Chapter 10

Isolation and Analysis of Detergent-Resistant Membrane Fractions

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Abstract

The hypothesis that the Golgi apparatus is capable of sorting proteins and sending them to the plasma membrane through "lipid rafts," membrane lipid domains highly enriched in glycosphingolipids, sphingomyelin, ceramide, and cholesterol, was formulated by van Meer and Simons in 1988 and came to a turning point when it was suggested that lipid rafts could be isolated thanks to their resistance to solubilization by some detergents, namely Triton X-100. An incredible number of papers have described the composition and properties of detergent-resistant membrane fractions. However, the use of this method has also raised the fiercest criticisms. In this chapter, we would like to discuss the most relevant methodological aspects related to the preparation of detergent-resistant membrane fractions, and to discuss the importance of discriminating between what is present on a cell membrane and what we can prepare from cell membranes in a laboratory tube.

Key words Detergent-resistant membrane, Lipid raft, Liquid-ordered phase, Membrane domain, Microdomain, Sphingolipid

1 Introduction

Amphipathic lipids represent the major structural lipids in all cellular membranes in eukaryotes; due to their aggregative properties, they form bilayers that represent the bulk structure of biological membranes, allowing the compartmentalization of the extra- and intra-cellular aqueous environments. Glycerophospholipids (GPL) are by far the major components of biological bilayers, the main bilayer-forming lipid being phosphatidylcholine (PC), which typically accounts for >50 % of all cell membrane GPL. In addition, cellular membranes contain cholesterol and sphingolipids (SL) (in different amounts, depending on the specific membrane, with the highest concentration associated with plasma membranes). Neither cholesterol nor SL are bilayer-forming lipids, however they can be inserted in GPL bilayers through their hydrophobic moieties. Singer and Nicholson [1] proposed that

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the GPL bilayer acts as two-dimensional solvent for the other membrane components, in particular for membrane-associated proteins, allowing in principle the unrestricted freedom of lateral movement of membrane components. Nevertheless, biological membranes are characterized by a high level of lateral order, and membrane components appear to be organized into "membrane domains," i.e., "ordered structures that differ in lipid and/or protein composition from the surrounding membrane" [2]. Lateral heterogeneity in the structure of cellular membranes is strikingly evident in polarized cells (such as epithelial cells, neurons, and oligodendrocytes), however it is present in virtually any cell type, and it is also present in membrane regions lacking a morphologically distinguishable architecture at the micron scale, as revealed by the finding that some membrane components are restricted in their lateral movements and are transiently confined to small domains at the submicron scale ("microdomains") [3]. According to the fluid mosaic model, short- and long range lateral order in biological membranes is due to the creation of a network of protein-driven lateral interactions among membrane components. Considering the wide variety of biologically relevant protein–protein interactions, protein-driven events such as the creation of membrane-associated multiprotein complexes (in some cases, organized by specific proteins able to act as scaffolds [2] such as clathrin, tetraspanins [4], caveolins [5, 6], and flotillins [7]) are undoubtedly major players in the creation and stabilization of membrane domains. A very sophisticated and comprehensive interpretation to protein-driven compartmentalization of membrane components is given by the membrane-skeleton "fence" model [8]. According to this model, limitations in lateral diffusion observed for some membrane-bound proteins might not necessarily imply direct interactions with other membrane components, but might be due to the formation of compartmental boundaries by actin-based membrane skeleton "fences" that are anchored to the membrane by "pickets" consisting of transmembrane proteins. Membrane components are transiently trapped within the compartment. In addition, hydrodynamic friction-like effect at the surface of the immobilized proteins reduces the diffusion rate of membrane components in the adjacent membrane region.

Nevertheless, the collective aggregational properties of membrane lipids might represent a further factor leading to lateral order within membranes. In fact, a series of experiments describing thermal effects on the behavior of lipid mixtures, published almost at the same time of the presentation of the fluid mosaic model, suggested that the limited solubility of lipids in complex lipid mixtures, leading to fluid–fluid phase separation, could be responsible for a certain degree of lateral organization, and could represent a major driving force behind the separation of distinct domains within cell membranes [9–12]. Liquid–liquid phase separation has

been observed in a variety of model systems (reviewed in [13]) and, with some limitations, in biological membranes, for example using fluorescence microscopic and spectroscopic analyses employing order-sensitive probes [14]. In 1982, Karnovsky had already hypothesized that the existence of multiple phases in the membrane lipid environment could drive the "organization of the lipid components of membranes into domains" [15]. However, this notion was applied to an actual cell biology problem only 6 years later, when it was invoked by Simons and van Meer to explain the different lipid composition of the apical and basolateral plasma membrane macrodomains of polarized epithelial cells [16]. Glycosphingolipids (GSL) and cholesterol, which are highly enriched in the apical macrodomain, need to be sorted from the lipids of the basolateral domain (mainly glycerophospholipids) at some intracellular site during their trafficking to the plasma membrane. Van Meer and Simons proposed that the self-associative properties of the apical lipids, leading to the formation of liquid-ordered (l*o*) phase [17–20] domains in intracellular membranes along the trafficking pathway, might be the driving force underlying the differential sorting of apical and basolateral membrane lipid components [21]. These authors introduced the term "lipid rafts" to describe l*o* phase-segregated domains. The concept of lipid rafts became intimately linked with resistance to detergent solubilization in 1992, when Brown and Rose demonstrated that GPI-anchored proteins can be recovered from lysates of epithelial cells in a low-density, detergent-insoluble (Triton X-100-insoluble) form. Detergent-resistant membranes (DRM) enriched in GPI-anchored proteins were also enriched in GSL, but not in basolateral marker proteins [22]. This was regarded as a strong experimental evidence supporting Simons and van Meer's hypothesis regarding the sorting of proteins to the apical domain of polarized cells as a consequence of their association with a GSL-enriched environment, and strongly influenced subsequent research in this field, which became enormously popular when Simons and Ikonen proposed that association with lipid rafts/detergent-resistant membranes might represent a general mechanism for the sorting, targeting and co-localization of membrane-associated proteins, and that lipid rafts might represent functional platforms for the segregation of proteins involved in signal transduction processes [23]. Since 1997, more than 5,000 papers have been published describing the putative structure and functions of lipid rafts (for a few examples, *see* [24–32]), and recently a database specifically dedicated to mammalian lipid raft-associated proteins (RaftProt, http://lipid-raft-database.di.uq.edu.au/) has been developed [33]. A consistent number of these papers relied on the use of resistance to detergent solubilization (based on Brown and Rose's method, sometimes with heavy modifications) for the preparation of fractions representing putative isolated lipid rafts, and many

investigators de facto equated lipid rafts with a membrane fraction characterized by its insolubility in non-ionic aqueous detergents [22]. Alongside enthusiasm, the concept of lipid rafts has elicited fierce criticisms (for an overview *see* [34, 35]), and in particular the notion that detergent-resistance might represent an adequate tool to isolate a fraction enriched in lipid rafts defined as areas of phase separation naturally occurring in biological membrane [2], has been strongly opposed by some authors [35].

In this chapter, we summarize the methodological aspects related to the preparation of detergent-insoluble membrane fractions, and critically review the usefulness of this method as a tool to investigate the supramolecular organization of biological membranes.

2 Preparation of Detergent-Resistant Membrane Fractions

Challenging cells with aqueous solutions containing detergents results in the effective solubilization of most components of the cell membranes (including GPL and intrinsic membrane glycoproteins), as the result of the formation of mixed micelles incorporating detergent and membrane component molecules [36, 37]. This feature has been exploited for the isolation and study of membrane associated proteins. On the other hand, it has been known for a long time that some cellular components are insoluble in non-ionic (Triton X-100) or zwitterionic (Empigin BB) detergents under certain experimental conditions. Indeed, detergent insolubility has been used as an analytical criterion or as a preparative tool long before the lipid raft hypothesis was formulated. The "detergent-insoluble material" (DIM), isolated by sedimentation or centrifugation, was originally shown to be enriched in pericellular matrix proteins (e.g., fibronectin, tenascin, Gp140), in cell attachment site components, including components of cytoskeleton ("detergent-insoluble substrate attachment matrix," DISAM) [38], and in glycosphingolipids, in particular GM1 ganglioside ("detergent-insoluble glycolipid-enriched material," DIG) [39, 40]. Subsequently, it has been shown that detergent-insoluble material was very heterogeneous, being enriched in other sphingolipids, not only gangliosides, (including sphingomyelin, SM, as well) [41–43], cholesterol [42], lipid-anchored proteins (proteins with a glycosylphosphatidylinositol (GPI) or linked with fatty acid residues) [22, 43–48] and other hydrophobic plasma membrane proteins, such as caveolin [49]. Thus, the concept gradually emerged that a peculiar lipid composition leading to the separation of a l*o* phase (the features corresponding to lipid rafts in biological membranes, as hypothesized by van Meer and Simons) could be responsible for the insolubility in aqueous non-ionic detergents, and that DIM is at least in part represented by "detergent-resistant membranes" (DRM), an isolated fraction corresponding to lipid

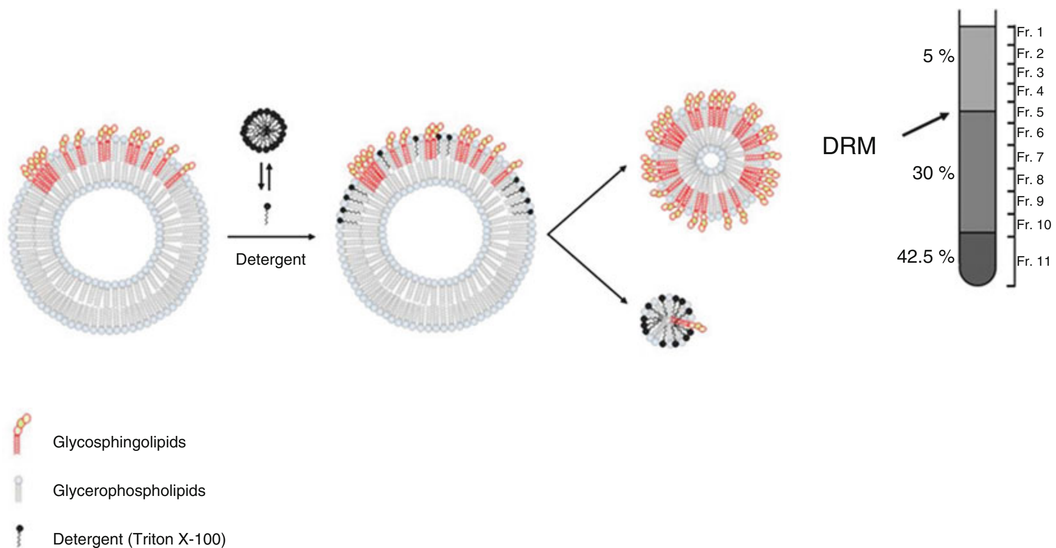

Fig. 1 Detergent insolubility and separation of membrane domains. Detergents in aqueous solutions at concentrations above the critical micellar concentration (CMC) form aggregates such as small micelles. CMC for Triton X-100 is 0.31 mM. Thus, in a 1 % solution, several detergent monomers are present, and can be inserted into the fluid portions of the membrane. Fluid membranes containing Triton X-100 are dissolved and form small mixed micelles enriched in detergent, glycerophospholipids, and proteins. On the other hand detergent is not able to penetrate into membrane areas highly enriched in sphingolipids and cholesterol, due to their high degree of lateral order. This membrane portions form microsome-like structures that can be separated by density gradient centrifugation

rafts, such as those belonging to the apical compartment of polarized epithelial cells (MDCK) or to the caveolar membrane system. Treatment with non-ionic detergent (the most widely used being Triton X-100) at low temperature allows to solubilize lipid components present in the membrane in a liquid-disordered phase (e.g., most glycerophospholipids). These lipids are subtracted from the membrane due to the formation of mixed micelles with the detergent ("solubilization"), while lipid raft components remains laterally organized, excluding detergent monomers ("detergent-insoluble") forming microsome-like or planar structures (Fig. 1). After detergent treatment, the detergent-insoluble membrane fraction can be separated due to its relative low density (buoyancy), likely due to its richness in lipids, i.e., to the high lipid-to-protein ratio [22], using continuous or discontinuous density gradients.

Applying the method originally described by Brown and Rose [22], or its modifications, DRM fractions were isolated from a wide variety of cultured mammalian cells (normal and tumor epithelial cells [22, 49–51], lymphocytes [52, 53] and lymphoid tumor cells [54], neutrophils [55], platelets [56], erythrocytes [57], fibroblasts [50, 58], neurons [59–62] and neuroblastoma

cells [63–65]), and tissues [66–73], plant cells [74, 75], yeast [76, 77], and protozoan [78–80]. We used this procedure to prepare DRM fractions from melanoma [81] and neuroblastoma cells [65], cultured neurons either at different stages of differentiation [59, 82, 83] or challenged with pro-apoptotic stimuli [84], ovarian cancer cells [85, 86], and mouse brain [73].

The original experimental procedure used for the preparation of Triton-insoluble membrane fractions is as follows:

- Cells ($5–8 \times 10^7$, usually corresponding to 4–7 mg cell proteins) are mechanically harvested in phosphate-buffered saline containing 0.4 mM Na_3VO_4, and pelleted.
- Cell pellet is suspended in 1 ml of lysis buffer containing 1 % Triton X-100, 10 mM Tris buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and protease inhibitors, allowed to stand on ice for 20 min, and homogeneized using a hand-driven tight Dounce homogenizer (ten strokes).
- Cell lysates are centrifuged (5 min at $1300 \times g$) to remove nuclei and large cellular debris.
- The post-nuclear fraction is mixed with an equal volume of 85 % sucrose (w/v) in 10 mM Tris buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1 mM Na_3VO_4 (the presence of phosphatase and protease inhibitors in the buffers is critical, since the association of certain proteins to the DRM can be modulated by phosphorylation, and DRM represent a site for active and regulated proteolysis).
- The resulting diluent is placed at the bottom of a continuous sucrose concentration gradient (30–5 %) in the same buffer and centrifuged (17 h at $200,000 \times g$) at 4 °C.
- The entire procedure is performed at 0–4 °C.
- After ultracentrifugation, the gradient is fractionated, and the white light-scattering band in the low density region of the gradient is regarded as the sphingolipid-enriched fraction (DRM). Fractions can be collected manually or automatically from the top or from the bottom of the gradient without changing the significance of the results.

Two apparently trivial factors deeply influence the reproducibly of the results and the possibility to compare the patterns of DRM-associated molecules reported in different papers. One is represented by the different methods used to collect the fractions. The other is represented by the need to carefully homogenize the compact pellet recovered in the bottom fraction, which contains the majority of sample protein. Substantially overlapping results can be obtained using discontinuous gradients (in our lab, we usually use a two step 5–30 % discontinuous sucrose gradient) or density media other than sucrose (for example, Optiprep).

However, the method has proven to be very sensitive to the specific experimental conditions (where temperature, detergent concentration and detergent-to-cell ratio seem the most critical parameters). Standardization of the experimental procedures is difficult sometime, and the overall composition of DRM fractions or the association of specific molecules with it seem to be affected even by tiny modifications of several conditions, including agents used for membrane disruption (different detergents or different detergent concentration [49, 87–89]), mechanical procedures used to obtain or aid membrane solubilization (sonication, homogeneization) [90], temperature [22, 89, 91, 92], pH, and ratio between detergent and biological material [88].

3 Temperature

As mentioned above, all steps in the DRM preparation should be performed between 0 and +4 °C [22]. Indeed, in our experience incubation at room temperature or at +37 °C before gradient fractionation [92] is one of the best methods to ensure the full solubilization of DRM components. Applying the Triton X-100 extraction procedure to purified myelin at 20 °C led to the formation of two distinct low density fractions [93], both fractions characterized by higher cholesterol/phospholipid and GalCer/phospholipid ratio than the starting myelin preparation. However, the two fractions were characterized by a different content of GM1 ganglioside and by a different enrichment in specific protein markers. Similar discrepancies between the results obtained performing the separation at different temperatures, together with the fact that the low temperature, usually maintained during DRM preparation, can be hardly be extrapolated to those of living cells, has raised serious criticisms about the biological relevance of detergent-insoluble fractions prepared under these experimental conditions. Nevertheless, separation of a l*o* phase in model membranes occurs at 37 °C [94], and DRM in some cases can be prepared from cells and tissues at 20 or 37 °C [88, 89, 91, 93]. In the case of mouse cerebral cortex, the lipid membrane domain markers flotillin, F3, prion protein and alkaline phosphatase were detergent-insoluble at both 4 and 37 °C. Proper adjustment of the ionic composition of the solubilization buffer (e.g., Mg^+ and K^+ concentrations similar to those in the intracellular environment and addition of EGTA to chelate Ca^{2+}) allows the preparation at 37 °C of DRMs that have many of the properties of lipid rafts isolated from brain membranes or cultured cells using Triton X-100 or Brij 96 [95]. These "37 °C DRMs" were larger than lipid rafts prepared at low temperature, indicating that some aggregation may have occurred during the purification. This phenomenon can be

avoided by replacing the flotation method for DRM isolation with a magnetic immunopurification procedure, which minimizes the time required for DRM isolation [96].

4 Type of Detergent

As mentioned above, the original method for DRM preparation used Triton X-100 as the detergent, and several papers published afterwards described the composition and feature of Triton X-100 DRM. However, early experiments indicated that similar results can be obtained with other non-ionic or zwitterionic detergents, and in the literature there are several comparative studies performed using a wide range of different detergents [83, 87, 89, 97], aimed at understanding a possible artifactual nature of cellular fractions prepared thanks to their resistance to detergent solubilization. These studies showed that, using detergents with different stringency, it is possible to prepare a DRM fraction enriched in cholesterol and sphingolipids, as well as in certain proteins usually regarded as lipid membrane domain markers, in particular GPI-anchored proteins and acylated proteins. We compared the composition of DRM prepared from differentiated cerebellar neurons in the presence of Triton X-100 or Brij 96 and found a totally overlapping distribution of different lipid classes (DRM being highly enriched in sphingolipids and cholesterol, and depleted of GPL) and of several lipid rafts protein markers, including GPI-anchored proteins (PrP, Thy-1) and Src family kinases [83]. However, the association of other proteins (especially integral membrane proteins) with the DRM fraction is strongly affected by the type of detergent used. We compared the features of DRM prepared using different detergents (Triton X-100 vs. Triton X-114, or Triton X-100 vs. Brij 98) (Fig. 2). All of the different detergent used allowed us to separate a DRM enriched in sphingolipids and cholesterol, and heavily depleted of transferrin receptor (usually regarded as a non-raft marker). However, the association of some proteins, usually regarded as resident in lipid rafts (e.g., uPAR, caveolin-1, integrin receptor subunits), with DRM was deeply affected by the type of detergent used for the separation. For example, uPAR was largely soluble in Triton X-100, but insoluble in Triton X-114, whereas integrin receptor subunits were largely soluble in Triton X-100, and insoluble in Brij 98 (Fig. 2). In general, comparing the behavior of different proteins respect to solubilization with different detergents allows to draw the conclusion that the detergent insolubility of a protein is determined mainly by the intrinsic structural features of the protein, in particular, by the mode of association with the plasma membrane [97], and, thus, detergent-insolubility per se is not a sufficient criterion to establish the association of a protein with a lipid raft. In our

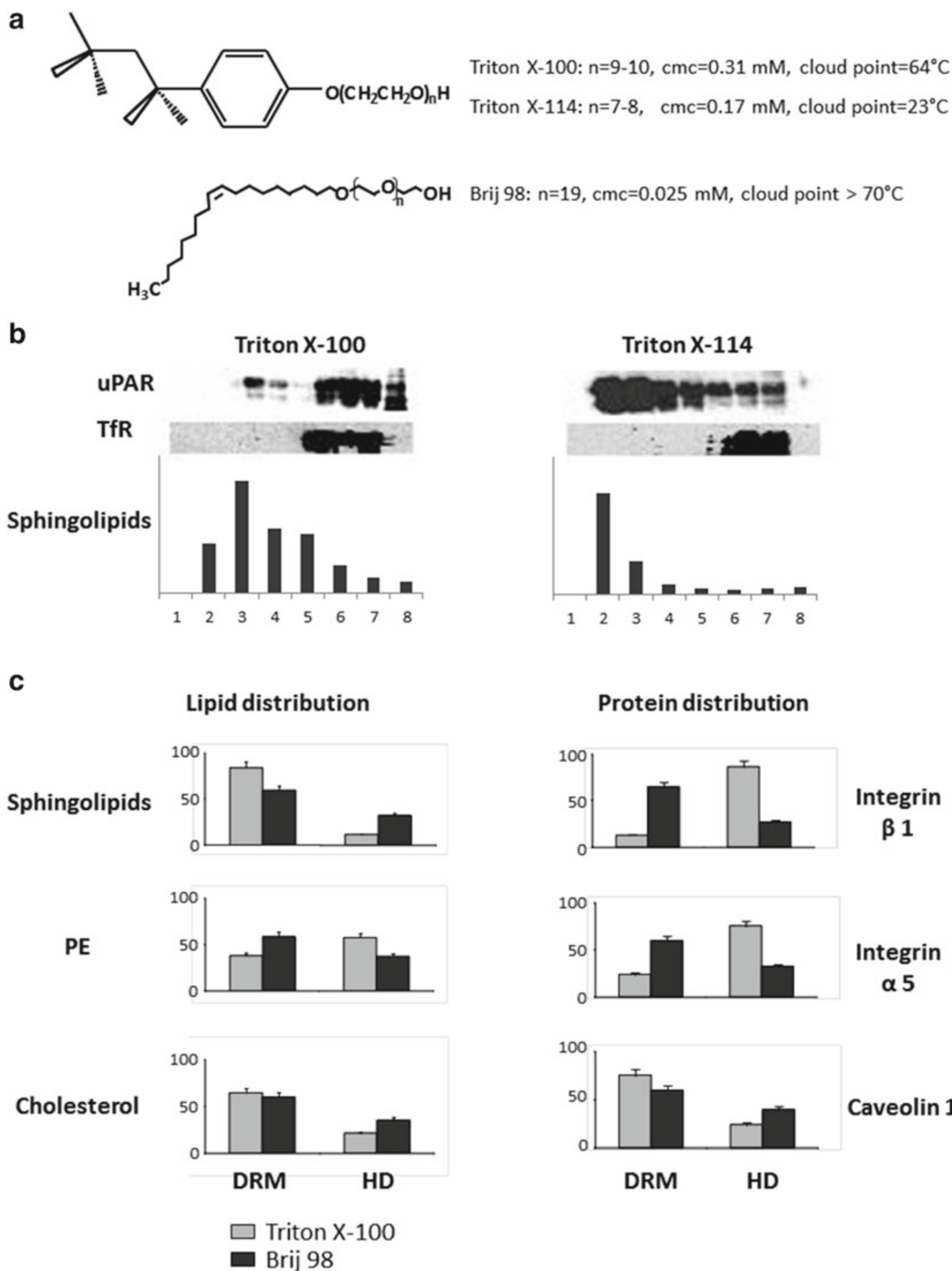

Fig. 2 Lipid and protein composition of DRM prepared using different detergents (Triton X-100 vs. Triton X-114, Triton X-100 vs. Brij 98). Panel (**a**): molecular structures and main physicochemical properties of Triton X-100, Triton X-114 and Brij 98. Panel (**b**): upper panels depict western blot analysis of uPAR (usually regarded as a raft marker), and TfR (usually regarded as a non-raft marker), in fractions prepared by lysis with Triton X-100 or Triton X-114 from HT1080 cells. Lower panels report the sphingolipid distribution along the gradient fractions. Panel (**c**): upper panels depict western blot analysis of integrin receptor subunits β1 and α5 and of caveolin-1 (usually regarded as a raft marker) in fractions prepared by lysis with Triton X-100 or Brij 98 from A2780 ovarian cancer cells. HD, High Density gradient fractions

opinion, the biochemical analysis of the complex environment of the protein, and especially of its lipid composition, remains essential to clarify the protein association with a lipid-rich, laterally organized membrane domain. On the other hand, some structural features that represent putative targeting signals to lipid rafts (in particular, the presence of a GPI anchor or a double fatty acylation) are usually associated with insolubility of the protein in nonionic detergents.

Many authors reported that the detergent-insoluble material obtained in the presence of different detergents floats at different densities, suggesting DRM prepared using different detergents might vary in their lipid composition or in their lipid-to-protein ratio. Again, this seems to point out the artifactual nature of these fractions; a concern further strengthened by the observation that DRM fractions may contain membrane fragments derived from the fusion of distinct lipid membrane domains [87], and that Triton X-100 treatment increased the average domain size by inducing the aggregation of preexisting domains [98] in a model membrane with a composition similar to that of the outer leaflet of plasma membranes. Remarkably, among different detergents Triton X-100 (the paradigmatic detergent used for DRM preparation) seems the one that more markedly alters the lateral organization of biological membranes [99]. Nevertheless, the separation of a l*o* phase in a membrane model was not affected by the treatment with Triton X-100 [98].

On the other hand, at least some studies seem to indicate that the different composition of DRM obtained by using different detergents might actually reflect the existence of biochemically distinct lipid membrane domains within the plasma membrane of the same cell, or the existence of different degrees of lateral order within the same lipid membrane domain [22, 89, 100–103], in agreement with an increasing number of studies indicating the presence of a high heterogeneity in membrane lateral organization in intact cells. In other words, the use of different detergents might represent an adequate tool to dissect the fine structure of membrane domains [89, 104]. For example, differential solubilization by Triton X-100 and Brij 96 has been used to show that two neuronal GPI-anchored proteins, Thy-1 and PrP, belong to structurally different lipid membrane domains characterized by a different degree of order [87]. The presence of two distinct domains characterized by a different detergent solubility has been related to differences in the lipid environment of these proteins [87]. In particular, mass spectrometry analysis of phosphatidylcholine, sphingomyelin and hexosylceramide [104], highlighted an enrichment in saturated fatty acids in the Thy-1 domain and in unsaturated fatty acids in the PrP domain. In addition, the use of different nonionic (Triton X-100, Brij 96, Triton X-102) or zwitterionic (CHAPS) detergents allowed to separate biochemically distinct detergent-specific domains from myelin membrane [89].

5 Protein/Detergent Ratio

Another experimental parameter that is critical in DRM preparation is the ratio between the amount of sample and the amount of detergent. DRM were originally prepared from MDCK cells using about 4 mg of cell protein/1 ml 1 % Triton X-100 [22]. We prepared DRM fractions starting from 0.5 to 6 mg neuronal cell proteins lysed in the same conditions, obtaining a constant composition of the DRM in terms of lipid content and patterns and of selection of proteins associated with this fraction [105]. The amount of lipid and protein material associated with the Triton X-100-insoluble fraction remains constant for a wide range of detergent-to-sample ratios, but it suddenly drops to barely detectable quantities above a certain value, suggesting that for a given type of biological material—cell or tissue—there is a certain threshold value for the detergent-to-sample ratio, above which it is impossible to prepare a low-density DRM fraction simply because the excess of detergent is able to solubilize even membrane domains with a high lateral order, that are normally resistant to detergent solubilization. These results are in agreement with those obtained by Parkin et al. [88, 106], who studied in detail the effect of various protein/Triton X-100 ratios on the isolation of a detergent-resistant fraction from mouse brain. Triton X-100 DRM could be prepared by sucrose gradient centrifugation after solubilization of mouse cerebral cortex with a fixed 1 % Triton X-100 concentration at different protein/detergent ratios, ranging from 15 to 2 mg of protein/ml. This fraction was enriched in two lipid membrane domain marker proteins (alkaline phosphatase and flotillin) regardless form the protein/detergent ratio. However, enrichment of some lipid raft markers (flotillin, prion protein and F3) in the DRM fraction increased when the protein/detergent ratio in the sample was decreased, while the association with this fraction of proteins, usually excluded from the lipid membrane domain, increased at high protein/detergent ratios.

6 Detergent-Free Methods

The fierce criticisms raised by the use of detergents in the isolation of laterally ordered (possibly l*o*) membrane domains stimulated the development of different "detergent-free" methods for the separation of low-density membrane fractions corresponding to lipid rafts. The rationale underlying these methods relies on the principle that resistance to solubilization of highly organized, "rigid," and thermodynamically favored lipid membrane domains should represent a particular aspect of a more general phenomenon, and thus ordered membrane domains should also be resistant to a variety of treatments able to disrupt the structure of less ordered membrane.

The disruption of cells in the presence of high pH or hypertonic sodium carbonate or by mechanical treatments (sonication under carefully controlled conditions, nitrogen cavitation) produces membrane fragments that can be separated by density gradient centrifugation [107–114]. The composition of the detergent-free low-density membrane fractions obtained as described above is very similar, but not identical [49, 59, 61, 65, 81, 90, 91, 115–122], to that of DRM obtained using Triton X-100 or other detergents, suggesting again that any experimental procedure used for membrane disruption alters at some extent the lateral organization of membrane components, while preserving the stable network of interactions underlying the formation of lipid rafts.

7 DRM from Tissues

DRM fractions have been isolated not only from cultured cells but also from various tissues, including chicken smooth muscle [67], mouse [97], rat and human [106, 123] cerebral cortex, mouse [87, 124–128], rat [104, 129–132] and human [133] brain, rat cerebellum [130], bovine and mouse brain myelin [89, 93, 126, 134], rat [135] and mouse [122] brain synaptosomes, rat [136], mouse [125] and rainbow trout [137] liver, rat [138], rabbit [139] and mouse [125] lung, rat lung endothelium [140], pig [88, 97] and mouse kidney [125]. However, as mentioned above, it should be kept in mind that detergent-insoluble material is represented not only by membrane components but also by some extracellular matrix components. Moreover, DRM fractions prepared from tissues originate from heterogeneous cell populations. Thus, the possibility that different lipid membrane domains could artefactually coalesce due to the presence of the detergent is particularly worrying in the case of preparations derived from tissues. Mixing together Triton X-100 DRM fractions obtained from rat and mouse brain resulted in a system where rat and mouse Thy-1 could be immunoprecipitated together, indicating that fusion of distinct lipid membrane domains did occur under these experimental conditions [87]. Nevertheless, we have not observed any fusion of PrP-rich and Thy-1-rich DRM prepared from cultured rat neurons [83]. Treatment of brain tissue sections with low concentrations of Triton X-100 at 4 °C resulted in the extensive redistribution of gangliosides and GPI-anchored proteins [141, 142] Moreover, addition of exogenous gangliosides to mouse brain sections in the presence of Triton X-100 at 4 °C, resulted in the incorporation of ganglioside molecules in white matter areas. Thus, the application of detergent-based methods for the preparation of lipid membrane domains from tissues still requires careful evaluation. In particular, only in a few cases, a partial characterization of the lipid composition of DRM obtained from tissues has been carried out [104, 106, 123].

We analyzed in detail the DRM prepared from mice brain [73]. Using 3–6 mg of proteins in 1 ml 1 % Triton X-100 (thus a protein-detergent ratio in the range usually applied for the preparation of DRM from cultured cells) we obtained a fraction that contained high amounts of Akt protein, that is usually regarded as a non-lipid raft protein marker [82, 143]. In addition, this fraction was highly enriched in lipids with respect to proteins, but did not show any enrichment in sphingolipids and cholesterol with respect to GPL. When we reduced to 1 mg protein the amount of brain subjected to lysis with 1 ml 1 % Triton X-100, all of the membrane were solubilized and no light fraction containing DRM could be separated by sucrose gradient ultracentrifugation. Only using the ratio of 1.3 mg of brain protein/1 ml of 1 % Triton X-100 we could isolate a DRM fraction with lipid and protein enrichments similar to those observed in DRM from neuronal cells in culture. This result suggested that the preparation of DRM from tissues requires careful validation by complex analytical controls, and in particular indicates that the simple use of protein markers to define the quality of a DRM preparation can be misleading, and that it is critical to analyze the enrichment in lipids that (sphingolipids, cholesterol and GPL) must be assessed to confirm the separation of a fraction containing lipid rafts from the bulk membrane.

8 Analysis of DRM Fraction: Importance of Lipid Analysis

Lipid membrane domains are defined on the basis of their peculiar lipid enrichment with respect to the whole cell or cell membranes. Thus, to validate the use of a method for the preparation of a lipid raft-enriched fraction, it is essential to quantitatively analyze the complete cholesterol, glycerolipid and sphingolipid profile of the fraction. Nevertheless, this type of accurate analysis has been performed only in a few papers using detergent insolubility as a tool to isolate lipid rafts, mainly due to the technical difficulties that are faced in the analysis of subcellular fractions with high contents of detergents, sucrose or other density media and salts. Chemical analysis of DRM lipids however usually requires complex purification of the lipid fraction of interest from the total extract, separation by HPTLC followed by colorimetric detection or immunostaining (using anti-glycolipid antibodies or staining with cholera toxin after treatment with bacterial sialidase to identify ganglio-series structures), or mass spectrometry analysis. Most works on DRM simply rely on the use of cholera toxin B subunit, a component of a heat-labile enterotoxin produced by *Vibrio cholerae* to detect GM1 as a putative DRM marker. Nevertheless, it is necessary to recall that GM1 is a very minor component in several cell lines, and that cholera toxin shows similar [144, 145] or higher affinity toward other gangliosides such as Fuc-GM1 [146, 147].

In addition to this, glycoproteins can be also recognized [147]. Thus, the use of cholera toxin alone in a simple immune dot blot identification experiment performed on membrane fractions is inconclusive [147]. In our hands, the most effective way to measure the relative enrichments of different lipid classes in DRM fractions relies on the use of metabolic radiolabeling procedures. Sphingolipids can be metabolically labeled with radioactive serine, palmitate or sphingosine/sphinganine. We have extensively used [1-^{3}H]sphingosine for steady-state metabolic labeling of sphingolipids in a wide variety of cultured cells (including neural and extraneural, normal and transformed, primary cultures and cell lines) [59, 82–86, 92, 148–153]. Using [1-^{3}H]sphingosine allows the simultaneous radiolabelling of phosphatidylethanolamine (PE) (due to the recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine). Using this procedure, we observed that the DRM fraction contained 50–65 % of the radioactivity associated with sphingolipids in the cell homogenate, and that less than 10 % of radioactive complex sphingolipids was present in the heavy density fraction of the gradient, which contained about 60 % of cell proteins. Radioactive PE, on the other hand, was predominantly recovered in the heavy fractions of the gradient, and only a low amount was detectable in DRM [59]. Thus, metabolic labeling with [1-^{3}H]sphingosine permits the simultaneous analysis of lipid components that are differently enriched in the DRM and non-DRM gradient fractions, representing a valuable analytical tool to check the efficiency of DRM separation under specific experimental conditions. In some cases we have performed a more detailed analysis of the GPL distribution in the gradient by metabolic labeling with [^{32}P]orthophosphate. Using this method, we showed that the DRM fraction from rat cerebellar neurons contained less than 10 % of the total cell GPL. However, about 22 % of PC was present in the DRM, with an enrichment of 13.2, which makes PC the most abundant lipid component of the DRM fraction [59, 82]. Based on our results, all sphingolipids are highly enriched in the DRM fraction (with an enrichment ranging from 30- to 40-fold respect to the cell lysate, depending on the specific sphingolipid). A similar enrichment has been calculated for cholesterol (that can be easily detected by colorimetric procedures after thin layer chromatography separation). In rat cerebellar neurons, the molar ratio between glycerophospholipids, cholesterol, sphingomyelin, ceramide and gangliosides was 41.6:6.1:1.3:0.3:1 in the cell homogenate and 8.3:4.0:1.4:0.2:1 in the DRM [59].

Nevertheless, papers reporting on lipidomics analysis of lipid rafts have recently appeared (and likely their number will greatly increase due to the wide diffusion of high sensitivity lipidomics tools), providing useful comparative sets of data [154, 155]. The analysis of the lipid composition of DRM by mass spectrometry has added very important information on DRM lipids, revealing that

DRM lipids are selected also on the basis of their fatty acid composition, being highly enriched in palmitic acid [82] (confirming the theoretical predictions based on the hypothesis that lipid rafts represent l*o* phase-separated domains), and that different detergent-resistant microenvironments are characterized by a different fatty acid composition: the Thy-1-rich and the PrP-rich microenvironments, separated from rat brain plasma membranes on the basis of their differential detergent solubility, are respectively enriched in saturated and unsaturated fatty acids [104].

9 Immunoseparation of DRM Complexes

In our opinion, the pieces of information discussed so far indicate quite convincingly that detergent membrane fractions contain different subpopulations of particles and supermolecular aggregates, and that some of these aggregates do actually reflect, in their composition and architecture, membrane domains actually existing at the cell surface. However, it is clear that DRM fractions as a whole do not represent isolated lipid rafts. The availability of antibodies toward specific components of DRM (both anti-protein and anti-lipid antibodies) has been sometimes exploited to develop highly specific methods for the immunoisolation of detergent-resistant membrane complexes from a "crude" DRM fraction [61, 81, 116, 118, 156–159]. Caveolin-1, the main structural protein present in *caveolae* and an important molecular organizer for membrane-associated multiprotein complexes [6] is usually highly enriched in lipid rafts (where it closely interacts with sphingolipids). Anti-caveolin-1 antibodies were used to discriminate between caveolar membrane domains and immunoaffinity-purified non-caveolar membrane domains, which seem to represent to distinct lipid raft subpopulations [55, 90, 92, 121, 160–162]. We used anti-caveolin-1 antibody to immunoisolate a multimolecular complex from DRM obtained from ovarian carcinoma cells characterized by high levels of GM3 ganglioside. Caveolin-1 in these cells tightly interacts with gangliosides and integrin receptor subunits, forming a signaling complex able to inhibit cell motility by negatively controlling the activity of Src kinase [85, 86]. We used immunoseparation of a PrP-rich detergent insoluble domain to study the organization of PrP microenvironment and the effect of a modification in membrane lipid composition on the association of PrP to neuronal membranes during apoptosis [83, 84].

Since a high enrichment in glycosphingolipids is a general feature of lipid membrane domains, particularly interesting are the immunoaffinity isolation methods relying on the use of anti-glycolipid antibodies [163]. Anti-GM3 ganglioside monoclonal antibody DH2 was used to immunoisolate GM3-enriched DRM from melanoma [162] and neuroblastoma cells [65]. Anti-GD3

ganglioside monoclonal antibody R24 was used to isolate a DRM fraction from rat cerebellum [129] and from differentiated rat cerebellar neurons [92]. Anti-LacCer monoclonal antibody Huly-m13 was used to isolate LacCer-enriched domains from human neutrophils, demonstrating the functional coupling between LacCer and Lyn [164]. Anti-sulfatide monoclonal antibody O4 was used to isolate lipid rafts from cultured rat immature oligodendrocytes [165].

Immunoisolation of detergent-insoluble complexes has the potential to discriminate between different subpopulations of lipid rafts. Using anti-GM3 ganglioside monoclonal antibody DH2 and anti-caveolin-1 antibody, it was possible to isolate two distinct Triton X-100-resistant membrane subpopulations from B16 melanoma cells by antibody, respectively [162]. Two distinct DRM subpopulations were immunoisolated from mouse brain using two different neuronal GPI-anchored proteins, Thy-1 and PrP, as the target [87]. Immunoprecipitation of two GPI-anchored proteins with different subcellular distribution in polarized epithelial cells allowed to conclude that the microenvironment of the two proteins is characterized by a different enrichment in lipids, and that there is no artificial lipid mixing or domain formation caused by Triton X-100 extraction (that has been shown in whole brain preparations, as discussed in the next paragraph), thus suggesting that the co-immunoprecipitated lipids represent the boundary lipids around each protein [166].

10 Conclusions

Several experimental techniques are currently available for the direct detection of lipid rafts or organized domains in intact cell membranes. These experimental tools were almost completely unavailable when the lipid raft hypothesis was formulated and when Brown and Rose developed the Triton X-100-based method for the preparation of DRM. Nevertheless, the highly diverse experimental methods used for the identification of lipid rafts on the cell surface are all based on the detection of a putative lipid raft marker (which is usually defined on the basis of the marker's enrichment in DRM fractions) and require the use of a physical, chemical, or biological probe whose nature depends on the experimental approach, making it difficult to compare results obtained with different techniques. When applied to the study of cell membrane heterogeneity, these techniques revealed a non-random distribution of cell surface molecules, leading to a highly hierarchical membrane organization that encompasses the existence of microdomains differing in their composition, size, and spatial and temporal dynamics (reviewed in ref. [13]). It is easy to predict that at some point Stimulated Emission Depletion (STED) microscopy shall become the golden standard in this sense. STED has the

potential to overcome the limit imposed by the diffraction barrier, thus scaling the resolution of fluorescence microscopy down to the nano level required for the study of the fine structure of cell membranes and of lipid rafts [167, 168]. STED microscopy demonstrated that putative lipid raft markers, including GPI-anchored proteins, SM, and GM1, were confined to molecular complexes that cover membrane areas with diameters <20 nm. These complexes were transient and had an average life span of 10–20 ms. The complexes appeared to be cholesterol-dependent, as the trapping was reduced upon cholesterol depletion [169, 170]. STED microscopy was also used to demonstrate that CD11b integrin and LacCer are associated with the same "nanodomain" in the membrane of living neutrophils and participate in LacCer-mediated phagocytosis of microorganisms [171].

The biochemical study of detergent-resistant membrane fractions has undoubtedly greatly contributed to our understanding of lateral organization of plasma membranes, and the core concepts elaborated on the basis of the data obtained using this approach have survived the test of modern technologies. It is clear that the association of a certain molecule with DRM does not automatically equate with its presence in lipid raft. DRM fractions represent an average preparation stabilized by the presence of the detergent, while lipid rafts are non-equilibrium entities, dynamic and heterogeneous in time and space. In our opinion, analysis of DRM can still provide useful information, but it is crucial to keep in mind that the methods for DRM preparation require a very tough standardization of the experimental procedures and careful analytical controls.

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