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Summary

A commonly-used method for analyzing raft membrane domains is based on their resistance to extraction by non-ionic detergents at 4°C. However, the selectivity of different detergents in defining raft membrane domains has been questioned. We have compared the lipid composition of detergent-resistant membranes (DRMs) obtained after Triton X-100 or Lubrol WX extraction in MDCK cells in order to understand the differential effect of these detergents on membranes and their selectivity in solubilizing or not proteins. Both Lubrol and Triton DRMs were enriched with cholesterol over the lysate, thus exhibiting characteristics consistent with the properties of membrane rafts. However, the two DRM fractions differed considerably in the ratio between lipids of the inner and outer membrane leaflets. Lubrol DRMs were especially enriched with phosphatidylethanolamine, including polyunsaturated species with long fatty acyl chains. Lubrol and Triton DRMs also differed in the amount of raft transmembrane proteins and raft proteins anchored to the cytoplasmic leaflet. Our results suggest that the inner side of rafts is enriched with phosphatidylethanolamine and cholesterol, and is more solubilized by Triton X-100 than by Lubrol WX.

1. Introduction

Evidence has now accumulated that the plasma membrane of mammalian cells is organized into microdomains [1-4]. The best characterized microdomains are membrane rafts which have been defined as “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” [5]. Rafts are thought to correspond to liquid-ordered (l_o) phases that are generated in model membranes composed of ternary mixtures of glycerophospholipids, sphingolipids and cholesterol [6]. However, their existence in biological membranes has remained controversial, especially because rafts cannot be viewed in resting cells by current fluorescent or electron microscopy techniques, except caveolae, which are considered as a special type of stabilized rafts [7]. Although it is now well recognized that membranes are not homogeneous, there is still controversy on the ability of lipids to form domains in biological membranes [3, 8, 9]. Based on techniques that measure lateral mobility of lipids and proteins in living cells, alternative models have been proposed. In the lipid shell hypothesis [10], the organization of rafts is driven by proteins that have affinity for sphingolipids and cholesterol. On the other hand, using high-speed single-molecule tracking, Kusumi and collegues have suggested that the membrane of living cells is predominantly compartimentalized by cytoskeleton fences that restrict diffusion of proteins and lipids [11]. Recent data obtained from fluorescence correlation spectroscopy have suggested that diffusion of proteins is both lipid and cytoskeleton dependant [12].

The commonly-used method for analyzing the domain organization of membranes biochemically is extraction by non-ionic detergents such as Triton X-100 at 4°C [13]. Most lipid–lipid and lipid–protein interactions are disrupted, but a fraction of cell membranes is preserved and can be isolated as detergent-resistant membranes (DRMs) due to their

buoyancy on sucrose density gradient. DRMs are thought to originate from rafts, which resist extraction due to the tight packing of raft lipids [14, 15]. DRMs also contain proteins that retain their association with membrane lipids. These proteins are mostly GPI-anchored proteins [13, 16], signaling proteins attached to the inner face of the plasma membrane such as the dually acylated Src family kinases [17-19], but only few transmembrane proteins, which are targeted to rafts through their transmembrane domain [20-22], or through membrane proximal determinants [22-26].

It is unclear how DRMs reflect the distribution of membrane components in raft domains. A failure to recover a protein in DRMs does not necessarily mean that it is not in rafts in living cells. For instance, the T-cell receptor has been shown to be in rafts by means of fluorescence microscopy patching experiments [27] although it is not usually present in DRMs after Triton X-100 extraction; the epidermal growth factor is found in a subset of raft domains by subcellular fractionation [28], although it is fully soluble in Triton X-100 [29]. Furthermore, in polarized cells, the apical plasma membrane is likely to contain a large fraction of raft domains because it is very rich in sphingolipids and cholesterol. Indeed, experiments performed in living cells have suggested that, in apical MDCK membranes, the raft phase is a continuous percolating phase with isolated domains of a non raft phase [30]. Yet, except GPI-anchored proteins, most apical transmembrane proteins are largely solubilized by Triton X-100.

A further development in the analysis of membrane domains has been brought by the use of different detergents. There are several examples of apical transmembrane proteins that are recovered in DRM fractions prepared with detergents other than Triton X-100 such as Lubrol WX [31-34]. Recently we have reported that the apical nucleotide pyrophosphatase/phosphodiesterase NPP3 was insoluble in Lubrol WX, in contrast to the closely related basolateral NPP1 [33]. Whether Lubrol DRMs reflect the existence of

membrane domains distinct from rafts or a different mode of protein-lipid interaction is not clear. The lipid composition of Lubrol DRMs has not been studied in details in polarized cells, and conflicting interpretations have been made [31, 35]. In order to get insight into the significance of the results obtained with different detergents, we have compared the lipid composition of DRMs obtained after Triton X-100 or Lubrol WX extraction in MDCK cells. Our results suggest that Lubrol WX preserves the membrane inner leaflet better than does Triton X-100, a property which may explain the high recovery of transmembrane proteins and proteins of the cytoplasmic leaflet, in Lubrol DRMs.

2. Materials and methods

2. 1. *Materials*

Triton X-100 and phospholipase C (from *Bacillus Cereus*) were from Sigma-Aldrich (St-Quentin-Fallavier, France). Lubrol WX, cell culture reagents, and PBS were from Invitrogen SARL (Cergy-Pontoise, France). Ganglioside standards were from Matreya LLC (Pleasant Gap, USA). Phospholipid standards were from Sigma-Aldrich. HPTLC silica gel 60 plates were from Merck (Whitehouse Station, USA). Monoclonal antibody anti-yes was from Transduction Laboratories (Lexington, USA). Anti-Lck monoclonal antibody clone 3A5 was from Tebu Bio (Le Perray-en-Yvelines, France). Anti-GFP monoclonal antibody was from Boehringer Roche Diagnostics France S.A (Meylan, France). Mouse monoclonal anti-Cav-1 (2234) and anti-flotillin-2/ESA (Reggie-1) antibodies were purchased from BD Transduction Laboratories (Lexington, USA). HRP-conjugated species-specific secondary antibodies and ECL reagent were from GE Healthcare Europe GmbH (Saclay, France).

2. 2. *Cell culture, detergent extraction and flotation gradients*

MDCK II cells were grown in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a 5% CO_2/air atmosphere. MDCK cells stably expressing NPP1 or NPP3 have been described [36]. The MDCK-GFP-GPI cells were obtained after transfection of a cDNA construct encoding EGFP fused to the 5'end of the mouse Thy-1 GPI-anchoring sequence [37], and selection of stable transfectants as described [36]. For the experiments, cells were seeded at $2x10^3$ cells/cm^2 in 100-mm dishes and grown for 8 days. The detergent solubility assay was performed as described by Brown and Rose [13]. Cells were washed with PBS, then lysed on ice for 30 min in 2 ml TNE buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.5 % (w/v) of either Triton X-100 or Lubrol WX. Lysates were passed through a 23G gauge needle, mixed with 80% sucrose to a final concentration of 40% sucrose, transferred into Ultraclear tubes (Beckman Coulter France S.A, Villepinte, France) and overlaid with 4 ml of 35% sucrose and 3.5 ml of 5% sucrose prepared in TNE buffer. Gradients were spun at 38,000 rpm for 18 h at 4°C without brake in a Beckman SW 41 rotor. Fractions were collected and processed for lipid or protein analyses. Material from six gradients, each from one 100 mm-plate of confluent MDCK cells were pooled for each point determination.

2. 3. HPLC analysis of phospholipid molecular species and fatty acids.

DRM fractions were collected, washed with TNE and pelleted at 49,000 rpm for 1 h at 4°C in a Beckman 50Ti rotor. Pellets were sonicated and lipids were extracted by the method of Bligh and Dyer [38]. The lower chloroform phase was evaporated under a stream of nitrogen and lipids were resuspended in 1:1 chloroform:methanol (v/v) and separated on HPTLC plates by migration in 65:25:4 chloroform:methanol:water (v/v/v). Glycerophospholipids and sphingomyelin (SM) were revealed on plates by iodine vapours and identified by comigration

with phospholipids standards. Silica powder corresponding to each lipid was scraped and lipids were extracted by the method of Bligh and Dyer [38]. An aliquot of lipid extract was evaporated, and the phosphorus content was determined by colorimetric assays according to the method of Rouser et al. [39]. Glycerophospholipids spots were scraped and extracted with chloroform/methanol (1:2), and saponified with potassium hydroxide in methanol at 56°C for 1 h. After neutralisation with HCl, the reaction mixture was extracted with hexane. Extracted free fatty acids were used to prepare phenacyl esters (FAPEs) according to the method of Chen and Anderson [40]. FAPEs were quantified by HPLC.

The different subclasses of glycerophospholipids were separated after the conversion to glycerobenzoate derivatives. The choline, ethanolamine and serine containing phospholipids were separated by HPTLC, extracted from silica gel, and then hydrolysed by phospholipase C from *Bacillus Cereus.* The diglycerides obtained were converted to benzoates derivatives, which were separated by HPTLC in the solvent mixture 50:45:4 benzene:hexane:diethyl ether (v/v/v) and visualized after spraying with dichlorofluorescein. Once extracted from silica gel, molecular species were analysed by reverse phase HPLC using acetonitrile/propanol-2 at a flow rate of 1 ml/min as already described [41].

2. 4. Cholesterol

The amount of cholesterol was quantified by the method of Gamble et al. [42] except that the assay did not include the cholesterol ester hydrolase reaction, thus measuring non-esterified cholesterol only. In order to calculate cholesterol enrichment in DRM fractions, cells were incubated with 1 µCi/ml [^{3}H]cholesterol for 20 h in complete culture medium. Cells were then processed for detergent extraction and analysis as described above. The amount of cholesterol in the gradient fractions was quantified by counting aliquots of the fractions by liquid scintillation in a LS 6000 SC (Beckman Coulter France S.A, Villepinte, France).

2. 5. Ganglioside analysis

Aqueous phases of DRM lipid extracts were evaporated under a stream of nitrogen. Lipids were resuspended in 3:7 methanol:water (v/v) and passed through a small reversed-phase C18 Sep-Pak cartridge (Waters, Milford, USA) pre-washed twice with 2:1chloroform:methanol (v/v) and equilibrated with 3:7methanol:water (v/v). Samples were passed 3 times through the C18 Sep-Pak cartridge. Then the column was washed 4 times with bidistillated water and the gangliosides were eluted in 2 ml 2:1 chloroform:methanol (v/v) and 1 ml chloroform. Once evaporated, samples were separated on HPTLC plates by migration in 5:4:1 chloroform:methanol:0.2M KCL (v/v/v). Plates were sprayed with resorcinol-hydrochloric acid followed by a 5-min incubation at 130°C to reveal sialic acid. Individual species of ganglioside were identified by comigration with ganglioside standards. Stained plates were scanned and bands were quantified using Image J software.

2. 6. Western Blot and protein assay

Fractions harvested from the gradients were brought to 1% Triton X-100 in the presence of protease inhibitors. All fractions were sonicated and warmed to 37°C for 30 min to solubilize detergent-resistant membranes. Aliquots were subjected to SDS-PAGE electrophoresis, transferred onto nitrocellulose membrane, and immunoblotting was performed as previously described [43] using the ECL Plus detection kit. Bands were scanned and quantified using Image J software.

Protein was quantified in homogenates and DRM fractions using the BCA method (Pierce).

3. Results

3. 1. Triton and Lubrol DRMs are both enriched with cholesterol but differ in their phosphatidylcholine/phosphatidylethanolamine ratio

In order to compare Triton and Lubrol DRMs, MDCK cells were lysed in Triton X-100 or Lubrol WX and lysates were resolved on sucrose gradients as described [33]. Fractions containing floating material (fractions 3-5 from the top of the gradient) were pooled to make the DRM fraction. Lipid and protein content was analysed in these pooled DRM fractions and in the lysates. Total lipid was calculated as the sum of the major membrane lipids (glycerophospholipids, sphingomyelin and cholesterol). The amount of floating detergent-resistant material was higher after Lubrol lysis than after Triton lysis. This was especially due to the presence of 3-fold more protein in Lubrol DRMs while there was 1.4-fold more lipid (Table 1). When the results were expressed as nmole lipid/mg DRM protein, as usually reported in the literature, Lubrol DRMs appeared to contain less lipid than Triton DRMs, especially less phosphatidylcholine (PC), sphingomyelin (SM) and cholesterol. However, the results are biased because of the higher amount of protein in Lubrol DRMs. When expressed as nmol/mg lysate protein, Lubrol and Triton DRMs contained equivalent amounts of PC and SM. Major differences concerned phosphatidylethanolamine (PE) which was recovered in higher amount in Lubrol DRMs (2-fold as compared to Triton). The amount of cholesterol was also 1.5-fold higher in the Lubrol DRM floating fraction. Phosphatidylserine (PS) was only slightly increased in Lubrol DRMs. When the relative molar composition (mol %) was compared, it was apparent that the major phospholipid in Triton DRMs was PC (~24%), while it was PE in Lubrol DRMs (~22%). Both Triton and Lubrol DRMs were much enriched with cholesterol, with relative mole content of 42% and 45% respectively (Table 1). Both had a higher mole percent of cholesterol than did the lysate. The ratio SM/GPL was higher in Triton

DRMs (0.21 and 0.16 for Triton and Lubrol DRMs respectively), but the ratio cholesterol/GPL was not significantly different (Triton: 0.87 and Lubrol: 0.96).

The fatty acid pattern of the main glycerophospholipids (PC and PE) was compared. Figure 1 shows typical HPLC elution profiles. The fatty acid composition of PC from Triton and Lubrol DRMs was very similar. PC mainly comprised saturated (16:0, 18:0) or mono-unsaturated (16:1, 18:1) fatty acids. By contrast, the fatty acid pattern of PE was different. PE contained more polyunsaturated fatty acids and these were increased in Lubrol DRMs as compared to Triton DRMs (Fig. 1). Analysis of the diacyl species (Table 2) showed minor differences in the PC diacyl species. By contrast, the diacyl species of PE differed, with a high enrichment of mono- and poly-unsaturated diacyl species in Lubrol DRMs. A remarkable difference was the high amount of 18:1/18:1 diacyl PE in Lubrol DRMs as well as the presence of diacyl species bearing long polyunsaturated fatty acyl chains (20:4, 22:6). Fewer differences were observed in PS although Lubrol DRMs also contained more 18:1/18:1 diacyl species than Triton DRMs.

The pattern of glycosphingolipids was not qualitatively different between Triton and Lubrol DRMs. In both types of DRMs, the ganglioside GM3 was the major glycosphingolipid species (Fig. 2).

Taken together, the lipid analysis showed both quantitative and qualitative differences between Triton and Lubrol DRMs, especially a high recovery of PE in Lubrol DRMs, a lipid which is generally considered to be localized preferentially if not exclusively in the inner plasma membrane leaflet.

3. 2. Lubrol DRMs are enriched with raft-associated transmembrane proteins and proteins associated with the inner leaflet of rafts

The lipid composition analysis suggested that Lubrol DRMs contained more lipids of the inner membrane leaflet than Triton X-100 DRMs. To investigate whether proteins anchored to the inner and outer leaflet would be also differentially solubilized by the two detergents, we analysed the partition of proteins with different membrane topologies, in Lubrol and Triton DRMs. GPI-anchored proteins are anchored to the external leaflet of the membrane and have affinity for rafts. MDCK cells stably transfected with the GPI-GFP cDNA expressed GPI-GFP exclusively at the plasma membrane (not shown). The protein partitioned essentially in DRMs both after Triton and Lubrol lysis (Fig. 3). On the other hand, doubly acylated kinases are anchored to the cytoplasmic leaflet only. MDCK cells endogenously express the kinases Lck and Yes which have been shown to localize to raft microdomains [19, 44]. Both Lck and Yes were relatively poorly detected in the Triton-DRM fractions while they were mainly recovered in the DRM fractions after Lubrol lysis (Fig. 3). Caveolin [45] and flotillin-2/reggie-1 [46] are also known as raft-associated proteins located at the cytosolic face of the membrane, but they are anchored both by acylation and membrane-spanning segments [47]. The bulk of these proteins was recovered in Triton as well as Lubrol DRMs. We also studied the partition of the transmembrane protein NPP3, which is a putative raft-associated protein [33]. NPP3 was ~10% insoluble in Triton X-100 but almost insoluble in Lubrol WX (Fig.3; [33]). As a control, the transmembrane protein NPP1, which is considered as non raft-associated was fully soluble in Triton X-100, as expected. However, ~10% of NPP1 was recovered in the Lubrol DRM fractions (Fig.3), indicating that Lubrol WX may not fully solubilize non raft-associated proteins. Quantification of the gels clearly shows that all proteins are recovered in higher amounts in Lubrol DRM fractions although to a variable extent (Fig. 3B). Major differences were observed for proteins of the inner membrane leaflet, and especially for the transmembrane protein NPP3.

4. Discussion

We have compared the composition of Lubrol and Triton DRMs in order to understand the differential effect of these detergents on membranes and their selectivity in solubilizing proteins. We observed that Lubrol DRMs yielded more insoluble floating material, and especially more protein than did Triton DRMs, a result that has already been reported in several cell types [35, 48]. The amount of protein in Lubrol DRMs was 3-fold that of Triton DRMs, which is far less from the 10-fold enrichment reported by Schuck et al [35] in MDCK cells, but close to the 2-fold difference reported by Hinrichs et al [48] in HT29 cells. Furthermore, compared to Triton, Lubrol DRMs contained more glycerophospholipids, especially unsaturated species, which are considered as non-raft lipids. These results suggest that Lubrol DRMs may be contaminated with non-raft membranes. Indeed, in a comparative study of the action of several detergents, it has been shown that calnexin and the transferrin receptor, which are not raft-associated, are partially recovered in Lubrol floating fraction [35]. Similarly, we show here that NPP1, a non raft-associated protein is partially recovered in Lubrol DRM fractions.

Despite the fact that Lubrol DRMs contain some non raft membranes, careful analysis of their lipid composition indicates that they are quite different from bulk membranes. Similar to Triton DRMs, Lubrol DRMs were highly enriched with cholesterol over the lysate, thus exhibiting characteristics consistent with the properties of membrane rafts. Both preparations were depleted with glycerophospholipids, but Triton DRMs contained especially less PE while Lubrol DRMs contained less PC. In fact, the two DRM fractions differed considerably in the ratio between lipids of the inner and outer membrane leaflets. In the plasma membrane of eukaryotic cells aminophospholipids are predominantly exposed on the cytosolic leaflet, whereas PC and sphingolipids are predominantly located in the outer leaflet [49-51]. The

absolute amount of the outer lipids, PC and sphingolipids, was slightly greater in Lubrol DRMs versus Triton DRMs, but there were only minor differences in the molecular species. By contrast, the amount of inner lipids, especially PE, and to a lesser extend PS, was very different. Lubrol DRMs contained more PE, and additional molecular species with long unsaturated fatty acid chains than were absent from Triton DRMs. The abundance of PE has not been noticed in previous analyses of Lubrol DRMs that focused on the relative composition of raft lipids [35, 48]. However, Pike et al [52] have already reported that detergent-free rafts or rafts obtained with the detergent Brij 98, are enriched with PE and contain a balance of inner and outer membrane lipids, whereas Triton-resistant rafts are relatively depleted of inner membrane lipids. Furthermore, lysing the cells with the detergent Brij58 yielded essentially similar amount of PE in DRMs as did Lubrol (our unpublished results).

Raft models predict that a liquid-ordered packing of lipids in the outer leaflet would be mirrored by an equivalent organisation in the inner leaflet of the membrane. This is indeed what is observed in artificial membranes in which the two leaflets are symmetrical. It is thought that coupling of the two raft leaflets occurs through overlapping of the long saturated fatty acid chains of sphingolipids. In biological membranes, the lipid composition of the inner side of rafts has not been adequately defined but has to be different because of the asymmetric distribution of lipids and the almost absence of sphingolipids in the cytoplasmic leaflet. The amount of PC that might be present in the inner side of rafts and the nature of the fatty acid chains are poorly known. The results obtained with non detergent methods suggest that the inner side of rafts might contain more PE than PC, with relatively more unsaturated chains [52]. While the ordering effect of cholesterol on PC molecules is well known, there are several data indicating that cholesterol also increases the order of the PE acyl chains very similarly to that observed with PC, especially at a high cholesterol concentration of 45 mol%

[53], which is the concentration we observed in Lubrol DRMs. Cholesterol distribution between the bilayers is not well known but is likely to be present in both leaflets and even in higher amount in the inner leaflet [54]. In a recent analysis of the formation of raft-like domains in asymmetric planar bilayers, Kiessling et al [55] observed that l_o phase domains in one asymmetric bilayer do not induce the formation of domains in the opposite leaflet when this leaflet is composed of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and cholesterol, but do induce domains when this leaflet is composed of a natural mixture of PC, PE, PS and cholesterol. These results make it plausible that the inner side of rafts would be enriched with PE. Coupling of the two leaflets could be realized by the high melting temperature of PE molecules which can hydrogen bound their headgroups and induce frustrated curvature strain into the lipid bilayer [55]. Furthermore, PE molecules have long fatty acyl chains at position sn-2 that could interdigitate with the long saturated fatty acyl chains of sphingolipids of the external leaflet.

L_o phases including PE are likely to differ in their detergent-resistance properties from l_o phases made of sphingolipids or PC. It has been shown that PE is less extracted by detergents over PC [56], and these properties may be amplified by the ordering of PE acyl chains in a cholesterol-rich environment. Strong detergents like Triton X-100 may be more effective at solubilizing raft PE than Lubrol WX and other weak detergents. These properties may have important consequences for the maintenance of bilayer integrity. They may explain why the major proteins recovered in Triton DRMs are raft proteins anchored to the external leaflet only, i.e. GPI-anchored proteins, while only selected proteins anchored to the internal leaflet are found in Triton DRMs. Among these are caveolins and flotillins, which are inserted by at least one membrane-integrated but not -spanning hairpin loop domain, and which have the particularity to form oligomers. Caveolins and flotillins associate with, and induce the formation of rather stable, morphologically identifiable specialized raft domains [57]. Doubly

acylated signalling molecules are also somewhat resistant to Triton X-100, but they are more resistant to Lubrol extraction, as we observed in the case of Lck and Yes. It is only after activation of cells that they become especially Triton-resistant [58]. Activation of cells might recruit and stabilize signalling molecules in rafts such that they form detergent resistant complexes. It is interesting to note that cross-linking of the immunoglobulin E receptor FcεRI in RBL-2H3 cells increased the proportion of polyunsaturated phospholipids recovered in the Triton-DRM fraction [59]. Transmembrane proteins are also likely to be destabilized if the inner leaflet is solubilized by Triton X-100. This may explain that raft-associated transmembrane proteins are solubilized by Triton X-100 but not by Lubrol WX ([31-33], this study). It must be noted that after cross-linking with antibodies, the MDCK endogenous protein gp114, an apical transmembrane protein, also becomes resistant to Triton extraction, while the basolateral LDL receptor remains soluble in similar conditions [60]. These observations suggest that Triton X-100 solubilizes a portion of proteins that have affinity for rafts, especially transmembrane proteins and proteins of the inner leaflet, unless they are oligomerized or engaged in molecular complexes.

Taken together, our results suggest that Triton X-100 would solubilize part of the inner leaflet lipids of rafts, especially unsaturated PE, and would partly solubilize certain raft-associated proteins. By contrast, Lubrol WX would preserve the internal leaflet of rafts, and maintain raft association of transmembrane proteins and proteins anchored to the cytoplasmic leaflet only. On the other hand, Lubrol WX would not fully solubilize non-raft associated proteins. Therefore, it may be interesting to make use of both detergents when studying the partitioning of proteins in membrane microdomains.

5. References

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Figure legends

Fig 1: Fatty acid composition of glycerophospholipids from Lubrol WX and Triton X-100 DRMs. Fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were extracted and separated by HPLC as indicated in *Materials and methods*. Typical chromatograms are shown. Fatty acids are indicated by numbers: 20:5 (1); 22:6 (2); 20:4 (3); 16:1 (4); 18:2 (5); 18:1+16:0 (6), 18:0 (7).

Fig 2 : Ganglioside distribution in Lubrol WX and Triton X-100 DRMs. Gangliosides of the aqueous phase of DRM lipid extracts were isolated and separated on HPTLC plates as indicated in *Materials and Methods.* The amount of each ganglioside species is expressed as percent of the sum of all identified gangliosides (GT1b, GD1b, GD3, GM1, GM2 and GM3). Results are the means of two determinations. Note that the total amount of gangliosides was 20% higher in Lubrol DRMs as compared to Triton.

Fig 3: DRM association of marker proteins from MDCK cells. (A) MDCK cell homogenates were extracted with 0.5% Triton X-100, or 0.5% Lubrol WX and separated on sucrose gradients. An equal volume of each gradient fraction was analyzed by SDS polyacrylamide gel electrophoresis followed by Western blotting with the indicated antibody. The light fractions from the top of the gradients are on the left, the heavy bottom fractions on the right. (B) The diagrams represent the percent of protein recovered in the DRM fractions (fractions 3-6). Results are means ± SD of three independent experiments. Cav-1, caveolin 1; Flot-2, flotillin 2; NPP1, nucleotide pyrophosphatase/phosphodiesterase 1; NPP3, nucleotide pyrophosphatase/phosphodiesterase 3.

Table 1. Protein and lipid content of lysates and DRMs

	Lysate		Triton X-100 DRM			Lubrol WX DRM		
Protein (mg/dish)	3.57 ± 0.29		0.046 ± 0.006			0.136 ± 0.028		
	nmol/mg lysate protein	mol%	nmol/mg lysate protein	nmol/mg DRM protein	mol%	nmol/mg lysate protein	nmol/mg DRM protein	mol%
PC	92.3 ± 5.1	36.0	11.6 ± 1.7	886.7 ± 56.7	24.2	12.1 ± 2.1	318.8 ± 29.9	17.2
PE	67.8 ± 2.2	26.4	7.2 ± 0.7	549.0 ± 19.1	15.0	15.4 ± 1.9	404.1 ± 17.2	21.8
PS	27.1 ± 3.4	10.6	4.2 ± 1.2	324.4 ± 66.5	8.7	5.7 ± 0.5	149.4 ± 18.0	8.1
SM	18.0 ± 0.4	7.0	4.9 ± 0.6	375.7 ± 16.1	10.2	5.5 ± 0.9	144.1 ± 15.0	7.8
Chol	51.5 ± 2.4	20.0	20.1 ± 2.8	1543.8 ± 89.5	41.9	31.8 ± 3.6	834.2 ± 28.3	45.0
Total lipid	256.7 ± 13.5	100	48.0 ± 7.0	3679.6 ± 237.9	100	70.5 ± 9.0	1850.1 ± 92.2	100

Quantitative lipid analysis was performed as described in *Materials and Methods.* Molar ratio of individual lipid classes to total lipids is indicated. Results are means ± SD of three independent experiments.

Table 2. Analysis of glycerophospholipid diacyl species in TX-100 and Lubrol DRMs

	Diacyl PC			Diacyl PE			Diacyl PS		
	Lysate	TX-100 DRM	Lubrol DRM	Lysate	TX-100 DRM	Lubrol DRM	Lysate	TX-100 DRM	Lubrol DRM
16:0/16:0	2.0 ± 0.3	2.2 ± 0.4	2.3 ± 0.4	4.9 ± 1.0	2.7 ± 0.3	2.3 ± 0.4	nd	nd	nd
18:0/18:0	nd	nd	nd	nd	nd	nd	nd	nd	nd
16:0/18:1	54.4 ± 5.2	59.6 ± 2.3	53.0 ± 3.7	24.6 ± 2.1	25.9 ± 5.0	23.2 ± 5.6	11.8 ± 1.5	12.9 ± 1.8	15.3 ± 0.5
18:0/18:1	21.5 ± 2.1	18.6 ± 2.6	26.1 ± 4.3	39.6 ± 3.2	57.6 ± 3.1	26.4 ± 0.8	79.6 ± 6.0	80.2 ± 1.2	73.1 ± 0.8
18:1/18:1	22.1 ± 2.2	19.7 ± 3.0	18.8 ± 4.9	23.5 ± 2.1	9.6 ± 0.2	26.0 ± 0.5	7.5 ± 0.5	4.0 ± 0.8	10.2 ± 0.7
16:0/18:2	nd	nd	nd	5.7 ± 1.5	2.5 ± 0.4	8.8 ± 0.3	nd	nd	nd
16:0/20:4	nd	nd	nd	nd	nd	5.6 ± 3.0	nd	nd	nd
18:0/20:4	nd	nd	nd	1.5 ± 0.5	1.6 ± 0.3	5.0 ± 1.4	1.1 ± 0.2	2.9 ± 0.2	1.4 ± 0.2
16:0/22:6	nd	nd	nd	nd	nd	2.6 ± 0.7	nd	nd	nd

Molecular species were separated by HPLC and quantified by absorbance as indicated in *Materials and Methods*. Values are expressed as percent of the major diacyl phospholipid species quantified in the considered fraction. Results are means ± SD of three separated experiments. nd: not determined

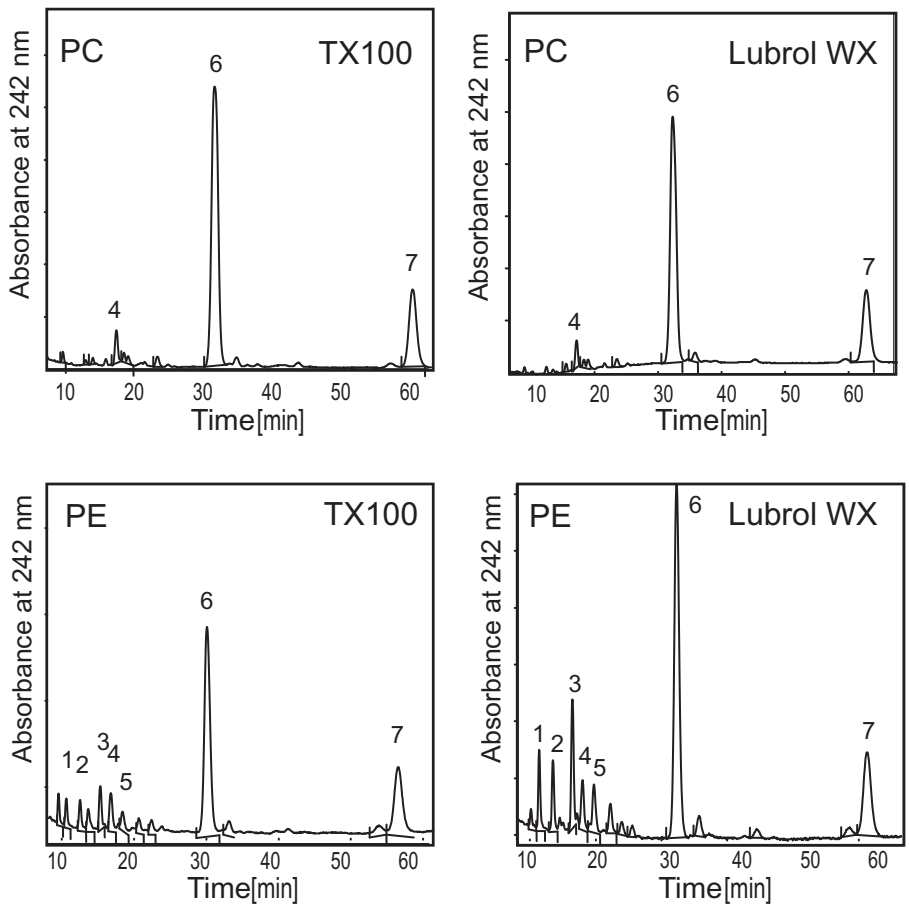
PC
TX100
Absorbance at 242 nm
Time[min]
PC
Lubrol WX
PE
TX100
PE
Lubrol WX

Figure 1

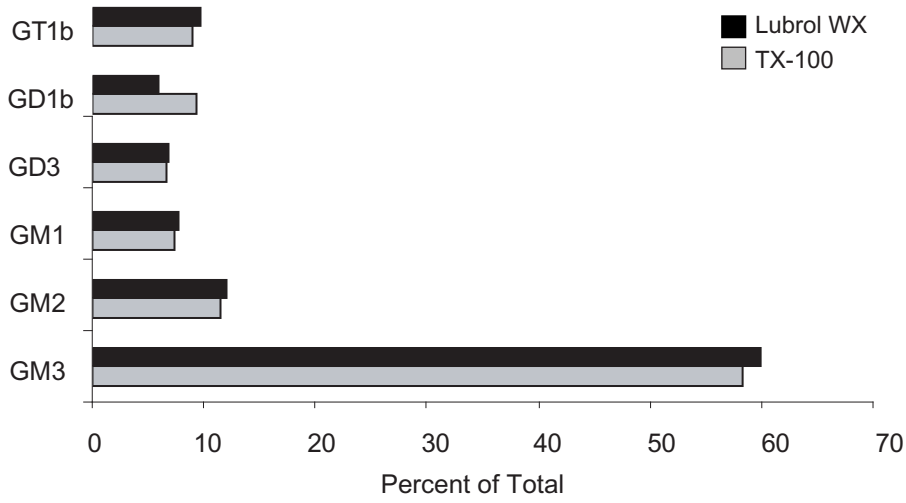
GT1b
GD1b
GD3
GM1
GM2
GM3
Lubrol WX
TX-100
0
10
20
30
40
50
60
70
Percent of Total

Figure 2

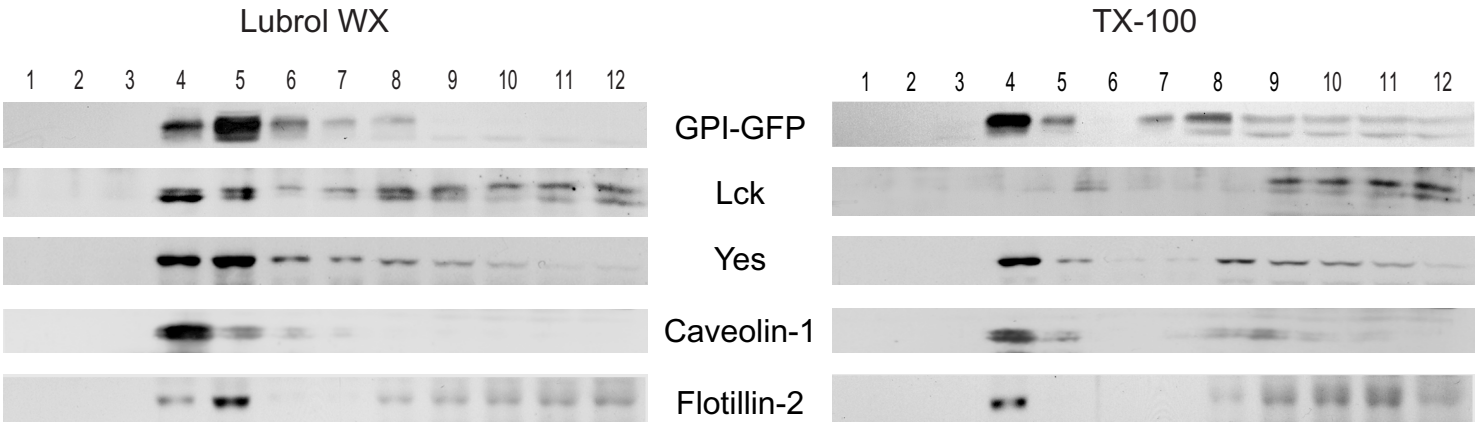
Lubrol WX
TX-100
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
GPI-GFP
Lck
Yes
Caveolin-1
Flotillin-2

Figure 3