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The order of rafts

Conference on Microdomains, Lipid Rafts and Caveolae

Chiara Zurzolo[1,2+], *Gerrit van Meer*[3] & *Satyajit Mayor*[4++]

[1]Università Federico II, Napoli, Italy, [2]Pasteur Institute, Paris, France, [3]Utrecht University, Utrecht, the Netherlands, and [4]Tata Institute of Fundamental Research, Bangalore, Karnataka, India

The EuroConference on Microdomains, Lipid Rafts and Caveolae was held in Tomar, Portugal, from 17 to 22 May 2003. This joint EURESCO Conference/EMBO workshop was organized by G. van Meer and K. Simons and was the second meeting in a series that was initiated by K. Fiedler in 2001.

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Introduction

The plasma membrane was described as a fluid mosaic in the early 1970s by Singer & Nicolson (1972). Since then, several studies that were designed to elucidate the temporal and spatial architecture of the plasma membrane have provided a more complicated picture (reviewed in Edidin, 2003a). Many of these studies indicate that the plasma membrane is at the very least a mosaic of compartments that is maintained by an active cytoskeleton mesh. The membrane raft hypothesis proposes another type of compartmentalization (Simons & Ikonen, 1997; Simons & van Meer, 1988), in which specific lipids may dynamically associate with each other to form platforms that are important for membrane protein sorting and the formation of signalling complexes.

This conference focused on the lateral domains that occur in biomembranes. In this report, we discuss new developments in the understanding of the lateral segregation of lipids that have been obtained from studies in artificial membranes and the parallel efforts to visualize lipidic assemblies in living cell membranes. We also report on new information about the roles of rafts in several cellular processes, such as in the sorting of membrane constituents during vesicular trafficking and in signal transduction, especially in immunological processes and caveolae formation and function.

Raft structure

In artificial membranes. To address the role of lipids in the formation of membrane rafts, studies have been performed with artificial membranes, and these suggest that homogeneous membrane bilayers might be an exception rather than a rule (Edidin, 2003b; McConnell & Vrljic, 2003). In general, the lipid bilayer can exist in three possible states, whose coexistence is temperature- and composition-dependent: gel (s_o, liquid ordered (l_o) and liquid disordered (l_d). Whereas the gel phase is generally observed below the melting temperature (T_m) of the constituent lipids, the two fluid (liquid) states can coexist at equlibrium at temperatures above the T_m or in the presence of cholesterol (Brown & London, 2000). This two-state coexistence for simple mixtures is well understood in terms of thermodynamics, and is described by a phase diagram (Fig. 1). It was recently proposed that the phase diagram that describes domain formation in cholesterol and sphingomyelin (SM) mixtures represents the formation of condensed complexes of the two lipids (Radhakrishnan *et al*., 2000), but P. Kinnunen (Helsinki, Finland) presented an alternative perspective. His studies of pyrene-labelled phospholipids and their interaction with cholesterol indicate that there is no specific interaction between SM and cholesterol in membrane bilayers. Instead, cholesterol accumulates underneath the strongly hydrated phospholipid head groups, which is evident as apparent condensation, or co-segregation, of cholesterol and sphingolipids (SLs) and increased acyl chain order in this region. The co-segregation of SLs

[1]Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, Via Pansini 5-80131, Napoli, Italy
[2]Pasteur Institute, 25 rue du Dr Roux 75724, Paris, France
[3]Department of Membrane Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands
[4]National Centre for Biological Sciences, Tata Institute of Fundamental Research, UAS-GKVK Campus, Bangalore 560 065, Karnataka, India

[+]Corresponding author. Tel: +39 081 545 3033; Fax: +39 081 770 1016;
E-mail: zurzolo@unina.it
[++]Corresponding author. Tel: +91 80 363 6421/29; Fax; +91 80 363 6662/75;
E-mail: mayor@ncbs.res.in

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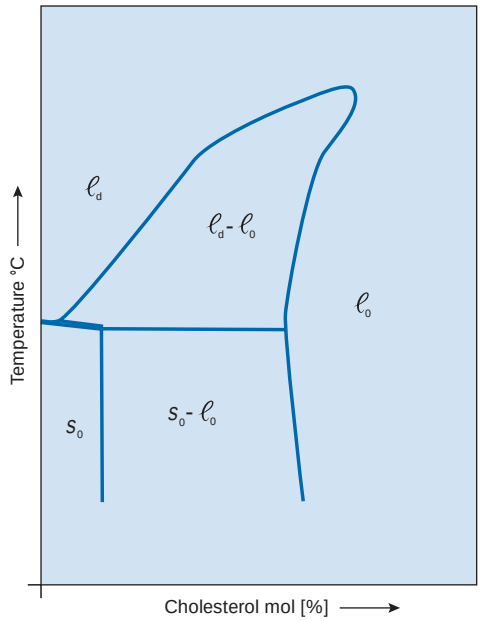

Fig. 1 | Phase diagram depicting liquid-ordered (l_o), liquid-disordered (l_d) and gel (s_o) phases and a region of phase coexistence for a dipalmitoylphosphatidylcholine–cholesterol membrane system. (Figure adapted from Sankaram & Thompson, 1991.)

and cholesterol is thus a result of the mismatch in the thicknesses of their hydrophobic parts with respect to unsaturated phospholipids (Fig. 2). Segregation minimizes the length of the energetically unfavourable boundary between the SL–cholesterol domains and the membrane that is composed of unsaturated phospholipids.

Several lipid probes that differentially partition into l_o or l_d domains have been used to visualize 'raft' formation or phase segregation in artificial membrane systems (Dietrich *et al*., 2001; Samsonov *et al*., 2001; Feigenson & Buboltz, 2001; Veatch & Keller, 2002). These domains range from several micrometres in size to nanometre-scale assemblies. The choice of probe seems to be crucial in identifying the size and character of these domains. W. Vaz (Coimbra, Portugal) presented two rules for the partitioning of lipid amphiphiles between l_o and l_d phases: (i) Partitioning into ordered phases is favoured in proportion to the length of the acyl chain and is disfavoured by chain unsaturation (Mesquita *et al*., 2000); (ii) orientation of the dipole moment of the probe in a direction parallel to the dipolar potential of the membrane surface promotes partitioning into ordered phases, whereas an anti-parallel orientation works against it (Estronca *et al*., 2002). These considerations bring to the fore another level of complexity in using probes to study membrane composition. K. Jacobson (Chapel Hill, NC, USA) showed that a cholesterol-binding protein preferentially associates with l_o domains in artificial membranes, which potentially provides a probe to visualize caveolae/raft domains that are enriched in cholesterol in live-cell membranes (Khan *et al*., 2003).

Relationship of detergent-resistant membranes and l_o *domains*. The use of non-ionic detergent extraction to generate low-density detergent-resistant membranes (DRMs) has had a major role in implicating rafts in cellular functions (Brown & London, 2000). DRMs have been correlated with the existence of l_o phases in artificial membranes and, by extension, l_o domains in cell membranes (London & Brown, 2000). However, the physical basis for the extraction of lipids with detergents is poorly understood. M. Prieto (Lisbon, Portugal) described the phase diagram for a three-component mixture that consists of cholesterol, SM and phosphatidylcholine (PC) at various temperatures. Prieto found that extraction of liposomes made up of a plasma-membrane-like lipid composition (Chol–SM–PC (1:1:1)) with Triton X-100 (TX100) at 4 °C generates DRMs with a composition that is remarkably similar to that of the l_o domain expected at 37 °C. This finding suggests that detergent extraction at 4 °C induces a strong perturbation of the bilayer and does not reflect an *a priori* structure (De Almeida *et al*., 2003). In conjunction with recent studies on the effect of detergents on membrane domains, in which it was observed that TX100 promotes domain formation in single-phase membranes (Heerklotz, 2002; Heerklotz *et al*., 2003), Prieto's work sends out a strong signal that the relationship between DRMs and pre-existing l_o structures is complex and still not understood.

In cell membranes. Unlike the artificial membranes that have been studied, the cell membrane has many lipid components and an active asymmetrical transbilayer. Thus, it is difficult to extrapolate principles that have been obtained from equilibrium phase separation observed in 'dead' artificial membranes to the situation in living cells. In the latter, raft association has been primarily defined by the partitioning of proteins and lipids into DRMs. In this way, glycosylphosphatidylinositol (GPI)-anchored proteins have a central role; they associate with DRMs in a cholesterol- and SL level-sensitive fashion in a variety of cells (Brown & London, 2000).

Many new approaches for detecting heterogeneity in cell membranes have emerged (Edidin, 2003b; Jacobson & Dietrich, 1999) that rely on the distinct diffusion characteristics or enhanced proximity between raft components. Single-particle tracking (SPT) studies at an unprecedented 25-μs timescale (Dietrich *et al*., 2002; Fujiwara *et al*., 2002) have enabled A. Kusumi (Nagoya, Japan) to measure the diffusion characteristics (hop diffusion and intra-compartmental diffusion) of GPI-anchored proteins. Results from these experiments suggest that the GPI-anchored proteins, attached to antibody (Fab)-colloidal gold conjugates, diffuse as extremely small species, which is consistent with them being monomers but inconsistent with any large-scale organization of stable rafts. Stable large-scale structures are formed only after cross-linking 'monomeric' raft components, which results in the assembly of a long-lived signalling platform.

Methods to measure lipid proximity, including chemical crosslinking and fluorescence resonance energy transfer (FRET), have been used but no consensus on the size of rafts has been reached (Edidin, 2003b). Using a combination of hetero-FRET and homo-FRET microscopy (Varma & Mayor, 1998), in conjunction with theoretical modelling of FRET efficiencies, S. Mayor (Bangalore, India) provided evidence that GPI-anchored proteins are present in extremely small cholesterol-sensitive nanoscale structures that consist of only a few molecules. This suggests that the lipid-dependent clusters in membranes of living cells are unexpectedly small, and resolves the contradictory findings that were obtained using different FRET methodologies, chemical crosslinking and diffusion studies (Edidin, 2003b). R. Parton (Brisbane, Australia) used electron microscope (EM) images and quantitative statistical analyses of cluster distributions to show the existence of separate clusters of inner leaflet signalling molecules H-Ras and K-Ras in 40-nm structures that cover ~35% of the inner leaflet (Prior *et al*., 2003). Clearly, understanding how these novel types of lipid–protein assemblies are formed, and how they

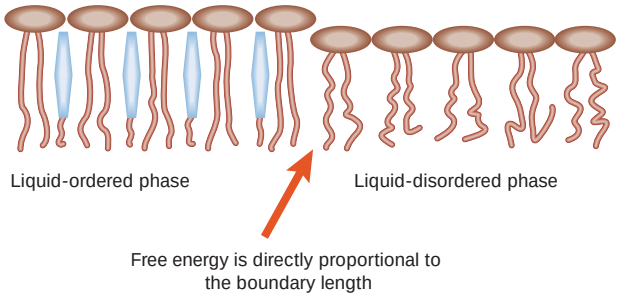

Fig. 2 | Mismatch of hydrated phospholipids and the intercalation of cholesterol as a driving force for a phase separation into liquid-ordered and -disordered phases. (Figure courtesy of P. Kinnunen.)

relate to l_o domains and their function will provide important clues on the structure and composition of membrane rafts.

Emerging raft definitions. At present, rafts are conceptualized by several hypotheses (Simons & Ikonen, 1997; Anderson & Jacobson, 2002; Maxfield, 2002). A general consensus that emerged at this meeting about the nature of a raft in a cell membrane is summarized as follows. Considering the complexity of the system and the poorly understood nature of DRM formation, it is unlikely that DRMs that are derived from cells reflect some pre-existing structure or organization of the membrane. However, the ability to partition with the DRM could reflect an important membrane-related biochemical property of the specific component in question. In living cells, equilibrium phase separation is unlikely, which further complicates the relationship between DRMs, l_o phases and rafts. Cellular lipid assemblies in their *a priori* state are likely to be small, indicating an intrinsic diversity of composition. Functional rafts (that is, larger platforms) are then induced as required and in specific cellular contexts of sorting or signalling. Understanding the mechanisms that govern the generation and use of these lipidic structures will no doubt occupy centre stage of the raft field in the coming years.

Rafts in sorting processes

Exocytosis. In contrast to mammalian cells (see below), GPI-anchored proteins are detergent-insoluble in the yeast endoplasmic reticulum (ER). DRMs might form in the ER because ceramides can mimic the function of SL due to their long chain (C26) fatty acid. Using a synthetic lethal screen in a yeast mutant deficient in fatty acid elongase, ELO3, R. Schneiter (Fribourg, Switzerland) found that a transmembrane protein (Pma1) became separated from DRMs and mis-targeted to the vacuole, whereas GPI-anchored Gas1 traffic was unaffected in a double mutant of *ELO3* and *ERG6*. *ERG6* encodes an enzyme that methylates C24 in the aliphatic side chain of ergosterol (the cholesterol homologue in yeast). He proposed that distinct rafts could be involved in protein sorting and exit from the ER in yeast (Eisenkolb *et al*., 2002), which is consistent with recent studies on protein secretion from the laboratory of K. Simons (Dresden, Germany; Bagnat & Simons, 2002a). According to H. Riezman (Geneva, Switzerland), GPI-anchored proteins exit from the ER in two steps. Initially, they are sorted in the ER membrane and then packaged in COP II vesicles, which are different from those that carry most other transmembrane proteins (Muniz *et al*., 2001). One hypothesis to explain this observation is that there is a physical separation of cargo molecules into distinct ER membrane domains before they are packaged into budding vesicles. Riezman showed that individual SNARE proteins (not a *cis*-SNARE complex) create sites for tethering factors to bind and aid in the initial sorting step (Morsomme *et al*., 2003). Whether these ER-distinct domains are raft-like domains is an open question. In a different assay for polarized protein delivery in yeast, Simons showed that when yeast undergoes membrane polarization and shmoo formation in response to pheromones, ergosterol and DRM-associated proteins localize to the tip of the shmoo (Bagnat & Simons, 2002b). Mutants in SL and ergosterol biosynthesis fail to polarize proteins to the tip of the shmoo and are deficient in mating. Simons therefore proposed that in addition to their role in ER sorting, lipid rafts are required for the functional segregation of different domains at the surface of mating cells.

The role of rafts in protein sorting in mammalian cells may be traced to the observation that apically targeted GPI-anchored proteins first associate with DRMs in the Golgi of polarized epithelia (Brown & Rose, 1992; Zurzolo *et al*., 1994), where (unlike yeast) SL biosynthesis also occurs (Holthuis *et al*., 2001). However, the role of the GPI anchor and its association with DRMs as the sole mechanism for apical sorting has been recently questioned (Lipardi *et al*., 2000). In some cases, oligosaccharide additions are also required (Alfalah *et al*., 1999; Benting *et al*., 1999), which suggests a hierarchical order in apical signals. Furthermore, C. Zurzolo (Naples, Italy) showed that some basolaterally targeted GPI-anchored proteins also associate with DRMs. For apical targeting to occur, she showed that in addition to DRM association, the formation of high-molecular-weight complexes is required. This suggests that oligomerization or association with a high-molecular-weight complex could stabilize raft association and cause the coalescence of small rafts into a functional apical sorting domain or signal.

Endocytosis. Although it has generally been found that association with DRMs is not sufficient to specify a particular pathway of endocytosis (Sharma *et al*., 2002), individual pathways may preferentially internalize distinct types of lipid organization. In this context, Mayor reported that GPI-anchored proteins are internalized through a distinct clathrin- and dynamin-independent pinocytic pathway (Sabharanjak *et al*., 2002) that is sensitive to cholesterol- and SL-level perturbations, which implicates rafts in endosome formation. Using a theoretical approach, M. Rao (Bangalore, India) proposed that if large-scale rafts form from components such as cholesterol and SLs that are able to impart a specific packing arrangement, this could provide a driving force for budding (Rao & Sarasij, 2001) and result in spherical, flask-shaped or grape-like invaginations.

G. van der Goot (Geneva, Switzerland) discussed a new clathrin-dependent 'raft' pathway that is used by anthrax toxin, which binds to the cell-surface anthrax-toxin receptor (ATR) and induces its oligomerization. As for many receptors, including the B-cell receptor (see below), this clustering induces DRM association and clathrin-dependent endocytosis (Abrami *et al*., 2003). The possibility of using toxins as selectable makers for cells that survive the intoxication process could provide an understanding of the molecular and genetic details of the different steps that are involved in internalization through this pathway.

Caveolae, caveolin and cholesterol homeostasis

Flask-shaped invaginations at the cell surface that are decorated by a caveolin 1 (Cav1) coat have been termed 'caveolae' (Anderson, 1998). Although these structures are morphologically well defined (unlike membrane rafts), there is no consensus on their primary function

(Parton, 2003); many aspects seem to be cell specific and dependent on the different methods used for their analysis and purification. The activation of caveolin or caveolae to trigger internalization was another issue at the meeting. This mechanism was proposed by A. Helenius (Zürich, Switzerland) to be important for simian virus 40 (SV40), which enters cells via caveolae and is then delivered to caveosomes (Pelkmans & Helenius, 2002; Pelkmans *et al*., 2002). Caveolar endocytosis of SV40 has revealed a new vesicular transport pathway to the ER, which requires dynamin and a complex orchestration of actin and tubulin networks (Pelkmans *et al*., 2002). Surprisingly, endocytosis of the virus also occurs in Cav1- deficient cells, and in these cells, dynamin 2 is also not required. In an interesting parallel, R. Pagano (Rochester, NY, USA) showed that fluorescent glycosphingolipid (GSL) analogues are selectively endocytosed via caveolae (Singh *et al*., 2003). Exogenously added GSLs stimulate a dynamin-dependent caveolae-mediated internalization pathway that is dependent on the activation of c-*src*. This activation is mediated by an uncharacterized mechanism, but it is lipid specific; ceramide backbone lipids, but not glycerolipids, are more potent activators of endocytosis through this pathway. As activation of the non-receptor tyrosine kinase Src is also required for SV40 internalization, this suggests that activation of tyrosine kinases might be required for triggering a variety of non-clathrin-mediated endocytic mechanisms that seem to involve DRM components.

In terms of understanding the molecular basis of the association of caveolin with DRMs, D. Brown (Stony Brook, NY, USA) showed that no specific domain of Cav1 determines its ability to partition with DRMs, but rather that this property is dispersed along the entire protein. Surprisingly, caveolin mutants that are more soluble in TX100 become 'stuck' in the Golgi, compared with the wild-type caveolin that is normally rapidly exported to the cell surface. She proposed that the association of Cav1 with rafts provides a mechanism for Golgi exit. Caveolin mutants with a low affinity for rafts would either be retained in the Golgi or recycled back to this compartment and therefore fail to assemble at the plasma membrane. This hypothesis is consistent with the concept that membrane thickness dictated by cholesterol levels has a role in transmembrane protein localization. Parton suggested that caveolin might also have a role in regulating intracellular free cholesterol distribution because a caveolin mutant (cav-DGV) associates with lipid bodies and decreases free cholesterol levels in the plasma membrane (Pol *et al*., 2001).

T. Kurzchalia (Dresden, Germany) provided even more food for thought on the subject of cholesterol and caveolin when he showed that *Caenorhabitis elegans,* an organism that lacks cholesterol synthesis, can survive with 'homeopathic' doses of cholesterol. Its membranes have 2–5% of the sterol content of mammalian cells but still form DRMs. Considering that *C. elegans* expresses a functional caveolin gene (Scheel *et al*., 1999), these observations provide a divergent evolutionary perspective on caveolae, cholesterol and rafts.

Rafts and signalling

The role of lipid rafts in signalling is a much touted topic (Simons & Toomre, 2000). However, the structural relationship of lipid rafts with signalling complexes in membranes and the functional significance of rafts in signalling have been hotly debated. Studies on the IgE receptor and the B-cell receptor (BCR) provide some of the strongest evidence that implicates rafts in signalling, but their role in T-cell receptor (TCR) signalling is still unresolved (Germain, 2001). H.T. He (Marseille, France) reported that similar to the TCR, the partitioning of the Fas receptor in DRMs and the lethal effect of the Fas ligand in mouse thymocytes are sensitive to cholesterol depletion (Hueber *et al*., 2002). This suggests that TCR signalling and the triggering of apoptosis may be intimately linked to raft environments. S. Pierce (Bethesda, MA, USA) presented data that support the general idea that oligomerization of the BCR enhances its ability to associate with DRMs (Stoddart *et al*., 2002). Therefore, another emerging concept at this meeting was that the formation of stable, long-lived and possibly large-scale l_o domains provide amplification sites for different receptor signals. Evidence in support of this concept was presented by B. Baird (Ithaca, NY, USA). She summarized data using different biochemical and biophysical approaches suggesting that the dynamic recruitment of lipid rafts is a crucial step in signalling through the IgE receptor. She presented a micro- (and nano)-biochemical approach using micro (and nano)-patterned surfaces that allow the attachment of IgE receptors to ligands with defined stoichiometries. This could be used to analyse the threshold effects of receptor crosslinking and the molecular consequences of crossing signalling thresholds with respect to assembling specific complexes. P. Draber (Prague, Czech Republic) was more circumspect on the role of rafts. He showed that many treatments that implicate lipid components in rafts result in artefacts. Cyclodextrin extracts IgE receptors and Thy1 from cell surfaces, which invalidates the idea that this treatment modifies cholesterol levels alone.

Another important signalling system that seems to be modulated by rafts is that of the EGF-receptor family, EGFR and ErbB proteins. P. van Bergen en Henegouwen (Utrecht, the Netherlands) suggested that high-affinity EGFR exists in cholesterol-enriched domains because cholesterol enhances the affinity of the EGFR when reconstituted in artificial membranes. Furthermore, these receptors show energy transfer with GPI-anchored proteins that are present in the same membranes, but not with transferrin receptors. M. del Pozo (La Jolla, CA, USA) proposed a role for membrane microdomains in Rac GTPase-mediated signalling. On cell adhesion, integrin receptors regulate Rac membrane recruitment and release it from RhoGDI. It is then targeted to lipid rafts in a GTP-dependent manner, from which it couples to downstream effectors (Del Pozo *et al*., 2002). He presented evidence that Rac1 activity depends on the DRM-associated GSL, GM1 and caveolin levels at the cell surface. Loss of adhesion resulted in the loss of GM1 and caveolin from the plasma membrane, due to a mechanism that involves the phosphorylation of Cav1 and resulted in the downregulation of activated Rac1.

This report raises many questions. We are looking forward to an exciting third meeting on microdomains, lipid rafts and caveolae in 2005 to be organized by K. Simons and G. van der Goot, where we hope to hear many of the answers.

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Gerrit van Meer, Chiara Zurzolo & Satyajit Mayor