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The differential miscibility of lipids as the basis for the formation of functional membrane rafts

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Abstract

The formation of sphingolipid-cholesterol microdomains in cellular membranes has been proposed to function in sorting and transport of lipids and proteins as well as in signal transduction. An increasing number of cell biological and biochemical studies now supports this concept. Here we discuss the structural properties of lipids in a cell biological context. The sphingolipid-cholesterol microdomains or rafts are described as dispersed liquid ordered phase domains. These domains are dynamic assemblies to which specific proteins are selectively sequestered while others are excluded. The proteins associated to rafts can act as organizers and can modulate raft size and function. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Sphingolipid-cholesterol domains; Membrane transport; Signalling

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1. Introduction

The astonishing variety of lipids as structural constituents of cell membranes reflects the multitude of processes and rearrangements that take place at membrane interfaces and gives reason to study lipids beyond their function in forming a two-dimensional liquid barrier. The fluid nature of cell membranes has been well documented and accepted [1-5] but whether the fluid mosaic bilayer possesses short range order has been extensively investigated and debated [6,7]. In the late 70s and early 80s considerable effort was spent in studying the so-called lipid annulus or boundary lipids, that is the layer of lipids surrounding an integral membrane protein that has a distinguishable mobility and phase transition enthalpy as compared to the bulk of membrane lipids [8– 11]. However, this issue has been surrounded by controversy. The numbers and properties of immobilized lipids that have been reported were variable and dependent on the detection technique employed [11-15]. Moreover, the biological significance of boundary lipids for cell membrane function could not be demonstrated ([16,17], but see [18,19]). A major limitation in the interpretation of the biological implications of these studies has been the use of model systems, often consisting of non-natural lipids. It is clear that intermolecular interactions among synthetic lipid components can give rise to ordering and segregation in microdomains [20-24]; however, their possible existence and function in cell membranes remain in dispute.

In recent years, a renewed interest in the differential miscibility of lipids and the formation of domains has emerged from specific biological observations in the field of membrane transport and signalling. A new concept has been put forward that describes the formation of glycosphingolipidcholesterol microdomains or rafts that function as platforms for protein and lipid transport from the trans-Golgi network to the plasma membrane [25]. This proposal was initially based on the observation that glycosphingolipids as well as glycosylphosphatidylinositol (GPI) anchored proteins are targeted to the apical membrane of polarized epithelial cells [26,27]. These proteins are linked to GPI on the luminal side of the endoplasmic reticulum (ER) [28] and therefore do not contain a cytoplasmic targeting signal. GPI anchoring appears to be sufficient for correct targeting [29]. Since glycosphingolipids can associate into clusters [30], they form a unit to which other apically destined molecules can associate. The poor solubility of glycosphingolipid-containing membranes in the non-ionic detergent Triton X-100 (TX-100) [31,32] has been used as a tool to analyse lipid rafts and identify associated proteins. Extraction of polarized cells with TX-100 indeed yielded a detergent insoluble glycolipid complex (DIG) in which GPI-anchored proteins were enriched together with sphingomyelin (SM) and cholesterol [33-35]. Surprisingly, a subset of proteins associated with the cytoplasmic face of the membrane was also found in DIGs, including signalling molecules such as srclike tyrosine kinases that are membrane associated through double acylation and implicated in T-cell activation [36-38].

Evidence has now accumulated that raft-based transport and signalling is not only employed by polarized epithelial cells but also by non-polarized cells such as fibroblasts [39,40] and lymphocytes [41]. Recently, Scheek et al. [42] reported a dramatic demonstration of the coupling of sphingolipids and cholesterol in cells. They showed that degradation of sphingomyelin at the cell surface of fibroblasts directly inhibits the cleavage of SREBP-2, the membrane bound transcription factor in the ER that regulates the biosynthesis and uptake of cholesterol. These and earlier observations demonstrate that plasma membrane cholesterol flows back to the ER when the sphingomyelin concentration at the cell surface is decreased [43,44] and argue for a functional interaction between cholesterol and sphingomyelin in intracellular membrane transport. This accumulation of new biological observations provides a stimulus for re-analysing the lipid biophysical data pertaining to lipid miscibility. For an excellent review on sphingolipid organization in the membrane see [45]. Here we explore whether the structural properties of lipids can be reconciled with the proposed formation of lipid rafts and their specific functions in membrane transport and signalling.

2. The structural properties of sphingolipids and cholesterol

The structural properties of sphingolipids and cholesterol in part explain their behaviour in a complex membrane. The majority of the sphingolipids is composed of a ceramide which commonly consists of a sphingosine, a dihydrosphingosine or a phytosphingosine (Fig. 1) in amide linkage to a long chain fatty acid which is often hydroxylated. The attachment of different types of head groups to the terminal hydroxyl of the ceramide then gives rise to different classes. Sphingomyelin, which contains a phosphorylcholine head group like PC, is the only phosphosphingolipid in mammals. The glycosphingolipids are particularly diverse in their head groups which may possess as many as 30 glycosyl residues per lipid [46,47]. The sphingolipids differ from the glycerolipids in that they possess both hydrogen bond-accepting and -donating groups, their amide and free hydroxyl groups respectively, which are in close proximity to one another in the vicinity of the lipid-water interface. Together with the hydroxyls of the sugar and the acyl chain (when present) these groups facilitate extensive formation of hydrogenbonded networks. The predominant glycerolipids in eukaryotic membranes, however, consist of a glycerol base with ester linked fatty acids in the sn-1 and sn-2 position (Fig. 1), offering only hydrogen bond-ac-



Fig. 1. The chemical structure of sphingomyelin (A), with a sphingosine backbone (4-sphingenine, an 18 carbon *trans*-monounsaturated amino diol). Other bases commonly found in sphingolipids are dihydrosphingosine (sphinganine), an 18 carbon saturated amino diol (B), or phytosphingosine (4-D-hydroxysphinganine), like sphinganine but with an additional hydroxyl group at carbon 4 (C). Phosphatidylcholine (D) and cholesterol (E).

cepting capacity. The most abundant head group moieties of mammalian glycerolipids are phosphate ester-linked choline, serine, ethanolamine and myoinositol, of which the latter also has a limited hydrogen bonding capability [48]. Further differences between sphingo- and glycerolipids can be found in their fatty acyl chains. The amide-linked acyl chains in natural sphingolipids vary among tissues but the predominant species in most tissues but brain are palmitic acid (16:0), nervonic acid (24:1), behenic acid (22:0) and lignoceric acid (24:0). In brain, nervonic and lignoceric acid are less abundant while stearic acid (18:0) dominates [49]. The acyl chains in the sn-1 and sn-2 positions of mammalian glycerolipids are typically 16 and 18 carbons long and frequently polyunsaturated in the sn-2 position [48]. Thus, on average the sphingolipids are more saturated, often asymmetric due to the very long amide-linked chain and more prone to hydrogen bonding. A striking

consequence of these structural differences is found in the gel to liquid crystalline phase transition temperatures (or melting temperature, $T_{\rm m}$) of the different lipid species. These range from 40°C in long chain sphingomyelin to 83°C for N-24:0 galactosylceramide [50] while 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, 16:0-18:1) melts at -3° C. Small changes in the acyl chain composition of PC bring major changes in the phase transition temperature. The replacement of the monounsaturated oleic acid (18:1 Δ^9) in the *sn*-2 position of POPC by a saturated 18 carbon chain increases the $T_{\rm m}$ by 52°C from -3to 49°C [51]. The introduction of the monounsaturated nervonic acid (24:1 Δ^{15}) in galactosylceramide brings the T_m down by only 13°C with respect to lignoceric acid (24:0), probably because the double bond in nervonic acid is localized closer to the bilayer mid-plane and does therefore not interfere so much with the lipid packing. In this respect it should be emphasized that non-natural PC species, such as dipalmitoyl PC (DPPC) that is frequently used in in vitro experiments, exhibit non-typical transition temperatures and therefore complicate biological interpretation.

Cholesterol (Fig. 1), which is present in mammalian cell membranes, is intercalated between the lipid acyl chains. The four fused rings of cholesterol have little conformational flexibility and can be accommodated very effectively, allowing cholesterol to alter the physical state of the membrane drastically. In addition to this planar steroid ring system, cholesterol possesses a polar 3β -hydroxyl group which causes it to orient in a lipid bilayer parallel to the lipid hydrocarbon chains with the hydroxyl towards the lipid-water interface [52] and the hydrophobic side chain towards the centre of the membrane. Cholesterol has the well known ability to condense the lipid bilayer by decreasing the amount of *trans-gauche* isomerizations and increasing the fraction of *trans* dihedrals in the lipid acyl chains. This leads to motional ordering of the chains in the liquid crystalline phase [53,54]. Cholesterol also diminishes the gel to liquid crystalline phase transition of glycero- and sphingolipids [55–57] and decreases the chain order of a lipid in comparison to its order in the gel phase in the absence of cholesterol [58]. It is the planar α -face of the steroid nucleus which allows it to affect lipid acyl chain order. When this planar face is perturbed by methyl groups like in lanosterol, a biosynthetic precursor of cholesterol, the ordering effect is diminished. Consistent with this, lanosterol fails to substitute for cholesterol in yeast sterol auxotrophs [59] and fibroblast demethylase mutants [60], which shows the biological importance of the physical properties of cholesterol.

3. Lipid packing and asymmetry as a driving force

As a consequence of minimizing free energies, lipid molecules prefer to organize in structures that best accommodate their dynamic shape, which means that the preferred organization of a given species is not necessarily a bilayer [61]. However, in the cell membrane it is the extended bilayer which forms the basis of membrane structure. When lipids that are prone to form a more curved non-lamellar phase or lamellar cubic phase [61,62] are constrained in a planar lamellar phase, this results in a storage of bending energy [63]. This so-called intrinsic curvature of lipids that cannot be expressed in a bilayer membrane gives rise to specific packing of the lipid acyl chains and allows for structural modifications with the expense of very little energy [64]. Membrane bending and subsequent budding could exploit the strain generated by the asymmetric distribution of lipid classes and species over the two opposing leaflets that occurs in many biological membranes [26,65]. Theoretically, local variations of bilayer asymmetry caused by lateral phase separation can cause local variations in surface curvature [66] and could lead to the formation of folded structures. At the edge of a flat compositionally distinct domain there is a line tension that is minimized by entropy. With a curved domain the interfacial length is further reduced by the invagination of the domain. In an experimental approach, mixed brain sphingomyelins were used that phase separate in the physiological temperature range. By fluctuating through a 30–50°C temperature interval, small vesicles pinched off that differ in their composition from the mother vesicle [67]. In cells, the budding of membranes is the first step in the production of vesicles for intracellular transport. Although this is a process regulated by proteins [68], the budding can nevertheless be driven by changes in lipid composition, even in cases when



Fig. 2. Schematic illustration of the initial steps in the development of a cubic membrane which is a possible basis for the formation of caveolae. This structure can grow by means of a membrane folding process and the symmetry can change during growth as is indicated in B. Modified from [70].

these lipid domains are covered by a protein coat. Moreover, the protein coat could regulate the composition of the domains and create the specificity towards the target membrane. Caveolae, the caveolin-coated invaginations of the plasma membrane [69], show a morphology at later stages of their development in which a bi-continuous cubic membrane can be recognized [70] (Fig. 2). Caveolin, which is a cholesterol binding protein [71], could recruit the membrane components required for the formation of a highly curved branched structure within a continuous bilayer.

4. Lateral organization of lipids: the probability of microdomain formation

Non-ideal lateral mixing of lipids with different packing properties can be seen in bilayers consisting of specific PC species [72,73] but is particularly strong in glycerolipid/(glyco)sphingolipid bilayers when there is a chain mismatch. *N*-16:0 SM mixes almost ideally with dimyristoyl PC (DMPC, di14:0) but *N*-24:0 SM segregates from DMPC, although the two SMs have a similar T_m . It should be noted that more than 50% of the natural sphingomyelins are asymmetric with respect to hydrocarbon chain length which causes considerable chain mismatch [74]. Cholesterol can cause or enhance lateral separation of lipids in bilayers of more complex composition. The concentration of cholesterol is low in the ER, where it is synthesized, and increases towards the plasma membrane where high levels are present. In the concentration range of 7-30%, cholesterol induces fluid phase immiscibility in dipalmitoyl PC (DPPC, di16:0) bilayers, with a cholesterol-rich liquid ordered (l_0) phase in which cholesterol is present in both leaflets and a cholesterol-poor liquid disordered (l_d) phase in which cholesterol is present in one leaflet and the tail penetrates into the other leaflet [56,75–78]. This effect clearly depends on the degree of unsaturation of the lipids since cholesterol has little effect on the chain order of the unsaturated dioleoyl PC (DOPC, di18:1) but rather forms small pure cholesterol domains causing reduced cholesterol mobility [79]. Schroeder et al. [32] have shown that lipids in the l_0 phase are resistant to extraction with TX-100. Given the composition of TX-100 insoluble complexes derived from whole cells [33], this would suggest that these membranes are in the l_0 phase. In an elegant study, Ahmed and co-workers [73] used a fluorescence quenching assay to show that the l_{o} phase occurs in membranes with a lipid composition similar to that of the plasma membrane. Using diphenylhexatriene and a PC-linked nitroxide quencher they could demonstrate that cholesterol at 33 mol% induced the formation of a SM-enriched liquid ordered phase at 37°C in SM/DOPC at a SM concentration of 10-13%. Furthermore they confirmed that the presence of a l_0 phase does indeed correlate with detergent insolubility.

Glycosphingolipids have an even stronger tendency than sphingomyelin to separate from the phospholipids. Mixtures of bovine cerebrosides and monounsaturated PC are significantly inhomogeneous at 37°C. This is observed without cholesterol but also at very high cholesterol concentrations (>50%) at which the lateral separation in phospholipid mixtures is abolished [72]. The ganglioside G_{M1} has a preference for the gel phase in 1:1 dielaidoyl PC (DEPC, di18:1 trans-unsaturated)/DPPC and stabilizes compositional domains [80,81]. This ability of G_{M1} decreases with decreasing chain length and increasing unsaturation. G_{M1} separates also from *N*-palmitoyl SM in a binary mixture, probably due to a chain mismatch such as described for N-lignoceryl SM and DMPC.

In a mixture of cholesterol, N-palmitoyl SM and

 G_{M1} formation of separate cholesterol-enriched SM and G_{M1} -enriched SM domains can be observed at 20 mol% cholesterol and 10–20 mol% of G_{M1} [82], indicating that compositionally different cholesterolenriched domains may exist in the cell. Whether this would occur with more asymmetric SM species and with physiological concentrations of G_{M1} remains to be proven.

5. The size of lipid microdomains

A matter of crucial importance for biological functions is the size and connectivity of lipid microdomains. It is obvious that a bulk phase separation could not be functional in a biological sense while dispersed microdomains could. In a three-dimensional system with immiscible components, surface tension leads to bulk separation of two phases. In a two-dimensional membrane, however, the reduction of surface tension to line tension at the interface between domains prevents bulk separation and instead leads to formation of dispersed microdomains [83]. With the phase specific dye Merocyanine 540, the size and shape of the domains in a two phase component system were shown to be dependent on the lipid species. This can be explained when the driving force for domain fission is the alignment tendency of a particular lipid species or mix of species. The dimensions of the domain would then be the result of a balance between alignment tendency and the line tension at their boundaries [84]. Using epifluorescence microscopy in DPPC or N-palmitoyl SM monolayers, Slotte [85] demonstrated that the cholesterol-induced domains are small and different in size, number and properties, dependent on the lipid class. Domains are significantly smaller in SM than in DPPC with distribution peaks around 25 μm^2 and 100 μm^2 respectively. A high lateral pressure was needed to cause SM domains to dissipate and to form one phase. Upon lowering to initial pressure, SM domains re-emerged. Clusters of much smaller size are formed by asialo-G_{M1} at low mol fractions in POPC above the transition temperature [86,87]. With Fab fragments and freeze-etch electron microscopy clusters of approx. 15 molecules could be visualized. From these observations it follows that the dimensions of lipid domains are

strongly dependent on the lipid composition and fluctuate in space and time. As will be discussed later, experiments performed in a cellular context strongly indicate that lipid-protein rafts are small but can be clustered into bigger assemblies, for instance by receptor-ligand interaction. Interesting in this respect is the theoretical analysis that predicts the accumulation of cholesterol molecules at the phase domain interface [88,89]. In this location, cholesterol could stabilize domains and increase their size in a concentration-dependent manner.

6. How lipid microdomains behave: the percolation model

It is important to analyse how the behaviour of dispersed microdomains in the membrane could affect biological functions. For a membrane in which microscopic domains exist as phase domains, phase percolation is an important physical parameter to describe the system [83,90]. Percolation is defined on the basis of studies on binary and ternary lipid mixtures with phase specific fluorescent tracers and fluorescence recovery after photo bleaching [91]. One should consider for example a bilayer in which different phases such as $l_0 + l_d$ or l_d +gel coexist. When the liquid disordered phase is continuous, the solid or l_0 phase exists as isolated domains. Upon decreasing the temperature or increasing the gel/l_o phase component mass fraction, the gel or l_0 phase becomes continuous at a certain point and the l_d phase will form isolated domains: this point is called the percolation threshold [92]. Although the situation where the liquid disordered phase is continuous seems most physiological, some specialized membranes such as the apical membrane in polarized epithelial cells contain such high amounts of (glyco)sphingolipids and cholesterol [26] that a percolating l_0 phase is rather likely. Since lipids in the l_0 phase have a reduced lateral mobility compared to lipids in the $l_{\rm d}$ phase [54], the l_0 domains restrict the diffusion of lipids and proteins [54]. As a consequence, the percolation properties of membranes with coexisting phase domains would strongly determine long range translational diffusion [93]. This was demonstrated experimentally with a lipid-bound fluorophore and a lipid-bound quencher that both prefer the l_d phase.

At the point of gel phase percolation, an abrupt decrease in quenching occurred, the extent of which depended on the size of the disconnected domains [94]. An interesting possibility is that cells metabolically connect and disconnect a phase domain by increasing either number or size of the disconnected domains [95]. With liquid-ordered and liquid-disordered phases co-existing close to the percolation threshold, small changes could induce the transition from percolating to non-percolating state of one phase. Consider a bimolecular reaction of which both components prefer the liquid disordered phase. The reaction rates would be greatly affected by a disconnection of the disordered phase [30]. This is particularly the case when the number of reacting molecules is limited, which is often true in biological systems. Transitions between percolating and nonpercolating states of a phase domain can therefore be employed as a trigger mechanism in membranebound processes [90] and function to enhance or rate-limit bi- or multimolecular reactions. Such a dynamic control of the system would rely on the lipid composition as well as on lipid-protein interactions.

7. Triton X-100 as a tool to characterize rafts

The resistance of glycosphingolipid-cholesterol rafts to Triton X-100 at 4°C has proven a powerful tool to study the composition and character of rafts. The detergent insoluble glycolipid-enriched complexes (DIGs) can be isolated by floatation in a density gradient due to their high lipid content [33]. With the detergent approach, however, it is not possible to determine the size and subcellular localization of rafts or to distinguish between rafts of different composition. Caveolae contain clusters of glycosphingolipids and cholesterol and are found in a detergent insoluble fraction [96] but the finding that DIGs exist in cells that do not express caveolin and do not have caveolae [41,97,98] indicated that the molecules present in a caveolin-enriched detergent insoluble fraction are not necessarily localized in caveolae. In recent years, detergent-free methods have been developed to isolate caveolae [99-101]. With these methods, it should be possible to define the relation between caveolae and rafts on the plasma membrane. One recent refinement of detergent insolubility as a

criterion for raft association is the use methyl- β -cyclodextrin to deplete cholesterol from membranes. Most DIG-associated proteins will dissociate from the lipids after cholesterol depletion.

8. The presence of rafts in the plasma membrane

An increasing number of studies now support the existence of rafts in the plasma membrane. A single particle tracking analysis with antibodies conjugated to colloidal gold [102] demonstrated that a large portion (35–37%) of the GPI-linked Thy-1 on the fibroblast cell surface undergoes confined diffusion. The protein is transiently confined to regions with an average size of 260-330 nm diameter. This is not an indication of the size of the raft but rather confirms its existence in terms of a distinguishable confined mobility. In contrast, the movement of a fluorescein-labelled analogue of phosphatidylethanolamine, a phospholipid which is not enriched in DIGs, was found to be significantly less restricted. The confinement of Thy-1 in glycosphingolipid-depleted cells was 1.5-fold reduced as compared to control cells. This strongly suggests that it is the lipid environment of the protein that influences its mobility. These experiments also indicate that the GPIanchored protein is not 100% present in rafts but rather has an affinity for raft lipids, stressing the dynamic properties of the assembly. In another study, surface exposed molecules that were considered raft markers on the basis of their behaviour in detergent were cross-linked with antibodies. The GPI-anchored protein Thy-1, glycolipids and sphingomyelin that were initially dispersed, redistributed upon cross-linking and were found concentrated in caveolae on the mouse keratinocyte cell surface [103]. A co-clustering of these molecules occurred only upon simultaneous antibody incubation prior to fixation and did not require an intact cytoskeleton. Cross-linking of the transferrin receptor, a protein found in clathrin-coated pits, did not cause a relocalization to caveolae. A similar behaviour was observed for the GPI-anchored placental alkaline phosphatase (PLAP) and influenza haemagglutinin (HA) [141]. When labelled after fixation, HA and PLAP were found evenly distributed over the membrane. Incubation with both HA and PLAP antibodies simultaneously prior to fixation showed redistribution of these markers in overlapping patches. Extraction of membrane cholesterol with cyclodextrin reduced the patching of HA and PLAP and caused a more diffuse distribution. The integrity of the patches obviously depends on the presence of cholesterol. The same co-clustering was observed for G_{M1} and PLAP. PLAP clusters, however, segregated from the transferrin and LDL receptors. The latter proteins do not associate with DIGs and are internalized in clathrincoated vesicles. These findings stress the dynamics of raft assembly and suggest that rafts are too small to be optically detected in the dispersed state but can be stabilized to form larger domains by protein-ligand interaction. This all conforms to the behaviour of dispersed l_0 lipid microdomains under the percolation threshold [90].

9. Rafts are involved in cell signalling

Antibody mediated cross-linking of GPI-anchored proteins induces the phosphorylation of various substrates on tyrosine residues [104]. This suggests that GPI-anchored proteins can interact with src-like kinases such as p56^{lck} or p59^{fyn}. p56^{lck} (Lck) is a *src*-like tyrosine kinase critical for T-cell development and activation and the protein is found partly associated with DIGs [105]. The raft-associated Lck is hyperphosphorylated which correlates with a lower kinase activity relative to the TX-100 soluble Lck. The membrane-bound tyrosine phosphatase CD45 partitioned in the TX-100 soluble fraction of the membrane. The exclusion of CD45 from the glycolipidenriched domains allows the segregation of an inactive pool of Lck that can be activated when needed. The mechanism by which the partitioning of Lck is governed is yet unclear but could be a matter of ligand binding [106], interaction with proteins in the Triton soluble fraction [107], or both. Another possibility to regulate raft association of Lck is reversible palmitoylation [38,108]. The biochemical difference between the soluble and the DIG-associated Lck supports the existence of separate domains in the plasma membrane. In Jurkat cells, the signalling through the GPI-anchored CD59 was strongly suppressed by reduction of the membrane cholesterol level [109], indicating that raft integrity is essential for this signalling process. The raft-mediated signalling pathway is not restricted to GPI-linked proteins. The transmembrane protease receptor tissue factor (TF) that triggers the coagulation cascade on endothelial cell surfaces, acquires TX-100 insolubility upon binding the serine protease factor VIIa [110]. The subsequent formation of a complex with tissue factor pathway inhibitor (TFPI), which is predominantly present in DIGs, leads to down-regulation of proteolytic activation. In granulocytes as well as mast cells and basophils, the high affinity IgE receptor FceRI aggregates upon activation. Aggregation and activation of FceRI is driven in vivo by receptor-bound IgE antibodies recognizing multivalent allergens or in vitro by several anti-IgE antibodies or synthetic antigens. The aggregation causes an efficient association with lipid domains in the membrane that are enriched in the src-family tyrosine kinase lyn. The subsequent tyrosine phosphorylation of the β and γ subunits of the receptor triggers the signalling cascade that leads to release of mediators of the allergic response [142,143]. In peripheral blood lymphocytes, the major ganglioside G_{M3} is distributed in dispersed clusters on the cell surface [111]. G_{M3} was found enriched in a TX-100 insoluble fraction, together with the T-cell specific co-receptor CD4 and Lck [112]. CD4 and Lck interact via unique domains in both proteins and are involved in signal transduction through the T-cell antigen receptor [113]. This suggests that a multimolecular signalling complex is constituted via raft association and additional protein-protein interaction. Thus, receptorligand interaction can trigger the redistribution of receptors into rafts or stabilize larger raft assemblies, leading to receptor sequestration and regulation of function.

10. Communication between inner and outer membrane leaflets

Several protein tyrosine kinases that are associated to the cytoplasmic face of the bilayer through double acylation have been found in DIGs, including Lck, Fyn and Lyn [37,114,115]. Recent evidence that the clustering of GPI-anchored PLAP leads to co-patching of the doubly acylated Fyn suggests that when rafts are clustered by antibodies from the external side, molecules like Fyn on the cytoplasmic side of rafts are dragged along [141]. Glycosphingolipids and sphingomyelin are concentrated in the exoplasmic leaflet of the membrane while the cytoplasmic leaflet consists mainly of glycerophospholipids [26,65]. How is the communication across the membrane achieved? A transmembrane adaptor protein may form the connection between the membrane leaflets, like with CD4-mediated signalling in T-cells. However, such adaptors have so far not been identified for GPI-anchored proteins [113]. Cholesterol could mediate the association of cytoplasmic proteins to the domain formed in the exoplasmic leaflet since it is most likely present in both leaflets and cholesterol can form dimers under certain conditions [77]. Interdigitation of long-chain sphingomyelins and glycosphingolipids into the opposing membrane leaflet could cause more saturated phospholipids in the inner leaflet to co-localize with the l_0 domains in the exoplasmic leaflet, creating conditions which lead to preferential binding of signalling proteins that are post-translationally modified with the attachment of saturated myristoyl or palmitoyl chains [113,116]. The issue of acyl chain interdigitation is surrounded by some controversy. It has been suggested that interdigitation of the long chain of cerebroside sulphate in DMPC and DPPC occurs only in gel phase bilayers [117], while mixtures of asymmetric cerebroside species do interdigitate in the liquid crystalline phase [118]. Cholesterol diminishes chain interdigitation at 50 mol% [119]. This is, however, a concentration at which cholesterol induces miscibility of lipids rather than lateral phase separation [72]. It would therefore be more interesting to analyse the effect of cholesterol on interdigitation at 30 mol% when it typically induces lateral domain formation. The question remains unanswered: do the lipid monolayers act independently or can phase domains be coupled? Although there is clearly no coupling between leaflets in DMPC and DPPC bilayers, this is different for sphingolipids. Schmidt et al. [120] have shown that the phase behaviour of the outer monolayer dictates the phase behaviour of the inner monolayer in N-lignoceryl SM vesicles using proton NMR. The transition onset temperature of the inner leaflet followed the changes in the outer leaflet, suggesting that the two layers are coupled. It is obvious again that one should be careful about drawing conclusions from model studies with synthetic PC species that do not occur in cell membranes.

11. Lipid rafts function in sorting and transport

Cholesterol and ceramide are synthesized in the ER and the ceramide is further modified in the Golgi complex by the addition of phosphorylcholine to form SM or monosaccharides to form glycosphingolipids. Cholesterol is present in the Golgi complex and can be stained with the polyene antibiotic filipin [121,122]. This would suggest that rafts can be formed in the Golgi apparatus and serve as platforms for delivery from the trans-Golgi network (TGN) (Fig. 3). This is confirmed by the observation that in epithelial cells apically destined proteins like influenza HA and the GPI-linked PLAP acquire detergent resistance in the Golgi complex [33,96]. These proteins are solubilized by TX-100 after extraction of cholesterol with saponin or cyclodextrin [123,124]. The transport of GPI-anchored proteins through the secretory pathway also depends on sphingolipids: when sphingolipid biosynthesis is blocked in polarized Madin-Darby canine kidney (MDCK) epithelial cells with fumonisin, the apical sorting of the GPIlinked protein GP2 is abolished [125]. The transport of E-cadherin to the basolateral surface is not affected, consistent with the notion that basolateral sorting depends on the rab-NSF-SNAP-SNARE mechanism [126,127] and signals in the cytoplasmic domains of proteins, usually tyrosine or dileucine motifs, related to clathrin coated pit signals [128]. In addition to GPI anchors, other signals can direct proteins to rafts. For HA, the raft association depends on amino acids in the transmembrane domain [124] while other proteins may use their N-glycans to associate [129,130], possibly through the binding to lectins like VIP36, which is found in DIGs and TGN-derived vesicles [129]. The integrity of rafts critically depends on cholesterol. Reduction of the cholesterol content of living cells with lovastatin, an inhibitor of cholesterol synthesis, and additional extraction with methyl-β-cyclodextrin reduced the TGN to surface transport of the apical marker protein influenza HA and caused missorting while the transport of the basolateral marker VSV-G was unaffected [122]. The preferential axonal delivery and



Fig. 3. Apical (light grey) and basolateral (dark grey) exocytic pathways in MDCK cells (A) and equivalent pathways in fibroblasts (B) and neurons (C). In resting fibroblasts apical and basolateral cognate pathways deliver cargo to the cell surface randomly while in neurons (C) the axonal and somatodendritic surfaces are considered equivalent to epithelial apical and basolateral domains respectively. Modified from [122].

detergent insolubility of influenza HA and the GPIlinked Thy-1 in fully polarized hippocampal neurons suggest an analogy with apical transport in MDCK cells [131]. As in MDCK cells the polarized delivery of HA and endogenous Thy-1 in neurons was disturbed by cholesterol extraction and by inhibition of sphingolipid biosynthesis whereas the transport of the dendritic marker GluR1 was not affected.

12. Caveolae

The cholesterol-binding protein caveolin-1 is considered as the structural component that is responsible for the morphology of caveolae [71,132]. It is inserted in the membrane as a hairpin with both termini in the cytosol and forms homo-oligomers in vivo [133]. During the transport to the cell surface, the protein forms oligomers of increasing size and after reaching the Golgi, the complex becomes resistant to extraction with Triton X-100. The oligomerization is enhanced by cholesterol and stabilized by the triple palmitoylation on cysteine residues in the C-terminal region of the protein [134]. Caveolin-1 provides us with some riddles. It is present in the apical and basolateral plasma membranes of polarized epithelial cells but caveolae can be found exclusively in the basolateral membrane. The recently identified member of the caveolin family, caveolin-2 [135], may provide an answer. This protein differs from caveolin-1 in its N terminus and its hyperphosphorylation but has the same hairpin conformation and shows a largely similar tissue specific expression. Caveolin-1 and -2 colocalize in the basolateral membrane while caveolin-2 seems to be largely excluded from the apical membrane [136]. This suggests that caveolin-1 is a raft organizer that induces the formation of raft clusters for apical transport from the TGN while the specific morphology and functions of caveolae require the presence of both caveolin-1 and -2.

Caveolin mRNA levels are up-regulated following uptake of free cholesterol from LDL [137]. The mature (cleaved) sterol regulatory element-binding protein (SREBP), required to up-regulate the transcription of the genes involved in cholesterol biosynthesis, was found to down-regulate caveolin gene transcription [138]. When high levels of cholesterol in the ER prevent cleavage and release of the SREBP, caveolin is concomitantly expressed and enhances cholesterol efflux to HDL [139], while de novo cholesterol biosynthesis is simultaneously down-regulated. The role of caveolin in the regulation of the cholesterol balance is strong evidence that proteins can act as sphingolipid-cholesterol raft organizers to regulate their function.

13. Conclusions

The observation that sorting and transport of lipids and proteins as well as cell signalling are mediated by the formation of small lipid-based domains or rafts is fully consistent with the described intrinsic tendency of cholesterol and sphingolipids to organize into microdomains in membranes. Recent studies demonstrate that these sphingolipid-cholesterol rafts should be considered as liquid ordered phase domains dispersed in a liquid crystalline bilayer [73,140]. The integrity of rafts is critically dependent on cholesterol. The issue of raft size remains an open question but theoretical considerations as well as experimental data suggest that rafts are small and dynamic but can be stabilized into bigger structures such as caveolae and apical transport carriers by specific proteins. The association of proteins to rafts modifies their functions. It will be one of the future challenges to analyse the mechanisms by which molecules are sequestered or excluded from rafts. Finally, it will be necessary to employ new methods to analyse the composition of raft lipids in detail to understand the structural basis of lipid immiscibility.

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