer. The higher affinity of specific lipids for such boundary region sometimes results in the observation of bound lipid molecules in the high-resolution structures of several membrane proteins, despite the presence of detergents [29]. Moreover, in cases such as that of Bacteriorhodopsin, the bound lipid seems to define a complete annular shell around the protein. A subtle variation of this concept are the recently proposed "lipid shells" [30]. These would consist of a more stable "lipid annulus", where long-term interactions take place between specific lipids and selected proteins. The lipid shells and the protein they surround would exist as mobile entities in the plane of the membrane and would be thermodynamically stable structures with large affinity for pre-existing caveolae/rafts, targeting the protein they encase to these membrane structures.

In both models, the lipid annulus or the lipid shells, only the properties of the lipids adjacent to the protein are expected to be modified. However, there are some examples where the effect of the protein goes beyond the boundary lipid layer. One of such examples is the Ca^{2+}-ATPase, an integral membrane protein composed of ten transmembrane segments accounting for up to 90% of the total protein of sarcoplasmic reticulum from skeletal muscle. Differential scanning calorimetry (DSC) studies on vesicles composed of a single lipid species suggest the presence of at least two types of lateral domains [31]. One would be formed by the annular lipids, which, as commented above, would consist of a layer of about 30 exchangeable, but conformationally inhibited lipid molecules directly adjacent to the protein. The other would be a secondary region of surrounding lipids with a disrupted packing, extending roughly 130–170 lipid molecules/protein. This concept of a disrupted secondary lipid layer may also apply to other transmembrane proteins, such as glycophorin [32] and bacteriorhodopsin [33]. In the case of glycophorin the boundary lipid could account at the most for 20 lipid molecules, not explaining the calculated 300 lipids which are perturbed by the protein [34]. The authors point to a hydrophilic interaction between lipid headgroups and the carbohydrate-carrying protein region. A report by Polozova and Litman [35] on rhodopsin is a good example of lateral lipid segregation in which the protein distinguishes between two lipid components. Rhodopsin is a G protein-coupled receptor and the major protein of retinal rod outer segment disk membranes, which is able to segregate domains enriched in polyunsaturated phospholipids. These domains are disposed around the protein and contain at least two layers of phospholipids, being enriched in polyunsaturated lipids by a factor of six approximately. Polyunsaturated lipids associate with rhodopsin, even when artificial membranes containing saturated and polyunsaturated lipids are in the liquid crystalline phase, this phenomenon being highly dependent on the presence of the protein itself and cholesterol. The authors point to a preferential interaction of cholesterol with saturated acyl chains as a key factor promoting membrane lateral separation, stressing the fact that not only lipid–protein interactions are important for a protein to segregate a lipid domain, but also adequate lipid–lipid

interactions are required. These results are supported by molecular dynamics simulations that reveal that rhodopsin has the ability to reorganize its solvation lipid shell having a 3-fold greater strength attraction for polyunsaturated acyl chains relative to saturated ones [36]. The extreme flexibility of polyunsaturated chains allows them to adapt to the ragged surface of the protein transmembrane α-helices and to penetrate between them at little or no intramolecular energy cost, that enables a large number of favourable contacts occurring at short distances, explaining their large affinity for the protein.

The glycoprotein of the vesicular stomatitis virus, named G protein, represents an example of a membrane protein able to segregate macroscopic lipid domains. It has a transmembrane domain as well as covalently bound fatty acids, although it is not a typical integral membrane protein since, in spite of its transmembrane domain, it is also stable in water solutions. This protein showed a preferential affinity for phosphatidic acid (PA) in multicomponent artificial lipid vesicles, causing the segregation of macroscopic domains enriched in this lipid as observed by fluorescence microscopy [37]. In different vesicles, these domains varied in size and in the degree of PA enrichment, but on the average they occupy 22% of the area of the vesicle and have 3–4-fold higher PA than its surroundings. Interestingly, the combination of this G protein with the other protein in the virus envelope, the soluble M protein, caused the formation of domains not only enriched in PA, but also in phosphatidylserine (PS), sphingomyelin (Sph), and cholesterol, being the presence of the two proteins and PA required for this to occur [4]. From experiments with different lipids, the authors conclude that the fatty acid composition was not as important for this process as the polar headgroup of the lipids. This example illustrates the complexity of this phenomenon, where the association of an integral membrane protein and a soluble one, modulates the composition of the segregated domain, although it is the strong interaction of PA and the G protein that originates the process. A synergistic effect has also been reported when cytochrome *c*, a peripheral protein, and cytochrome oxidase, a transmembrane enzyme, are reconstituted in the same lipid vesicle [38]. It was found that cytochrome oxidase restricts the motion of the first lipid shell around it, the so-called lipid annulus, but the presence of cytochrome *c* extends the effect to the second and even the third shell of lipids around this protein, possibly caused by the simultaneous binding of cytochrome *c* to these three lipid shells.

Another interesting example is that of the Folch–Lees proteolipid protein (PLP), since it was studied in native membranes derived from cells, rather than in artificial systems [39]. The role of this protein was studied in monolayers of solvent-solubilized whole myelin membranes. At low-surface pressures, the protein-depleted mixture of myelin lipids shows coexistence of cholesterol-enriched and cholesterol-depleted liquid phases organized as round domains of homogeneous size distributed in rather regular lattices, but fails to undergo the topographic changes involving the formation of fractal domains under increasing compression, characteristic of whole myelin monolayers. The addition to myelin lipids of PLP, one of the major protein components of myelin membranes, is capable of reproducing the topographic organization of the whole myelin monolayer in a concentration-dependent manner. It is concluded that PLP preserves the liquid character of the coexisting phases at low-surface pressures, but modifies the size and shape distribution of domains. At high-surface pressures, PLP overrides the tendency of the lipids to merge and the surface aggregation of PLP-enriched fractal domains provides a topographic explanation for the heterogeneity of the monolayers. The authors, however, do not attribute the effect of PLP to any particular lipid–protein interaction.

Oligomerization of membrane proteins have also been proposed as an important factor in lipid domain formation. This is the case of caveolin-1, an integral membrane protein and principal component of caveolae membranes “in vivo”. Its strong tendency to homo-oligomerize and its affinity for specific lipid components are envisaged as critical points in the caveolae origin [40].

A membrane protein could also perturb its surrounding lipids through hydrophobic matching [19,41]. This phenomenon results as a consequence of the energetically unfavourable exposure of the hydrophobic portion of a lipid or a membrane protein and may extend up to 10 to 20 surrounding layers of phospholipids into the bilayer [42,43]. To accomplish matching, the protein could minimize the exposed hydrophobic area either by reducing its effective hydrophobic length, or by clustering, or by changing its conformation [44]. In regard to the former, the hydrophobic length could be reduced by rotating the tryptophan residues located at the end of the transmembrane α-helices about their $C\alpha–C_\beta$ bonds. Alternatively the whole transmembrane helices might be tilted. Lipids in turn, could modulate the membrane thickness by stretching or disordering their acyl chains or even assembling into non-lamellar structures, thereby disrupting the bilayer organization. Finally, when a heterogeneous mixture of lipids surrounds the protein, as in a biological membrane, mismatch may be relieved by preferential interaction of proteins and lipids with matching hydrophobic lengths, leading to microdomain formation and molecular sorting. Recent works suggest that the opposite could also be true, that is, some membrane protein could vary their assembling and packing as to maximize the hydrophobic matching, depending on the lipid domain where they are located [45,46]. The hydrophobic matching principle was initially a theoretical concept [19], later supported by some experimental evidences. This is the case of Lactose Permease [47], a protein with twelve α-helical transmembrane segments. Through the use of pyrene-labeled lipids able to form excimers, the lipid–lipid and lipid–protein interactions were studied for Lactose Permease reconstituted in artificial vesicles. This study supports hydrophobic matching as responsible for the 10-fold or higher enrichment of lipids around the protein which best match its hydrophobic length, without showing any preference for specific lipid headgroups. Another similar example is provided by Bacteriorhodopsin (BR), a protein that performs molecular sorting of the lipids in its neighbourhood to satisfy the hydrophobic matching condition [48]. Finally, a pulmonary surfactant protein, SP-C, forms aggregates in lipid mixtures in the gel phase, whose surroundings are enriched in shorter chain length and unsaturated lipids [49], satisfying again the

hydrophobic matching condition. From systematic studies with artificial hydrophobic peptides [44,50,51], it has been concluded that hydrophobic matching can induce preferential protein–lipid interactions, although it depends among other factors, on the hydrophobic length and diameter of the protein, the amino acid composition of the protein transmembrane segments (especially the interfacial amino acids), the type of lipid and the differences in hydrophobic length between the protein and the lipids. This conclusion stresses again the multifactorial nature of lipid–protein interactions: in spite of the existence of a predominant factor such as the hydrophobic mismatch in the above cases, it is its coupling with other factors what determines whether or not lipid domain segregation would take place.

Recently, others physical features of the membrane, the area compressibility and the bending moduli of the bilayer, have been proposed to direct lipid–protein interactions [52,53], especially in cases related to protein sorting to raft domains.

3. nAChR directs the formation of a PA-enriched lipid domain

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel composed of five subunits which, upon the binding of two agonist molecules, opens a channel allowing cations such as sodium, potassium or calcium to go through the membrane. Due to the ease of purification in high quantities, this protein has been used as a model to study different aspects of integral membrane proteins including lipid–protein interactions. These studies showed that there are independent binding sites for phospholipids and sterol, both accessible to fatty acids, in the vicinity of the nAChR, and that those lipids forming the "annular shell" are relatively immobile with respect to the rest of the membrane [see [54] for a review]. Interestingly, anionic lipids bind preferentially nAChR, which are also positive modulators of its function. Among them, phosphatidic acid (PA) seems to interact in a special fashion with this protein. "In vitro" studies with nAChR reconstituted in lipid vesicles of controlled composition show that PA is among those phospholipids that bind the protein with a higher affinity, and it is also most effective in preserving nAChR function [55–60], possibly through the stabilization of the resting versus the desensitised state of the protein [61]. On the other hand, as if a bi-directional coupling would take place, nAChR in PA-containing membranes leads to a dramatic increase in both, the lateral packing density and the gel-to-liquid crystal phase transition temperature of the lipid bilayer [61,62]. This strong interaction leads to the segregation of a PA-enriched domain from a complex mixture of lipids at certain lipid to protein ratios [62,63]. However, nAChR has no detectable effect on the lateral distribution of lipids when PA is substituted by other zwitterrionic or anionic phospholipids such as phosphatidylcholine (PC), phosphatidylglycerol (PG) or phosphatidylserine (PS) [63,64]. Also, it has been recently reported the segregation of a saturated PC from an unsaturated one by nAChR [62]. In this latter case the authors suggest that the maintenance of this domain is predominantly due to lipid–lipid interactions opposite to that with PA from above, which is more stable and mainly maintained by protein–lipid interactions. The PA domain has been detected using fluorescence, FT-IR and DSC techniques (Fig. 1). Such techniques are mostly sensitive to macroscopic events, thus indicating that

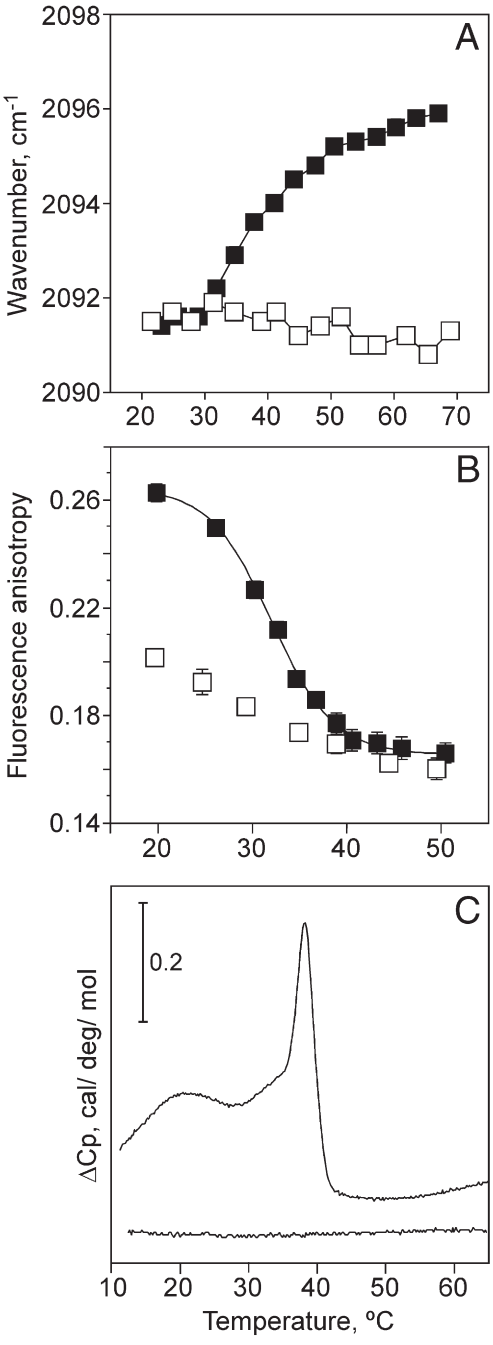

Fig. 1. nAChR induces the segregation of PA-enriched lipid domains. Lipid vesicles composed of 25 mol % cholesterol, 50 mol % egg PC and 25 mol % of DMPA with or without nAChR, were used in FT-IR (A), fluorescence anisotropy (B) and DSC (C) experiments. In the presence of nAChR, closed symbols in panel A, B and upper thermogram in C, a temperature-induced phase transition assigned to lipid domains enriched in DMPA appears. Empty symbol in A, B and the lower thermogram in C correspond to plain lipid vesicles. FT-IR experiments follow the temperature dependence of the CD_2 symmetric stretching vibration from perdeuterated DMPA, while in the fluorescence experiments, the anisotropy of the *trans*-parinaric acid probe incorporated into the membrane is measured.

macrodomains should be formed. In addition, resonance energy transfer experiments have shown that these domains are located next to the protein [63].

Phospholipid in membranes, including those interacting with membrane proteins, diffuse very fast, around 10^8 cm^2/s [57,65], so the ability of nAChR to sequester PA around it, will dramatically enhance its possibilities of interaction with this phospholipid, hence explaining the strong modulating effect of PA on nAChR.

Interestingly, the PA modulation of nAChR observed on "in vitro" systems has been confirmed "in vivo" using the *Xenopus* oocyte model [66]. In these experiments, purified nAChRs reconstituted in either PA:PC:Cholesterol (25:50:25 molar ratio), PC:Cholesterol (75:25 molar ratio), or soybean lipids are injected in oocytes, where they efficiently insert into the plasma membrane. Then, the functional activity and properties of the transplanted nAChRs are assessed using the voltage-clamp technique. A higher activity was found when the nAChR was reconstituted in the PA mixture than in soybean or the PC: Cholesterol mixture, which were very similar to each other. This effect was not due to a different fusion efficiency of the different proteoliposomes to the oocyte membrane. Interestingly, when nAChR is reconstituted with those lipid mixtures "in vitro", the activity is always higher in soybean lipids than in the PA mixture, while no activity is found in PC:Cholesterol. The fact that in the cell membrane the nAChR in PC:Cholesterol reversibly recovers its function suggests that the system is sufficiently dynamic as to allow the injected lipid around nAChR be exchanged for endogenous oocyte membrane lipids. Then, why do oocytes injected with nAChR reconstituted in PA display larger currents? Taking into account the above PA segregation results [56–58], one possibility would be that nAChR binds PA tightly, hindering its free exchange with other bulk membrane lipids, and leading to the formation of a PA-rich domain segregated around the protein. The permanent interaction with PA, a positive modulator, would result in the enhanced protein activity. An interesting observation that supports this hypothesis is that the PA content of the lipids which accompany the protein is progressively increased from 0.5–1.6% up to 2.2–2.9% during its purification from the *Torpedo* electric organ [67].

Another open question on the segregation of the PA domain relates to the molecular determinants in both the protein and the phospholipid, responsible for this strong interaction leading to domain segregation. Calorimetric studies using protease-cleaved nAChR point out to the transmembrane segments as mainly responsible for domain segregation [63]. It has been proposed that positively-charged amino acid present at the end of the nAChR transmembrane helices, such as Arg-429 or His-408 at the M4 could be responsible for the stronger binding to the anionic phospholipids [68–70]. As to the possible determinants in the PA molecule, only general properties of this phospholipid, such as its negative charge, its very small headgroup or its high capacity to form hydrogen bonds, have been invoked [71–74]. In this regard, a recent NMR study on PA-containing lipid bilayers in interaction with positively-charged model peptides, suggested a feedback process with an initial electrostatic interaction and a subsequent H-bonding with the phosphomonoester of the PA headgroup. This leads to further deprotonation of the lipid headgroup, which in turn enhances the electrostatic attraction, thereby stabilizing the protein–lipid interaction [75]. In agreement with these ideas pointing to a specific interaction between PA and nAChR, neither salt screening nor pH titration could destabilize the domain, nor can anionic lipids other than PA be segregated by nAChR. At this point it is important to stress that PA has a higher pK_a when the protein is present, so its anionic charge is diminished [63]. This could facilitate the formation of the PA domains since a lower repulsion and an enhanced attraction through hydrogen bonds between PA molecules would occur [76]. In turn, there would be a decrease in the electrostatic contribution to the free energy of the system [77], as to overcome the entropic effect that favours the homogeneous mixing of lipid components. Again, this emphasizes the notion that, in order to stabilize a protein-directed lipid domain, not only lipid–protein interactions are important, but also lipid–lipid interactions.

4. Mechanisms of lipid domain formation

The above examples on lipid domains segregated by membrane proteins, evidence the complexity and variety of factors involved. In spite of this variability, there is a common pattern for the lipid domain origin: a strong interaction between the transmembrane protein and certain lipid species. The attractive forces facilitating this initial step range from stronger electrostatic or hydrogen-bond interactions, mostly arising from polar and charged amino acids near the interfacial region of the protein and from lipid headgroups, to weaker Van der Waals and steric interactions, occurring in the protein transmembrane region and in the lipid interfacial and hydrophobic area. Experimental evidences and molecular dynamics simulations show that basic residues, especially lysine, thanks to its long and flexible side chain, can interact over a wide interfacial region, "snorkelling" deeply into the hydrophobic core of the membrane, while locating its basic group close to the lipid phosphate [78–80]. Aromatic residues located at the ends of protein transmembrane segments, mostly tryptophan, have also been proposed as key elements in this process [78,79]. In fact, molecular dynamics simulations in two transmembrane proteins indicate that aromatic side chains, located at the ends of transmembrane portions, are oriented so that their polar moieties are nearest to the lipid headgroup and solvent, while their hydrophobic regions interact with the lipid acyl chains of the bilayer [81]. Overall, the highest selectivity is found for anionic lipids, although different lipids with the same charge are not equivalent in their interaction with different proteins. In regard to the hydrophobic transmembrane protein region, it represents the most conserved sequences of membrane-spanning proteins. Although this may relate to conserved protein–protein interactions within the membrane, it seems plausible to assume that evolutionally conserved lipid–protein interactions could also be involved. Whatever it may be, direct interaction between lipid and protein is not enough to make thermodynamically favourable the entropically unfavourable lipid demixing. Adequate lipid–lipid interactions are also necessary for the process to occur.

These ideas are supported by Monte Carlo simulations which indicate that lipid and protein clustering are highly coupled [82]. This results from thermodynamic coupling between the intrinsic tendency for lipid clustering in the absence of any protein, and preferential binding of the protein to a given lipid. In these simulations the interaction between like and unlike lipids results to be determinant. The small difference between the energy interaction of like and unlike lipids, typically on the order of a few hundred calories per mole, together with the large number of lipid–lipid interaction and the cooperativity of the process, offers enormous possibilities for lipid lateral distributions on the membrane, which can be significantly varied by small structural changes in one or more lipid species, while maintaining the reversibility of the ordering processes. For example, very small changes in unlike lipid–lipid interactions provoke large changes in domain segregation. Altogether, slight perturbations in the concentration of lipids and proteins, pH, concentration of divalent cations, or electrostatic interactions may locally modify or even trigger component demixing and domain formation. Thus, the fine modulation of lipid domain composition and size is achievable, and the associated protein compartmentalization on the membrane surface becomes possible.

With the above ideas in mind a hypothesis on the formation of protein-directed lipid domains have been proposed [27], where specific lipids are accumulated around the protein because of favourable lipid–protein interactions. This variation of the local lipid composition causes other proteins to be recruited to the domain, thus inducing further lipid demixing. Then, the signal is amplified, since every lipid interacts with several near neighbours, finally leading to domain formation.

Other hypotheses on the origin of membrane domains have been entertained, both enhancing the role of other cell structures and the non-equilibrium nature of the process. One of such hypotheses is based on a theoretical model which suggests that, although membrane domains may arise due to specific molecular associations, much of the lateral heterogeneity detected in any membrane, particularly that observed by microscopy, may be non-specific, arising from the combination of a given vesicle traffic to and from the plasma membrane and from the hindered lateral diffusion of transmembrane proteins [83]. Alternatively, the other hypothesis based on experimental work with GPI-anchored protein in cells [84], proposes that clusters or membrane domains, at least the macroscopic ones where these proteins are located, should be maintained actively by the cell, discarding the possibility that they are formed by simple segregation of immiscible membrane components. Accordingly, artificial membranes would not be good model systems to describe the physical properties of the cell surface.

In conclusion, although the principles that govern the origin of protein-directed lipid domains begin to be known, it is still a controversial subject, where the exact role of lipid, protein and perhaps other cell components remain to be fully established.

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