Sphingolipids and Membrane Domains: Recent Advances

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Contents

Abstract There is growing evidence that cell membranes can contain domains with different lipid and protein compositions and with different physical properties. Furthermore, it is increasingly appreciated that sphingolipids play a crucial role in the formation and properties of ordered lipid domains (rafts) in cell membranes. This review describes recent advances in our understanding of ordered membrane domains in both cells and model membranes. In addition, how the structure of sphingolipids influences their ability to participate in the formation of ordered domains, as well as how sphingolipid structure alters ordered domain properties, is described. The diversity of sphingolipid structure is likely to play an important role in modulating the biologically relevant properties of "rafts" in cell membranes.

Keywords Rafts • Domains • Sphingolipids • Ceramide • Cholesterol

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1 Membrane Physical State and Membrane Domain Formation

Phospholipids and sphingolipids can form bilayers that exist in either a tightly packed solid-like (gel) state or a loosely packed fluid (liquid disordered, Ld) state (London 2005). For vesicles composed of a specific lipid, the gel state melts to form the Ld state above a characteristic melting temperature (Tm). In mixtures of high-Tm lipids (e.g., natural sphingolipids, which easily form a tightly packed, ordered state due to having saturated acyl chains and no cis double bonds) and low-Tm lipids (natural phospholipids which generally have one acyl chain with one or more cis double bonds), gel and Ld phases can coexist (Korlach et al. 1999). Although the potential biological importance of lipid phase or lipid domain formation involving coexisting ordered and disordered domains was considered in early studies, the biological implications of this possibility were only first seriously considered when it was proposed that sphingolipid domains might have an important role in sorting of molecules between membranes (Simons and van Meer 1988; van Meer and Simons 1988). Such domains were later named "lipid rafts" (Simons and Ikonen 1997). The idea that sphingolipid domains might exist in cells gained an important impetus when it was found that sphingolipid- and cholesterol-rich detergent-resistant membranes (DRM) could be isolated from mammalian cells (Brown and Rose 1992). When cholesterol is present, the tightly packed liquid ordered (Lo) state tends to form in place of the gel state (London 2005) and, based on model membrane studies, the hypothesis was proposed that lipid domains in cells might be sphingolipid- and cholesterol-rich Lo domains coexisting with Ld domains (Ahmed et al. 1997; Schroeder et al. 1994). It has now been widely shown that in vesicles composed of mixtures of sphingolipids, unsaturated phospholipids, and cholesterol, coexisting Lo and Ld domains can be observed by microscopy at conditions close to physiological (Hammond et al. 2005; Veatch and Keller 2003), and Lo domain formation is the working model for the physical state of "membrane rafts/lipid rafts" in cells (Dietrich et al. 2001a, b; Lingwood et al. 2008; London 2005; Sengupta et al. 2007a, b). Rafts are of biological importance because, by co-clustering membrane lipids and specific proteins in a domain or by segregating them into different domains, specific sets of protein–protein interactions can form or be regulated. In addition, the differences in lipid physical properties and composition in raft and non-raft domains could influence protein function via lipid environment-induced changes in protein conformation.

Raft domains have been proposed to play a crucial role in many important processes that take place within eukaryotic cell membranes, including not only protein and lipid sorting into different membranes but also modulation of signal transduction, especially in the immune system, many types of bacterial and viral infections, including HIV and influenza, and amyloid formation (e.g., Cuadras and Greenberg 2003; Drevot et al. 2002; Gulbins and Kolesnick 2003; Kamiyama et al. 2009; Klemm et al. 2009; Lafont et al. 2002; Lu et al. 2008; Lyman et al. 2008; Manneville et al. 2008; Mukherjee et al. 1999; Murphy et al. 2006; Persaud-Sawin

et al. 2009; Rentero et al. 2008; Riethmuller et al. 2006; Scheiffele et al. 1999; Scolari et al. 2009; Simons and Toomre 2000; Sohn et al. 2008a, b; Taylor and Hooper 2007; Williamson et al. 2008; Young et al. 2003, 2005; Zech et al. 2009).

There are many important issues that must be considered to understand the principles and details of domain formation and properties. We refer the interested reader to previous reviews that describe these issues and summarize earlier studies (Feigenson 2006, 2009; Heberle and Feigenson 2011; London 2005; Quinn 2010; Veatch and Keller 2005). In this review we concentrate on topics in lipid domain formation that have seen recent advances and also on studies that specifically focus on sphingolipids.

2 Growing Evidence for Rafts in Cells

Unlike the situation in model membranes, whether "lipid rafts" form in cells has been controversial. It appears that domains are hard to study in living cells due to their small size under basal physiological conditions (Lingwood et al. 2008; Lingwood and Simons 2010; Veatch et al. 2007, 2008). The presence of the cytoskeleton may limit raft size, or even inhibit domain formation (Baumgart et al. 2007; Ehrig et al. 2011). It is important to point out that to be functionally important, domains need not be large or permanent. They only need to be large enough to cluster or segregate proteins, do so for long enough to affect protein activity, and form upon physiological-triggering, e.g., by clustering raft-associating components (Hammond et al. 2005; Lingwood et al. 2008). Indeed, what appear to be much larger domains, or membrane regions enriched in one type of small domains, can be induced to form in cells under activated conditions (see below).

Despite the difficulty of detecting domains under most conditions, recent studies have strengthened the hypothesis that membrane domains do form in cells. Novel fluorescence microscopy probes to visualize membrane order (e.g., using Laurdan or its derivatives, or using di-4-ANEPPDHQ) (Gaus et al. 2003, 2005, 2006a, b; Harder et al. 2007; Kim et al. 2007; Owen et al. 2006, 2007; Zech et al. 2009) detect lipid domains in living cells. The domains these methods detect may also be raft-rich regions in which ordered raft nanodomains are especially abundant rather than single uniform domains. Recent advances in single molecule (Pinaud et al. 2009) and other fluorescence methods also provide additional evidence for raft-like cellular lipid domains (Lenne et al. 2006; Pinaud et al. 2009), including studies using advanced superhigh-resolution light microscopy that suggest the formation of very small domains (Eggeling et al. 2009; Sahl et al. 2010; van Zanten et al. 2010) as did early studies (Varma and Mayor 1998). However, the perturbations arising from the labeled lipids (Zhao et al. 2007) and the high laser powers used for super-resolution microscopy may complicate interpretation (Mueller et al. 2011). Evidence that ordered domains control protein–protein interaction, especially in the immune system, is also growing. Mast cell studies show that domains control kinase function by segregation from phosphatases (Young et al. 2003, 2005). Recent studies using sterol modification,

probes of membrane order, and lipidomic analysis of plasma membrane domains all indicate that lipid domain formation accompanies both T cell activation (Rentero et al. 2008; Zech et al. 2009), and B cell activation (Sohn et al. 2008a, b). Studies of membrane budding strongly support the hypothesis that traffic of lipids and proteins to different membranes is also dependent upon domain segregation (Brugger et al. 2000; Klemm et al. 2009; Manneville et al. 2008; Mukherjee et al. 1999) and that in at least one case, viral proteins exploit Ld/Lo domain boundaries to induce viral budding (Rossmann et al. 2010). Other recent papers also support an important role for rafts in membrane sorting of proteins (Klemm et al. 2009; Norambuena and Schwartz 2011; Refaei et al. 2011) while in vivo studies show a role of glycosphingolipids in sorting (Zhang et al. 2011). The difference between Lo vs. Ld bending modulus has been shown to be able to drive membrane segregation (Baumgart et al. 2003; Manneville et al. 2008; Rossmann et al. 2010; Sorre et al. 2009).

The argument that the compositional complexity of natural membranes in terms of their numerous lipid and protein species would prevent domain formation is ruled out by studies detecting large domain formation in two types of plasma membrane preparations, giant membrane vesicles, and plasma membrane spheres (Baumgart et al. 2007; Lingwood et al. 2008; Veatch et al. 2008). It is possible that a partial loss of membrane asymmetry and loss of cytoskeletal connections influence domain size in such preparations (Ehrig et al. 2011; Keller et al. 2009). Finally, raft-like domains have even been detected in a bacterium that contains cholesterol obtained from their hosts (LaRocca et al. 2010). Other bacteria may also have raft-like membrane domains (Lopez and Kolter 2010).

3 Recent Advances in Our Understanding of Raft Formation Principles from Model Membrane Studies: Domain Size

Several important raft properties have been the focus of recent model membrane studies. One is domain size. Although Lo domains can be very large in model membranes, and easily detected by light microscopy, it is clear that in cells they are often very tiny, perhaps on the order of a few to 100 nm. Recent studies have shown that in model membranes with realistic plasma membrane outer leaflet lipid compositions, i.e., sphingomyelin/1-palmitoyl, 2-oleoyl phosphatidylcholine/cholesterol (SM/POPC/cholesterol), Lo domains can also be very small (Pathak and London 2011). Under some conditions, ordered domains that are too small to be detected with FRET pairs having a large (~50 Å) donor-acceptor interaction radius (Ro) can be identified in SM/POPC/cholesterol vesicles. These domains are detected using FRET pairs and short-range quenchers with small (12–25 Å) interaction radii (Pathak and London 2011). These studies indicate that domain sizes with estimated radii as small as ~40 Å form in this mixture at 37 °C.

Why do these tiny domains fail to fuse into large ones? The origin of the size stability of such small "nanodomains" is an active area of research. It has been

proposed that domains are small because they exist at conditions close to the critical point of the ternary lipid mixtures investigated (Honerkamp-Smith et al. 2008; Veatch et al. 2007, 2008). Consistent with the predictions of this model, nanodomains gradually decrease in size in SM/POPC/cholesterol mixtures as temperature increases (Pathak and London 2011). It has also been proposed that the presence of molecules that prefer locating at domain edges may contribute to small domain size (Brown and London 1998; Chiantia et al. 2007; Mitchell and Litman 1998). Several studies have shown that the presence of lipids with one saturated and one unsaturated acyl chain (e.g., POPC), which are likely to have some affinity for domain edges, produce smaller domains than lipids with two unsaturated acyl chains (Brewster et al. 2009; Brewster and Safran 2010; de Almeida et al. 2003; Heberle et al. 2010; Pokorny et al. 2006; Schafer and Marrink 2010).

Such nanodomains may not have all the properties of true Lo phases, but retention of tight packing and protein binding specificity similar to that of large Lo domains, combined with a sufficient time persistence (under cellular conditions, e.g., in the presence of proteins), would be sufficient for them to be of biological relevance.

4 Recent Advances in Our Understanding of Raft Formation Principles from Model Membrane Studies: Evidence that Detergent Does not Induce Raft Formation

Ordered domains are detergent insoluble and, as noted above, detergent (e.g., TX-100)-insoluble sphingolipid- and cholesterol-rich ordered membranes (DRM) can be isolated from cells (Brown and Rose 1992; Schroeder et al. 1994). However, the isolation of such domains has not been sufficient to prove that DRM arise from preexisting rafts because detergent could alter domain formation (Brown and London 2000). Many studies have confirmed that when Lo and Ld domains coexist, the DRM arise from the Lo region of the membrane (Ahmed et al. 1997; Dietrich et al. 2001a, b; El Kirat and Morandat 2007; Garner et al. 2008). However, it has been reported by one group that domain formation in SM/POPC/chol vesicles can be stabilized by TX-100, so that they form at higher temperatures only in the presence of TX-100 (Heerklotz 2002; Heerklotz et al. 2003). This observation has been frequently cited as evidence that DRM obtained from cells may be a detergent artifact. However, a reinvestigation of the effect of TX-100 upon domains in SM/POPC/chol vesicles has found that TX-100 does NOT stabilize domain formation, but rather induces the coalescence of preexisting nanodomains into larger domains (Pathak and London 2011). This greatly reduces the concern that domains are an artifact of detergent treatment. Nevertheless, it must be kept in mind that solubilization studies carried out at 4 °C cannot be used to argue that ordered domains are present at 37 °C. Less perturbing methods to obtain domains by physical membrane fragmentation or detergent solubilization at 37 °C should be helpful in this regard (Ayuyan and Cohen 2008; Chen et al. 2009; Drevot et al. 2002; Macdonald and Pike 2005; Morris et al. 2011; Smart et al. 1995; Song et al. 1996).

5 Recent Advances in Our Understanding of Raft Formation Principles from Model Membrane Studies: Role of Lipid Asymmetry in Domain Formation

In the vast majority of studies the model membranes used are symmetric in terms of having identical lipid compositions in each leaflet (monolayer). This limits the ability to extrapolate from model membrane vesicles to real membranes, which have a distinct, if poorly characterized, degree of lipid asymmetry. In the case of the mammalian plasma membrane, it is known that the outer (exoplasmic, exofacial) leaflet is rich in sphingolipids, including sphingomyelin (SM) and glycosphingolipids (GSL), as well as phosphatidylcholine (PC). The inner (cytoplasmic, cytofacial) leaflet is rich in phosphatidylethanolamine (PE) and anionic lipids such as phosphatidylserine (PS) and phosphatidylinositides (Verkleij et al. 1973). Cholesterol is found in both leaflets (in relative amounts that are still disputed) (Mondal et al. 2009). In other membranes, much less is known about asymmetry due to the technical difficulty of asymmetry measurements.

An important asymmetry issue complicating our understanding of membrane domain formation is that inner leaflet lipids have little to no sphingolipid, although this is not entirely certain (van Meer 2011). This raises the question: How could ordered domains form in the inner leaflet? The solution may be that the outer leaflet lipids influence inner leaflet physical properties, i.e., inner and outer leaflet physical states may be coupled (Collins 2008; Kiessling et al. 2009). Coupling could provide a mechanism by which information is transferred across the membrane via lipids. For example, inner leaflet domains induced by outer leaflet domains could act by concentrating cytosolic-surface membrane proteins with a high affinity for ordered domains (e.g., proteins anchored by saturated acyl chains (London 2005; Melkonian et al. 1999)).

Until recently, few methods to prepare suitable asymmetric membranes have been available. Asymmetry has been most readily achieved with planar bilayers (Honerkamp-Smith et al. 2008; Kiessling et al. 2009; Wan et al. 2008). However, asymmetric closed lipid vesicles would have an even wider utility for a variety of applications. Past attempts to make asymmetric lipid vesicles have not come into wide use, perhaps because of limited control over asymmetry, applicability to a limited number of lipids (Everett et al. 1986; Hope and Cullis 1987; Hope et al. 1989; Malewicz et al. 2005; Pagano et al. 1981), and for those methods involving a leaflet-by-leaflet assembly method, unsuitability for membrane protein incorporation, and residual-contaminating oils (Hamada et al. 2008; Hu et al. 2011; Pautot et al. 2003).

Studies (Anderson et al. 2004; Niu and Litman 2002) showing that high concentrations of methyl-β-cyclodextrin (MβCD) can bind phospholipids tightly have opened up new possibilities for preparing asymmetric phospholipid and sphingolipid lipid vesicles, removing the original lipid in the outer leaflet of an acceptor membrane while a new lipid replaces it (Cheng et al. 2009). Asymmetric small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and giant

unilamellar vesicles (GUV) have now been prepared with this lipid exchange approach (Cheng and London 2011; Cheng et al. 2009; Chiantia et al. 2011). Exchange protocols have been used to produce asymmetric vesicles with various lipid compositions, including vesicles containing cholesterol.

Studies of asymmetric planar bilayers and vesicles have shown that leaflets rich in sphingolipids or other high Tm membrane lipids (i.e., with long, linear saturated acyl chains) can indeed induce reorganization/domain formation in the opposite membrane leaflet (Collins and Keller 2008; Kiessling et al. 2006; Wan et al. 2008). This ability depends on experimental conditions including the type of lipid in the leaflet opposite that in the high Tm lipid-rich leaflet (Cheng et al. 2009; Chiantia et al. 2011; Collins and Keller 2008; Kiessling et al. 2006; Wan et al. 2008). The exact physical state of the opposite leaflet (composed of low Tm lipid) is not clear; it may be fully or partly ordered (Cheng and London 2011; Cheng et al. 2009; Chiantia et al. 2011; Kiessling et al. 2006; Wan et al. 2008). This suggests that there is some degree of coupling between the inner and outer lipid physical states. However, this interleaflet coupling is not "strong," as strong coupling would imply that the thermal melting of the bilayer occurs at a Tm that is intermediate between that of the inner and outer leaflet lipids, and this is not what is observed (Cheng and London 2011; Cheng et al. 2009). Instead, the high Tm-lipid-rich leaflet has a Tm similar to that in vesicles composed of pure high Tm lipid. There may be weaker coupling that breaks down as temperature increases (Cheng and London 2011), although further studies are needed to confirm this. In any case, our knowledge of the behavior of asymmetric bilayers is very incomplete. Further progress awaits extension of studies to an even wider variety of lipid compositions, and the development and application of additional assays to define the physical state of the individual leaflets.

The role of membrane proteins and high cholesterol concentrations are other areas that need further investigation. We will not cover these topics here, but simply note that studies of the relationship of protein structure to raft affinity and raft properties in model membrane systems are beginning to yield important conclusions (Baumgart et al. 2007; Coskun et al. 2011; Fastenberg et al. 2003; Johnson et al. 2010; Kaiser et al. 2011; Nelson et al. 2008, 2010; Nikolaus et al. 2010; Sengupta et al. 2008; Shogomori et al. 2005; Tong et al. 2009). Computational methods are another important area seeing important advances (Perlmutter and Sachs 2011) that will not be covered here.

6 Effect of Sphingolipid Structure on Domain Formation

General Properties of Sphingolipids: Model membrane studies have shown that, in general, any lipid with high melting temperature can separate from lipids in an Ld phase to form ordered domains. Sphingolipids are particularly important in this context as the main source of lipids with high Tm that are found in cellular membranes. The tendency of this class of lipids to form ordered domains derives

from their peculiar chemical structure, very different from glycerophospholipids. Sphingolipids are composed of a long-chain base and an amide-linked acyl chain. The base, which is the analog of the glycerol backbone attached to a fatty acid on the 1-position portion of a typical glycerophospholipid (e.g., PC), is in most cases a sphingosine molecule. Sphingosine has 18 (or 20) carbon atoms with a trans double bond between the carbon atoms 4 and 5, plus 2 OH groups on carbon atoms 1 and 3 and an amino group on carbon atom 2. Sphingosine variants (e.g., saturated sphinganine or the 4-hydroxylated and saturated phytosphingosine) can also be found in nature (Goni and Alonso 2009). It is worth noting that this double bond should have a much smaller effect on Tm than the double bonds found in fatty acids for two reasons. First, a trans double bond only causes a small kink relative to that formed by a cis double bond. Secondly, it is near one end of the hydrocarbon chain, while double bonds in fatty acyl chains of a membrane lipid interfere with tight packing (as judged by low Tm values) most strongly when they are located near the center of the chain (Barton and Gunstone 1975), as is the case with the cis double bonds of natural fatty acids.

Most sphingolipids have a fatty acid (typically 16–24 carbon atoms long with the exception of skin lipids) N-linked to the sphingosine. This fatty acid is the analog of the 2-position fatty acid of glycerophospholipids. However, unlike the cis double bond containing fatty acids common in glycerophospholipids, this acyl chain is usually fully saturated in eukaryotes and is a major factor imparting a high Tm to sphingolipids. Even in the one common case in which eukaryotic sphingolipids have an unsaturated fatty acid, that fatty acid is usually a 24 carbon chain with just one double bond, which still supports a high Tm value. On the other hand, sphingolipids with very long polyunsaturated acyl chains are present in spermatozoa (Sandhoff 2010), suggestive of some specialized function.

In the discussions below on the effects of fatty acid chain structure and length upon domain-forming properties in model membranes it should be kept in mind that almost all studies to date involve bilayers with symmetric bilayer compositions, i.e., in which the lipid composition of the inner and outer monolayers/leaflets are identical. Such bilayers may have different properties than bilayers with lipid asymmetry, as found in natural membranes, especially in cases in which a lipid molecule has two hydrocarbon chains of unequal lengths (i.e., "chain asymmetry"), which gives rise to the possibility of penetration of the longer chain into the opposite leaflet (interdigitation).

At the level of the polar head of the lipid there are other important structural features which may contribute to a high Tm: while each glycerophospholipid can accept two hydrogen bonds (via its carbonyl groups), sphingolipid hydroxy and amide functional groups can act both as donors and acceptors for hydrogen bonding (Mombelli et al. 2003). This feature, together with the presence of long saturated acyl chains, may confer upon sphingolipids the ability to interact strongly with other (similar) lipids. It should be pointed out that hydrogen bonding between two groups in a membrane does not face as much competition with hydrogen bonding to water as in aqueous solution, and so may be a strong driving force for interactions between lipids.

There are a large variety of sphingolipids that can be found in cellular membranes, ranging from the simplest ceramides to more complex glycosphingolipids. In the next paragraphs, we review recent studies regarding the main sphingolipid species.

Sphingosine: Sphingosine (Sph) is by definition the simplest sphingolipid, but was shown to influence a variety of cellular processes, including cell growth and differentiation, receptor modulation, and cytotoxicity (Hannun and Bell 1989; Merrill and Stevens 1989). It is only a minor component of membranes, and the effects of Sph on membrane organization have received little attention. Work by Mustonen et al. (Mustonen et al. 1993) investigated the effects of Sph on membrane–protein electrostatic interactions, since Sph is positively charged at physiological pH (Lopezgarcia et al. 1995). More recently, Contreras et al. (Contreras et al. 2006) showed that Sph is not able to produce gel domains by itself but can reinforce the existing ones, and due to the generation of solid–fluid interfaces and the consequent packing defects, can also produce permeabilization in lipid vesicles. The anti-apoptotic Sph derivative Sph-1-phosphate was shown to have a stabilizing effect on lipid lamellar structures (versus negatively curved, inverted phases) and to be the only sphingolipid-signaling molecule that can be found dissociated from the cell membranes in the cytosol (Garcia-Pacios et al. 2009).

Ceramide: Ceramide (Cer) is another very simple sphingolipid, constituted by the sphingosine base (sphinganine in the case of dihydroceramide) linked to an acyl chain via its amide group. This molecule can be produced in mammalian cells either via de novo synthesis (via a family of six ceramide synthases) or through hydrolysis of SM phosphocholine group, mediated by sphingomyelinases. In response to specific stimuli, ceramide concentration in physiological contexts can reach 10–20 % of the total lipid content (Cremesti et al. 2002; Hannun 1996). Cer is considered both an important second messenger and a membrane structural component involved in several biological processes, such as cell growth, differentiation, apoptosis, senescence, and bacterial and viral pathogenesis (Bollinger et al. 2005; Kolesnick et al. 2000). It has a high Tm (which can be as high at 90 °C (Shah et al. 1995)) and high hydrophobicity (due to its small polar headgroup). These properties are important reasons Cer is one of the main components of the water-impermeable extracellular matrix of the stratum corneum of the skin (Shah et al. 1995; tenGrotenhuis et al. 1996; Wartewig and Neubert 2007).

Although Cer can exert some of its biological functions via direct interaction with specific target proteins (Grosch et al. 2012), we will focus on its effects on the lateral organization of the plasma membrane. It was observed that Cer accumulation in the plasma membrane leads to the formation of large lipid–protein domains ("platforms") involved, for example, in the internalization of viruses and parasites and in the induction of apoptosis (Gulbins and Grassme 2002). Cer-rich platforms may act in these contexts as sorting locations for membrane receptors, inhibitors, and other membrane components involved in signaling (Gulbins et al. 2004). For example, receptor clustering and trapping in Cer-rich domains have been suggested by experiments performed with the receptors Fcγ II (Shakor et al. 2004) and CD95

and CD40 (Grassme et al. 2001, 2002). Similarly, Cer-rich domains seem to recruit the receptors mediating the internalization of *Neisseria gonorrhoeae* (Grassmé et al. 2007).

The molecular mechanisms behind the formation of such Cer-rich domains and their relation to the SM–cholesterol raft domains were investigated in recent years using model membranes with controlled compositions. These studies showed that long-chain Cer which are symmetric in the sense that the acyl chain and sphingoid base have similar effective hydrocarbon lengths (C16:0, C18:0 N-linked acyl chains) can strongly interact with SM, forming a highly ordered Cer-rich phase (Boulgaropoulos et al. 2011; Chiantia et al. 2006; Megha and London 2004; Silva et al. 2007). The interplay between Cer, SM, and cholesterol can be understood in the context of the "umbrella model" (Huang and Feigenson 1999). Several studies have shown that cholesterol and Cer can compete for the interaction with SM, since both molecules have small headgroups that can be shielded from (unfavorable) interactions with water molecules by lipids with large headgroups like SM, which act as umbrellas (Alanko et al. 2005; Megha and London 2004; Nyholm et al. 2010; Sot et al. 2008). As a result, Cer can readily displace cholesterol from ordered domains, and the reverse is also possible (Megha and London 2004; Silva et al. 2007). Further work showed that, in analogy with Cer-rich platforms observed in cells, Cer homeostasis and the presence of Cer-rich domains affect the lateral organization of membrane proteins in model membranes (Chiantia et al. 2008; Dasgupta et al. 2009; Pabst et al. 2009). Very-long-chain Cer (with saturated C20–C24 N-linked acyl chain) promote in general ordering of the membrane, and formation of gel domains (Pinto et al. 2008, 2011). They also may have interesting functions due to the mismatch between the lengths of the hydrocarbon of the sphingoid base and acyl chain (see below).

Unnatural short-chain Cer (with C2–C12 N-linked acyl chains) are used extensively in in vivo experiments to replace their long-chain analogs. They were shown to have very different effects than long-chain Cer on the lateral organization of the bilayer (Chiantia et al. 2007; Megha et al. 2007; Nybond et al. 2005; Westerlund et al. 2010). Unlike long-chain Cer, these molecules disorder membranes (Gidwani et al. 2003; Sengupta et al. 2007a, b). Nevertheless, short-chain Cer often mimic natural Cer in their functional properties (see, e.g., (Bektas et al. 1998; Kolesnick et al. 2000)). This may reflect their remodeling into natural acyl chain Cer in cells, or effects not dependent upon membrane domain formation, e.g., interaction with specific proteins or functions arising from their "umbrella effect."

Another mystery is the origin of the functional differences between biologically active Cer and dihydroCer, which lacks the sphingoid base double bond. It is not yet clear if this reflects a difference in the domain-forming properties of these molecules, in other biophysical properties, or in functional interactions with proteins. However, functional differences between short-chain Cer and short-chain dihydroCer suggest that the physiological effects of Cer are not exclusively related to lipid domain formation (Simon and Gear 1998).

In addition to the effects upon the formation of lipid–protein domains, Cer can influence the properties of a lipid bilayer due to its intrinsic negative curvature and

tendency to form non-lamellar inverted phases. Increased lipid flip-flop, bending, and vesiculation of the membrane can be observed when Cer is produced in one leaflet of the bilayer (Contreras et al. 2005; Holopainen et al. 2000). This may provide a protein-free mechanism for the sorting of the membrane into the different populations of intraluminal vesicles in vivo (Trajkovic et al. 2008). Cer can also affect the properties of a lipid membrane via the formation of transmembrane channels (formed by stacked Cer molecules all parallel to the plane of the bilayer). These have been proposed to allow the passage of certain proteins initiating apoptosis (Colombini 2010; Siskind and Colombini 2000).

Sphingomyelin: SM (i.e., Cer phosphorylcholine) is one of the most abundant phospholipids in eukaryotic membranes and, in particular, an important component of raft domains in the outer leaflet of the plasma membrane together with cholesterol. The stability of such domains depends on SM–SM and SM–cholesterol interactions which, in turn, have been shown to be determined by details of the chemical structure of the SM molecule. Several studies have thoroughly investigated the role of many SM structural properties at the level of the membrane interfacial region, e.g., polar head size (Bjorkbom et al. 2011), specific stereoconfiguration (Ramstedt and Slotte 1999), the presence of the phosphocholine methyl group (Terova et al. 2005), the sphingosine double bond (Kuikka et al. 2001; Vieira et al. 2010), the hydroxylation of the N-linked acyl chain (Ekholm et al. 2011), and the role of the 3-hydroxyl group or the amide-linkage in establishing hydrogen bonds (Bittman et al. 1994; Bjorkbom et al. 2011; Kan et al. 1991). Other studies investigated the molecular requirements at the level of the hydrophobic region of SM that allow it to participate in the formation of ordered membrane domains. For example, it was observed that SMs from different natural sources (chicken egg, bovine milk, and porcine brain), which have different acyl chain composition, differ in their ability to segregate into ordered domains in the bilayer (Filippov et al. 2006). Saturated N-linked acyl chains with length similar to that of the sphingoid base have strong lipid–lipid interactions (Epand and Epand 2004; Jaikishan et al. 2010). Increasing the length of the N-linked acyl chain above that of the base increases (although not dramatically) the lipid packing abilities of SM, but decreases its interactions with cholesterol (Jaikishan and Slotte 2011; Niemela et al. 2006).

GSL: GSL are sphingolipids covalently bound to oligosaccharidic groups of different dimensions and complexity (Pontier and Schweisguth 2012). These molecules can be usually found in the plasma membrane. They are involved in cell–cell interaction and are often receptors for viruses and toxins (Hakomori et al. 1998; Lingwood et al. 2000; Viard et al. 2003). From a structural point of view, all GSL usually share a Cer backbone, i.e., a sphingoid base with an N-linked long saturated acyl chain. The heterogeneity of the lipids belonging to this group derives then mostly from the polar moiety bound to the Cer backbone, including (in mammals) small monosaccharides (e.g., glucosylceramide), charged groups (e.g., sulfatides), and large assembles of polysaccharides and sialic acid (e.g., gangliosides) (Westerlund and Slotte 2009). Very different phosphate-containing GSL-like sphingolipids are found in plants and fungi (Rhome and Del Poeta 2010; Sperling and Heinz 2003).

Of interest, GSL often have a remarkably high Tm and packing density compared to the corresponding acyl chain-matched SMs or PCs (Ruocco et al. 1981; Smaby et al. 1996). For this reason, they are often found in DRMs together with SM and cholesterol, but without necessarily implying that all these lipids co-localize always in the same membrane domains (Arvanitis et al. 2005; Braccia et al. 2003). It was shown in fact that GSLs can also form specialized domains (glycosignaling domains) involved in cell–cell recognition, independently from the presence of cholesterol (Hakomori 2004). Several studies in model membranes have investigated the domain forming abilities of GSL (see, e.g., (Bjorkbom et al. 2010a, b; Blanchette et al. 2006; Lin et al. 2007; Maunula et al. 2007)) concluding that the Cer backbone structure as well as the number of sugar units and presence of charge in the GSL headgroup can influence the partitioning of these lipids between lateral membrane domains. For a more thorough discussion, the reader is referred to the extensive review of Westerlund and Slotte (2009).

It is beyond the scope of this review to describe the vast literature on GSL physical and biological properties. However, one case worthy of specific comment involves the physical properties of the lactosylceramide (LacCer). LacCer is one of the most abundant neutral GSL and is expressed in particular in the lipid membranes of human neutrophils (Kniep and Skubitz 1998). In these cells, LacCer acts as one of the several pattern recognition receptors involved in the detection of infectious microorganisms and can bind specifically to various pathogens (e.g., *Escherichia coli* or *Candida albicans*) (Iwabuchi et al. 2010; Sato et al. 2006; Teneberg et al. 2004). Experiments by Pagano and coworkers addressed caveolae-dependent membrane trafficking of LacCer in human skin fibroblasts, proposing that LacCer with natural stereochemistry participate in the formation of membrane microdomains that endocytose via a caveolar pathway and promote β1 integrin signaling (Singh et al. 2006, 2007). Interestingly, LacCer analogs with unnatural stereochemistry were found not to support, or even to inhibit, these processes. It should be noted that the LacCer molecules used had either labeled or shortened acyl chains, which could perturb domain-forming properties.

Other studies show that acyl chain length is important for some of the biological functions of LacCer. LacCer has been found to mediate several biological processes (including chemotaxis, phagocytosis, and superoxide generation) that depend on the Src kinase Lyn (Iwabuchi et al. 2010). The interaction between LacCer in the outer plasma membrane and the Lyn molecules, which are anchored to the cytoplasmic side of the bilayer, was shown in turn to be dependent on the presence of long *N*-acyl molecular species (i.e., 24:0 and 24:1 LacCer). Antibody-mediated cross-linking of LacCer in neutrophils (which naturally possess C24:0 and C24:1 LacCer acyl chain species) caused the lipid to colocalize in DRMs and co-immunoprecipitate with the activated form of Lyn. If the experiment was repeated on a neutrophil-differentiated cell line (D-HL-60) that possesses only shorter C16–18 acyl chain species of LacCer, neither co-localization or activation of Lyn could be observed, unless the cells were loaded with exogenous long-chain LacCer (Iwabuchi and Nagaoka 2002; Nakayama et al. 2008). Analogously, the presence of C24 LacCer was shown to be necessary for generation of superoxide, for

chemotaxis towards ß-glucan or anti-LacCer antibodies, and for CD11b/CD18-mediated phagocytosis of non-opsonized microorganisms (Nakayama et al. 2008). These studies suggest that the long *N*-acyl chain of certain sphingolipids can cross the midplane of the lipid bilayer (i.e., interdigitate), to influence proteins on the cytosolic leaflet in order to couple external stimuli with intracellular signal cascades (Iwabuchi et al. 2010; Sonnino et al. 2009). An interesting question that is yet to be answered is whether this involves coupling between rafts in the outer and inner leaflet.

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