Domain-specific lipid distribution in macrophage plasma membranes

Katharina Gaus,[1,*] Macarena Rodriguez,[*] Kalani R. Ruberu,[*] Ingrid Gelissen,[*] Timothy M. Sloane,[*] Leonard Kritharides,[*,†] and Wendy Jessup[*]

Centre for Vascular Research at the School of Medical Sciences,* University of New South Wales, and Department of Haematology, Prince of Wales Hospital, Sydney, NSW Australia; and Department of Cardiology,† Concord Hospital, Concord, NSW Australia

Abstract **Lipid rafts, defined as cholesterol- and sphingolipid-rich domains, provide specialized lipid environments understood to regulate the organization and function of many plasma membrane proteins. Growing evidence of their existence, protein cargo, and regulation is based largely on the study of isolated lipid rafts; however, the consistency and validity of common isolation methods is controversial. Here, we provide a detailed and direct comparison of the lipid and protein composition of plasma membrane "rafts" prepared from human macrophages by different methods, including several detergent-based isolations and a detergent-free method. We find that detergent-based and detergent-free methods can generate raft fractions with similar lipid contents and a biophysical structure close to that previously found on living cells, even in cells not expressing caveolin-1, such as primary human macrophages. However, important differences between isolation methods are demonstrated. Triton X-100-resistant rafts are less sensitive to cholesterol or sphingomyelin depletion than those prepared by detergent-free methods. Moreover, we show that detergent-based methods can scramble membrane lipids during the isolation process, reorganizing lipids previously in sonication-derived nonraft domains to generate new detergent-resistant rafts. The role of rafts in regulating the biological activities of macrophage plasma membrane proteins may require careful reevaluation using multiple isolation procedures, analyses of lipids, and microscopic techniques.**—Gaus, K., M. Rodriguez, K. R. Ruberu, I. Gelissen, T. M. Sloane, L. Kritharides, and W. Jessup. **Domain-specific lipid distribution in macrophage plasma membranes.** ***J. Lipid Res.*** **2005.** 46: **1526–1538.**

Supplementary key words lipid rafts • detergent-resistant membranes • lipid order

Membranes of eukaryotic cells comprise an immense diversity of lipid species whose purpose and function are poorly understood. The simple model of biological membranes as two-dimensional lipid bilayers has recently been modified to recognize that the self-organizing properties of some lipids drive the formation of specialized domains within cellular membranes (1). Although biological membranes are typically in a fluid (liquid-disordered) state at physiological temperatures, cholesterol and sphingolipids self-associate to form condensed, liquid-ordered domains, or "lipid rafts," within the more fluid "sea" of the rest of the membrane (2–4).

Recent interest in lipid rafts comes from the observation that some membrane proteins appear to preferentially partition into raft domains, whereas others are excluded from them. Hence, the structure of lipid rafts, their distribution, and their abundance could control key biological events dependent on the functional organization of the plasma membrane, such as signaling cascades (2), protein and lipid sorting and trafficking (5), cell adhesion and migration (6), entry of viruses (7), bacteria, and toxins (8), and immune responses (9). Lipid rafts have been implicated in a range of macrophage functions, including endotoxin-mediated activation and cytokine production, major histocompatibility complex (MHC) class II antigen presentation (10), phagocytosis (11), and cholesterol export (12). These cells are subject to large variations in cholesterol status during normal phagocytic clearance of damaged, apoptotic, and foreign cells as well as to pathological accumulation of lipoprotein-derived sterol in atherosclerotic lesions. Because raft structure and function depend on cholesterol, it is likely that changes in

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Abbreviations: CH/PL ratio, cholesterol-phospholipid ratio; DRM, detergent-resistant membranes; GP, generalized polarization; HMDM, human monocyte-derived macrophage; mβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; SM, sphingomyelin; SMase, sphingomyelinase; SR-BI, scavenger receptor class B type I; TfR, transferrin receptor.

[1] To whom correspondence should be addressed.
e-mail: k.gaus@unsw.edu.au

macrophage cholesterol status affect raft-associated functions. To date, surprisingly little is known about the lipid and protein composition of membrane domains in human macrophages and which factors govern the formation and maintenance of their lipid rafts.

Despite their importance, reliable tools to investigate lipid raft abundance, size, and composition are scarce and controversial (13). This is attributable partly to the small diameter and dynamic nature of some rafts, making direct visualization difficult. Indirect estimates of lipid raft diameter vary from <70 nm to 2 μm, and total surface coverage varies from 13% to 50% (3). However, it is becoming clear that in some cells, including macrophages, more stable concentrations of lipid rafts are associated with specific structures, such as microvilli (14), adhesion points, and filipodia (15).

Most biochemical analyses rely on an initial physical separation of membrane raft and nonraft domains, which also presents practical problems. The most widely used method depends on the preferential solubilization of nonraft domains by mild nonionic detergents, such as Triton X-100, at 4°C. The residual, insoluble, or detergent-resistant membrane (DRM) domains are more buoyant than the solubilized membrane components and so can be purified by density gradient centrifugation. Evidence that isolated DRMs represent lipid rafts arose from the initial observation that glycosylphosphatidylinositol-anchored proteins moving through the secretory pathway become increasingly Triton-insoluble as they acquire their lipid anchor, which targets them into a fraction enriched in cholesterol and sphingolipids (16). Importantly, DRMs can be isolated from liposomes containing liquid-ordered cholesterol and sphingolipid domains (17–21). It is assumed that proteins associated with DRMs are also present in lipid rafts in the intact cell. However, significant concerns about the relationship between true cell membrane lipid rafts and isolated DRMs have been raised (13, 22). Detergent solubilization requires low temperatures that are likely to induce alterations in membrane lipid phase organization. This is likely to increase the amount of lipid in ordered domains. In model membranes, Triton X-100 treatment can induce major changes in bilayer architecture, creating ordered, detergent-resistant domains in a previously homogeneous fluid membrane (23–25). Furthermore, individual detergent types and concentrations differ markedly in their ability to solubilize membrane domains and membrane proteins, generating DRMs with very different lipid and protein compositions (26).

An alternative, detergent-free isolation method fragments membranes by sonication into small fragments of buoyant "raft" membrane domains and heavier "nonraft" membranes that can be separated by centrifugation (27). This method avoids the problems of membrane mixing and selective lipid extraction associated with detergents. However, it is possible that physical disruption associated with the procedure could also lead to lipid randomization.

The aim of the present study was to comprehensively characterize domain-specific lipid distribution in macrophage plasma membranes, which requires physical separation of raft and nonraft domains. We directly compared several different detergent and nondetergent methods for raft isolation, making detailed analysis of the lipid and protein composition of rafts/DRMs produced by each method and comparing their lipid order with membrane domains on intact cells (15). As shown previously for other cell types, the types of rafts or DRMs isolated by different methods varied considerably in their lipid and protein content. Comparison between different isolation methods and cross-reference to data from whole cells allowed us to identify the raft isolation conditions that most closely resemble the situation in the intact cell.

MATERIALS AND METHODS

Antibodies

Rabbit anti-human caveolin-1, mouse anti-human flotillin-1, mouse anti-Rac1, mouse anti-ATPase, mouse anti-CD14, mouse anti-dynamin II, mouse anti-Munc18, mouse anti-Ras, and mouse anti-syntaxin 4 were all from Transduction Laboratories. Mouse anti-human transferrin receptor (TfR) was from Zymed Laboratories, mouse anti-CDC42 was from Pierce, mouse anti-SNP23 was from Abcam, and rabbit anti-SRB-1 was from Novus. Secondary antibodies were peroxidase-conjugated donkey anti-rabbit or peroxidase-conjugated donkey anti-mouse (both from Jackson ImmunoResearch).

Phospholipids

Synthetic, symmetric phospholipids purchased from Avanti Polar Lipids were used for HPLC standards: 18:1 phosphatidylcholine (PC; 1,2-diacyl-*sn*-glycero-3-phosphocholine 9-*cis*-octadeconoic acid), 18:1 phosphatitic acid (1,2-diacyl-*sn*-glycero-3-phosphate dioleoyl), 18:1 lysophosphatidylcholine (1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine oleoyl), 18:1 phosphatidylethanolamine (PE; 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine dioleoyl), 18:1 phosphatidylserine (PS; 1,2-diacyl-*sn*-glycero-3-[phospho-L-serine]dioleoyl), 18:1 phosphatidylglycerol (PG; 1,2-diacyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]dioleoyl), 18:1 1,1′2,2′-tetra-acyl-cardiolipin tetraoleoyl, and 18:0. In addition, egg sphingomyelin (SM), liver phosphatidylinositol (PI), and cholesterol (all from Avanti Polar Lipids) were used.

Reagents

All solvents were HPLC grade (Mallinckrodt). BSA (essentially fatty acid-free), Dulbecco's PBS, chloramphenicol, and cholesterol were purchased from Sigma. [^{3}H]cholesterol (48 Ci/mmol) was from Amersham. RPMI-1640 (Trace Biosciences) was supplemented with 2 mM L-glutamine (Trace Biosciences) and penicillin/streptomycin (Sigma). Heat-inactivated FBS was from Trace Biosciences. White cell concentrates and human serum were kindly supplied by the Red Cross Blood Bank (Sydney, NSW, Australia). Triton X-100, methyl-β-cyclodextrin (mβCD), and sphingomyelinase (SMase; sphingomyelin phosphodiesterase) from *Staphylococcus aureus* were from Sigma; Lubrol WX was from Serva.

Cell culture and [^{3}H]cholesterol labeling

THP-1 monocytes were cultured in RPMI containing 10% (v/v) FBS at 37°C in 5% CO_2. For experiments, THP-1 monocytes were labeled with 1.0 μCi/ml [^{3}H]cholesterol (from a stock solution of 1 mCi/ml in ethanol) in 1% (v/v) FBS in RPMI for 24 h. For macrophage experiments, THP-1 cells were differentiated into macrophages for 72 h at a density of 1.2×10^6 cells/ml (in 150 cm^2

flasks) in RPMI with 10% (v/v) FBS and phorbol 12-myristate 13-acetate [PMA; 50 ng/ml (Sigma)]. Differentiated THP-1 macrophages were incubated with RPMI containing PMA (50 ng/ml), 1% (v/v) FBS, and 1.0 μCi/ml [^{3}H]cholesterol overnight. RAW 264.7 cells were plated at a density of 1.0×10^6 cells/ml in RPMI with 1% (v/v) FBS and 1.0 μCi/ml [^{3}H]cholesterol for 24 h.

Cyclodextrin and SMase treatments

Cyclodextrin and SMase treatments were carried out as described previously (26). Briefly, whole cell homogenates were incubated with 10 mM mβCD or 0.5 U/ml SMase for 60 min at 37°C. After cooling the samples on ice, the plasma membrane was isolated, followed by lipid raft isolation as described below.

Cell homogenization and plasma membrane and lipid raft isolation

Cell homogenates were prepared by shear-lysis from 50×10^6 cells in 20 mM HEPES, pH 7.4, with 0.25 M sucrose and protease inhibitors (28). Samples were spun at 16,000 *g* to remove unbroken cells and nuclei, and the supernatant was taken as whole cell homogenate. Plasma membranes were isolated as described previously (28). Briefly, whole cell homogenates were loaded onto a 1–22% (w/v) Ficoll gradient with a 45% (w/v) Nycodenz cushion and centrifuged at 228,000 *g* (average) for 90 min, and 26 × 200 μl fractions were collected from the bottom. Fractions 15–20 were pooled as plasma membranes (29). Equal samples of plasma membranes were then either sonicated on ice four times for 30 s each with a 3 mm titanium probe (frequency, 23 kHz; amplitude, 26 μm) or alternatively treated with 0.2–1.0% (v/v) Triton X-100 or Lubrol WX for 30 min on ice. In all cases, the samples were then mixed with an equal volume of 90% (w/v) sucrose in MBS [25 mM MES (pH 6.5) and 150 mM NaCl]. Two milliliters of the mixture was overlaid with 2.0 ml each of 35, 30, 25, and 5% (w/v) sucrose (all in MBS) (30). The sucrose gradient was spun at 198,000 *g* (average) in a Beckman SW41 rotor for 16 h. Ten fractions of 1.0 ml were collected from the top and analyzed for protein, radioactivity, and lipid content. Throughout this article, we use the terms "lipid rafts" or DRMs as the membrane material that floats on a sucrose gradient in the density range 1.060–1.090 g/ml; nonraft material was collected in the density range 1.140–1.180 g/ml.

Protein assay

BCA Protein Assay and BCA Micro Assay (both Pierce) or Protein Reagent (Bio-Rad) were used for whole cell homogenates, sucrose, and plasma membrane gradient fractions, according to their manufacturers' instructions.

Immunoblotting

Equal volumes (200 μl) of each lipid raft fraction were delipidated by adding methanol (800 μl), chloroform (200 μl), and water (600 μl; vortexed after each addition) and spun for 5 min at 6,000 *g* (31). The bottom phase was precipitated with methanol (600 μl), vortexed, and spun for 5 min at 6,000 *g*. The pellet was resuspended in SDS sample buffer [2% (w/v) SDS, 100 mM DTT, 50 mM Tris-HCl, pH 6.8, 0.1% (w/v) bromophenol blue, and 10% glycerol], boiled for 5 min, resolved by SDS-PAGE, and transferred to nitrocellulose (Amersham). Membranes were blocked by incubation for 1 h in PBS containing 5% (w/v) nonfat milk powder and 0.1% (v/v) Tween 20, then incubated with primary antibodies in PBS, 1% (w/v) nonfat milk powder, and 0.1% (v/v) Tween 20 containing rabbit anti-caveolin-1 (1:1,000), mouse anti-flotillin-1 (1:1,000), or mouse anti-TfR (1:2,000) for 1 h at room temperature. After washing three times for 10 min each in PBS with 0.1% (v/v) Tween 20, the membranes were incubated with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature (1:5,000) followed by three more washes. Membranes were developed with the ECL-Plus Western blotting detection system (Amersham) and analyzed by densitometry (Gel Doc; Bio-Rad).

Lipid extraction

To determine specific activities of cholesterol, membrane samples (1 ml) were mixed with methanol (2.5 ml) and extracted into hexane (5.0 ml) (32). For phospholipid determination, samples were extracted by Folch extraction (1.5 ml of sample, 2.0 ml of methanol, and 4.0 ml of chloroform). The solvents were evaporated and resuspended in a small volume of mobile phase solvent mixture. The extraction yield was 89–95% as determined by extracting known quantities of lipids.

HPLC analysis

Cholesterol was analyzed by reverse-phase HPLC as described previously (33, 34). Phospholipid subclasses were separated using an Astec diol silica column (5 μm, 250 × 4.6 mm; Alltech) and a gradient solvent system of mobile phase A [80% chloroform, 19% methanol, and 1% ammonium hydroxide (NH_3 content, 30%; Aldrich)] and mobile phase B (60% chloroform, 39% methanol, and 1% ammonium hydroxide). With a constant flow rate of 1 ml/min, mobile phase A was run for 5 min; from 5 to 30 min, the mobile phases were linearly switched from A to B; 100% mobile phase B was run from 30 to 55 min before switching back to mobile phase A over the next 3 min (55–58 min) and reequilibration with mobile phase A to 65 min. Detection used an in-line evaporative light-scattering detector (ELSD 2000; Alltech) run with high-purity nitrogen (1.7 l/min; BOC) and an evaporation temperature of 53°C. Peak areas were compared with known quantities of standards. Phospholipid standards were made up to 10 mg/ml in chloroform and stored at −80°C. Detection limits were typically 40 ng of phospholipid.

Laurdan labeling of membranes

Isolated membranes obtained from sucrose gradients (200 μl) were incubated with 5 μM Laurdan (6-acyl-2-dimethylaminonepthalene; Molecular Probes) for 30 min at 37°C. Intensities were determined on a temperature-controlled fluorometer (Perkin-Elmer) at 22°C. Generalized polarization (GP) values were calculated as

$$GP = \frac{I_{(400-460)} - G \times I_{(470-530)}}{I_{(400-460)} + G \times I_{(470-530)}}$$

ranging from −1 (most fluid) to +1 (most condensed). G-factors to correct for the experimental setup were obtained from blank gradients. Laurdan fluorescence, G-factors, and GP distributions for intact, live THP-1 cells were obtained as described previously (15).

Statistics

Statistically significant differences were tested with unpaired, two-tailed *t*-tests assuming equal variances. Comparisons of more than two groups were performed using one-way ANOVA with Tukey's post hoc test for multiple comparisons between all groups.

RESULTS

Protein analysis of plasma membrane lipid rafts prepared by different methods

We first compared the ability of sonication and various detergents to separate protein markers of raft and nonraft

membrane domains from macrophage membranes. Plasma membranes were used rather than whole cells to avoid interference from lipid storage bodies (which are often present in macrophages and have a similar density to raft domains) and cytoskeletal components (also detergent-insoluble). Pooled plasma membranes were isolated from PMA-differentiated THP-1 macrophages, and identical samples (1 ml; 1.460 ± 0.11 mg protein/ml, 115.2 ± 9.6 nmol phospholipid/ml, 62.1 ± 4.8 nmol cholesterol/ml; n = 6) were sonicated (27) or treated with detergent on ice for 30 min (26) before separation by centrifugation on a discontinuous sucrose gradient. Lipid rafts (generated by detergent-free sonication) or DRMs were defined as the buoyant material present in fractions 2–4 (d = 1.06–1.09 g/ml), and nonraft membranes were defined as the more dense material in fractions 8–10 (d = 1.14–1.18 g/ml). The majority of plasma membrane protein and lipids was found within these regions of the density gradient (**Fig. 1**).

We compared membrane disruption by sonication with detergent extraction by 0.2% Triton X-100, 1.0% Triton X-100, or 1% Lubrol WX. Domain separation was determined by the distribution of the raft marker proteins caveolin-1 and flotillin-1 and the raft-excluded/detergent-soluble TfR (26) (Fig. 1). TfR has been established by detergent-independent methods to be excluded from raft domains. As such, it can be used as a control for complete nonraft membrane solubilization. As others have found (30), the relative distribution of caveolin-1 and flotillin-1 across the density range was not always identical. For example, sonication left some caveolin-1 in the heavy, nonraft membranes, although all flotillin-1 was recovered in raft fractions. In contrast, essentially all caveolin-1 and flotillin-1 remained in DRMs generated from membrane solubilized by 0.2% Triton or 1.0% Lubrol. However, increasing the concentration of Triton X-100 to 1.0% completely solubilized caveolin-1 and flotillin-1, redistributing them to the bottom of the gradient (Fig. 1C). This suggests that the detergent-membrane protein ratio is a crucial determi-

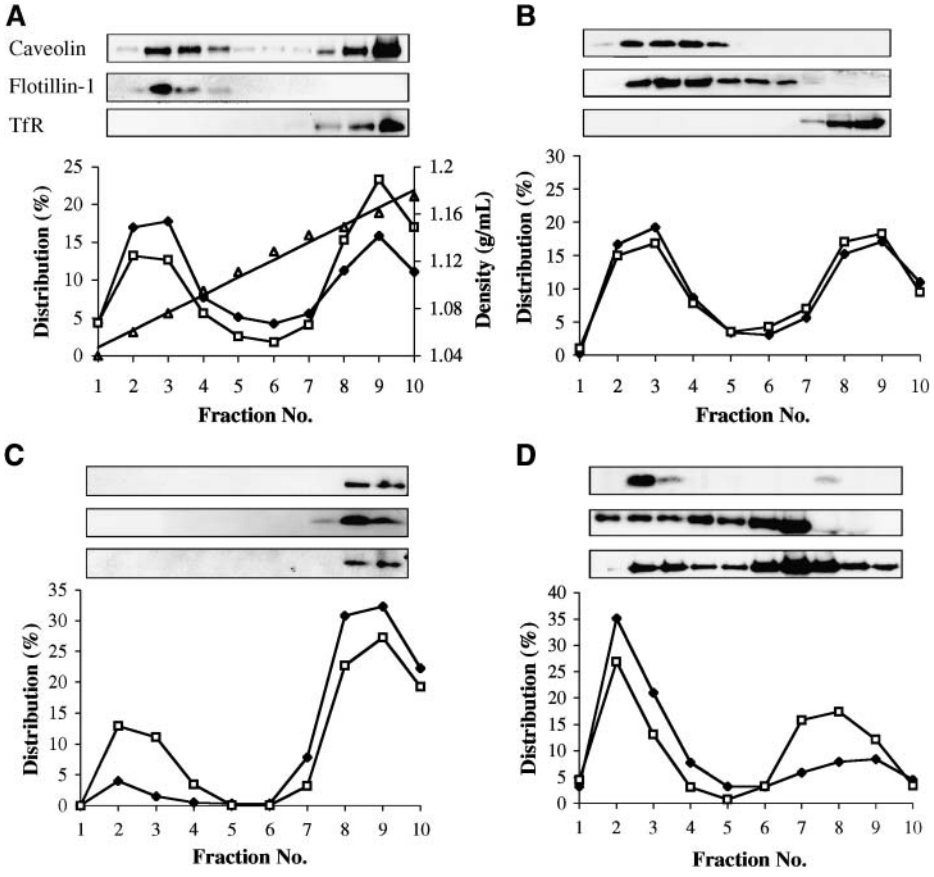

Fig. 1. Distribution of raft/detergent-resistant membrane (DRM) and nonraft marker proteins and lipids. Plasma membranes of differentiated THP-1 macrophages were either sonicated (A) or treated at 4°C with 0.2% Triton X-100 (B), 1% Triton X-100 (C), or 1% Lubrol WX (D) for 30 min before 5–45% sucrose density gradient centrifugation. Density (closed triangles) is shown in (A); fraction 1 is the lightest and fraction 10 is the heaviest. Equal volumes of each fraction were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed for caveolin-1, flotillin-1, or transferrin receptor (TfR). Typical protein concentrations (μg/ml) of fractions 3 and 9 were 4.4 ± 1.1 and 159.3 ± 12.5 (A), 6.7 ± 1.8 and 165.7 ± 27.6 (B), 2.4 ± 0.8 and 194.4 ± 25.8 (C), and 16.2 ± 5.0 and 130.6 ± 17.3 (D). The blots are representative of at least three independent experiments. Cholesterol (closed diamonds) and phospholipid (open squares) are means ± SD of three independent experiments.

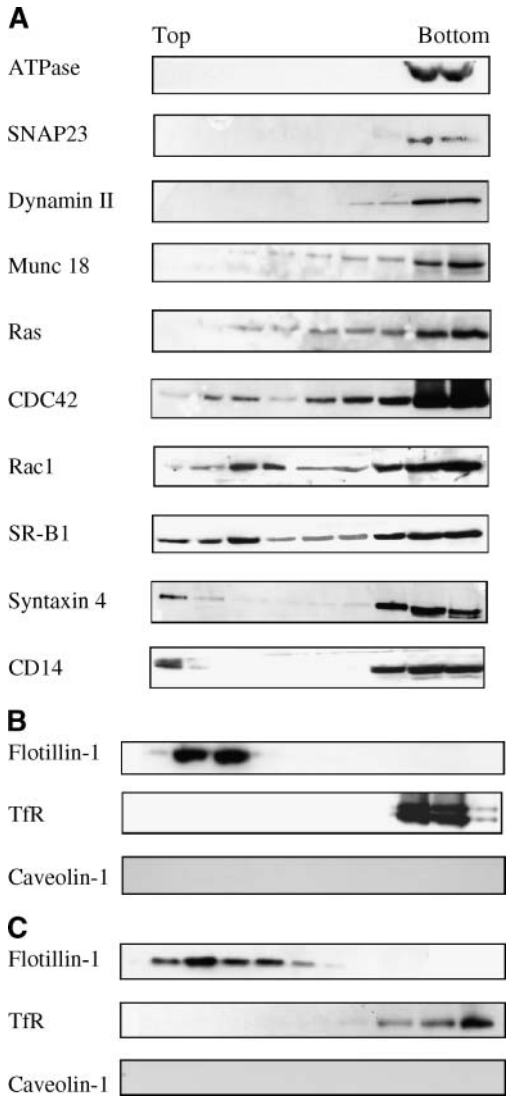

Fig. 2. Distribution of macrophage membrane proteins. Plasma membranes of THP-1 macrophages (A), primary human monocyte-derived macrophages (B), and THP-1 monocytes (C) were treated at 4°C with 0.2% Triton X-100 (A, C) or sonicated (B) and fractionated and immunoblotted as described for Fig. 1. Samples were separated and probed by Western blotting. Data shown are representative of at least three separate experiments. Similar distributions were obtained for all cell types for membranes fractionated by both sonication and 0.2% Triton X-100. SR-BI, scavenger receptor class B type I.

nant in the isolation of lipid rafts: whereas too little may incompletely solubilize nonraft membranes (22), an excess may also disrupt raft domains (26).

Previous studies have shown that Lubrol DRM contains a number of proteins that are Triton-soluble (35, 36). In agreement, we found that 1.0% Lubrol only partially solubilized TfR from macrophage membranes (Fig. 1D). However, all of the methods tested identified the majority of TfR as nonraft, as reported previously (17, 20, 26).

Figure 2A shows the protein profiles for a range of THP-1 macrophage proteins. CD14, syntaxin 4, scavenger receptor class B type I (SR-BI), and Rac1 were partially lipid raft-associated. CDC42 and Ras were predominantly found in nonraft fractions. Dynamin II, SNAP23, and ATPase were not found in raft fractions under nonstimulated conditions. In previous studies, an association of SR-BI with light, caveolin-1-containing membranes in differentiated THP-1 macrophages was shown (37), as was Rac-1 association in DRMs of monocytic THP-1 cells (38) and CDC42 association with Lubrol-DRMs but not Triton-DRMs in cholesterol-loaded human monocytes (36). In MonoMac-6 cells, complete sequestering of CD14 into DRMs was reported (39) but was not confirmed in human monocyte-derived macrophages (HMDMs) (40). Both syntaxin 4 and SNAP23 have been found in Triton-DRMs in adipocytes (41). Differences in raft association are attributed to different cell types, starting material (plasma membrane or whole cell homogenate), and, of course, raft isolation methods (26). Our data confirm the general trend of raft association of specific macrophage proteins.

Lipid composition of plasma membrane lipid rafts prepared by detergent extraction or sonication

Although the enrichment of lipid rafts relative to whole plasma membrane with cholesterol and sphingolipids is well established, relatively few studies have directly quantified the lipid composition of isolated lipid rafts (12, 42). We determined the cholesterol and phospholipid distribution in macrophage membrane fractions after disruption by sonication or detergent extraction (Fig. 1) and calculated the proportion of plasma membrane lipids present in rafts/DRMs prepared by the different methods (**Table 1**). In general, the majority of plasma membrane lipids colocalized with raft/DRM and nonraft marker proteins in fractions 2–4 (d = 1.06–1.09) and 8–10 (d = 1.14–1.18), respectively (Fig. 1). However there were some differences in the relative distribution of cholesterol and total phospholipids between raft/DRM and nonraft fractions between the various preparation methods (Fig. 1, Table 1). Extraction with 0.2% Triton generated DRMs with similar proportions of phospholipid (38.9 ± 6.2%) and cholesterol (44.0 ± 2.6%). Rafts prepared by sonication contained a similar proportion of cholesterol (42.6 ± 8.9%) but less phospholipid (33.0 ± 1.9%). More marked differences were found for 1.0% Triton DRM and 1.0% Lubrol DRM. Consistent with the profound effects of 1.0% Triton on membrane protein distribution, the majority of raft cholesterol was also solubilized, leaving only 5.8 ± 1.4% in the detergent-resistant raft fraction. Surprisingly, phospholipids were less affected, with 20.0 ± 6.7% remaining in the detergent-resistant fraction. In contrast, DRMs prepared with 1% Lubrol contained a greater proportion of plasma membrane cholesterol (63.8 ± 3.2%) than did those prepared by other methods. Lubrol nonraft membranes also floated at a lower density (fractions 7–10; Fig. 1D) compared with Triton-soluble material. Lipid rafts prepared by most of the isolation procedures (with the exception of 1.0% Triton) were enriched in cholesterol over phospholipid, relative to the whole plasma membrane.

TABLE 1. Lipid content of raft and nonraft domains prepared from macrophage plasma membranes

Lipid	Plasma Membrane	Sonication		0.2% Triton		1% Triton		1% Lubrol	
		LR	NR	LR	NR	LR	NR	LR	NR
					nmol/ml				
CH	62.1 ± 4.8	26.4 ± 5.5 (42.6%)	24.0 ± 4.2 (38.7%)	27.3 ± 1.6 (44.0%)	27.0 ± 5.3 (43.5%)	3.6 ± 0.9 (5.8%)[a]	52.8 ± 2.9 (85.0%)	39.6 ± 2.0 (63.8%)[b]	16.5 ± 0.2 (26.6%)
PL	115.2 ± 9.6	38.0 ± 2.2 (33.0%)	65.1 ± 10.5 (56.6%)	44.9 ± 7.2 (38.9%)	56.6 ± 7.1 (49.2%)	23.5 ± 7.7 (20.4%)[c]	86.5 ± 10.8 (75.1%)	43.6 ± 8.4 (37.8)	57.1 ± 6.8 (49.6)
CH/PL	35:65	41:59	27:73	38:62	32:68	13:86	38:62	48:82	22:78

Lipid rafts were isolated from plasma membranes of differentiated THP-1 macrophages (plasma membrane, 1.46 ± 0.11 mg protein/ml). Samples (1 ml) of the plasma membrane suspensions were either sonicated or treated with 0.2% Triton, 1.0% Triton, or 1% Lubrol before separation on a 5–45% sucrose gradient as described in Materials and Methods. Cholesterol (CH) and phospholipid (PL) content of the plasma membrane, lipid rafts (LR), and nonraft membrane fractions (NR) are given as mass (nmol). Lipid rafts are defined as the material in fractions 2–4, and nonraft membranes are defined as the material in fractions 8–10 (or 7–10 for 1% Lubrol; see Fig. 2). Values in parentheses are percentages of the total plasma membrane cholesterol and phospholipid recovered in raft and nonraft fractions, respectively. CH/PL is the molar ratio of cholesterol to phospholipid in the sample. The data are means ± SD of three independent experiments.

[a] Significantly lower than the corresponding data for sonication ($P < 0.001$).

[b] Significantly higher than the corresponding data for sonication ($P < 0.001$).

[c] Significantly lower than the corresponding data for sonication ($P < 0.001$).

Phospholipid subclasses of plasma membrane, raft, and nonraft membranes

The physical and functional properties of lipid rafts are likely to be differentially affected by individual phospholipid subclasses. For example, SM has important lipid-condensing properties that are modulated by its depletion with SMase (26). The predominantly unsaturated acyl chains of sphingolipids favor the denser packing of the raft environment, and cholesterol preferentially associates with some phospholipids with a specificity based on their head groups (SM > PE > PS). Other phospholipids, such as PS and PE, have potential roles in apoptosis or coagulation.

Individual phospholipid subclasses were separated and quantified using a normal-phase HPLC with a gradient solvent system and an evaporative light-scattering detector (**Fig. 3**). Note that SM elutes as a double peak, as reported previously (43). By comparing peak areas with known quantities of standards (see Materials and Methods), we determined the masses of PG, PE, PI, PS, PC, and SM in the plasma membrane and individual density gradient fractions for raft isolation (**Table 2**). Lysophosphatidylcholine and cardiolipin were not detectable in macrophage plasma membrane. Comparison between the raft/DRM domains prepared by the different protocols revealed both similarities and points of difference (Table 2). Consistent with previous reports, lipid rafts prepared by all methods were enriched in SM, relative to whole plasma membrane. Lipid rafts prepared using 0.2% Triton or sonication contained ~60% of plasma membrane SM, whereas those generated by 1.0% Triton or 1.0% Lubrol treatment contained even more (80–90% of total). Most rafts were relatively depleted of PC (16–20% total PC), except for those prepared with 0.2% Triton (~40% of plasma membrane PC); 0.2% Triton rafts also contained the highest proportion of plasma membrane PE (~56%). The proportion of anionic phospholipids (PS plus PI) in raft fractions was comparable for all four procedures, with 20–28% of plasma membrane anionic phospholipids in the raft fractions.

In summary, sonication and 0.2% Triton generated raft fractions with roughly similar protein and lipid contents. Increasing the Triton concentration to 1.0% caused extensive additional solubilization of the "detergent-resistant" raft domains, leading to loss of most cholesterol and proteins from this fraction. In contrast, 1.0% Lubrol DRMs covered a broader density range and contained a higher proportion of plasma membrane lipids and some proteins normally associated with nonraft domains. For subsequent studies, we limited comparisons to nondetergent rafts and 0.2% Triton DRMs.

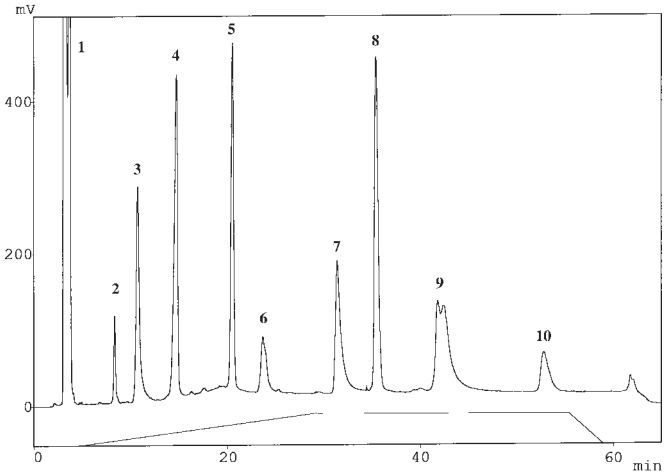

Fig. 3. HPLC of phospholipids. Separation of a standard mixture of cholesterol (peak 1), ceramide (peak 2), phosphatidylglycerol (peak 3), cardiolipin (peak 4), phosphatidylethanolamine (peak 5), phosphatidylinositol (peak 6), phosphatidylserine (peak 7), phosphatidylcholine (peak 8), sphingomyelin (SM; peak 9), and lysophosphatidylcholine (peak 10). The concentration of solvent B (see Materials and Methods) is shown schematically at the bottom of the chromatograph.

TABLE 2. Distribution of phospholipid subclasses in THP-1 macrophage membrane domains

Fraction	Plasma Membrane	Sonication		0.2% Triton		1% Triton		1% Lubrol	
		LR	NR	LR	NR	LR	NR	LR	NR
PG	12.3 ± 2.0	8.2 ± 1.4[a] (22.0%)	9.4 ± 3.4[a] (59.5%)	9.7 ± 5.8 (33.8%)	12.2 ± 3.8 (45.0%)	14.4 ± 7.9 (22.9%)	15.8 ± 9.5 (71.4%)	20.4 ± 3.8[a] (35.3%)	5.0 ± 2.3[a] (45.0%)
PE	15.0 ± 2.1	20.1 ± 1.3[a] (37.2%)	11.7 ± 1.7[a] (48.2%)	18.2 ± 0.8[a] (55.6%)	12.4 ± 4.6 (30.7%)	2.3 ± 2.9[a] (5.7%)	21.2 ± 2.8[a] (92.4%)	14.3 ± 6.1 (37.3%)	17.0 ± 2.9 (57.3%)
PI	8.5 ± 2.5	9.3 ± 1.1 (22.0%)	12.5 ± 3.3 (70.1%)	4.5 ± 1.9[a] (20.8%)	11.6 ± 2.6 (71.5%)	5.7 ± 1.1[a] (22.9%)	7.9 ± 5.8 (71.7%)	5.1 ± 2.4[a] (25.6)	10.8 ± 4.7 (69.0%)
PS	5.1 ± 2.5	3.1 ± 2.8 (24.5%)	3.3 ± 2.4 (63.0%)	3.1 ± 2.5 (14.3%)	12.2 ± 7.9 (59.4%)	1.5 ± 1.2[a] (29.0%)	2.2 ± 1.2 (66.0%)	2.3 ± 1.2[a] (14.6%)	9.5 ± 4.1 (77.1%)
PC	43.2 ± 6.4	24.5 ± 5.7[a] (15.8%)	53.8 ± 3.0[a] (79.0%)	37.5 ± 7.2 (40.6%)	44.5 ± 9.5 (46.7%)	30.6 ± 3.2[a] (16.2%)	48.6 ± 9.0 (70.0%)	21.4 ± 7.6[a] (20.6%)	57.7 ± 9.9[a] (73.0%)
SM	15.8 ± 2.5	34.9 ± 5.9[a] (54.6%)	9.3 ± 2.9[a] (29.4%)	26.2 ± 6.9[a] (63.3%)	7.1 ± 1.7[a] (23.9%)	44.6 ± 9.5[a] (80.7%)	4.4 ± 1.8[a] (15.4%)	36.6 ± 3.3[a] (90.0%)	<0.1[a] (<0.0%)

Plasma membrane was isolated from THP-1 macrophages as described in Materials and Methods, and lipid raft (LR) and nonraft (NR) membranes are defined as for Table 1. Data are expressed as molar ratios (mol%) and are means ± SD of three independent experiments; the lipid masses of plasma membrane, raft, and nonraft membranes are given in Table 1. Values in parentheses are percentages of plasma membrane lipid in that fraction. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

[a] Statistically significant difference ($P < 0.05$) compared with plasma membrane.

Lipid order of membranes in intact cells and isolated rafts/DRMs

To establish a link between isolated lipid rafts/DRMs and condensed membrane domains previously found on living, intact macrophages (15), we compared the GP (as an index of lipid order) of plasma membrane fractions separated on sucrose gradients (**Fig. 4A**) with the GP distribution of whole THP-1 cells (Fig. 4B). We used a fluorescent probe (Laurdan) that exhibits a 50 nm red shift as membranes undergo phase transition from gel and fluid and that we have used previously for direct visualization of the distribution of liquid-ordered domains on the plasma membranes of living cells (15). GP values of isolated lipid rafts (prepared by either 0.2% Triton or sonication; GP = 0.40–0.55; Fig. 4A) agreed well with the mean GP values of condensed membranes in intact cells (GP = 0.50–0.65; Fig. 4B). GP values of nonraft domains of isolated domains ranged from 0.08 to 0.22, and the mean values of the fluid center of intact cells varied from 0.01 to 0.17. We conclude that rafts/DRMs prepared by both 0.2% Triton and sonication yield a similar lipid structure to the liquid-ordered domains on whole live cells.

Effect of differentiation on THP-1 lipid raft composition

Different cell types can yield lipid rafts/DRMs with different protein and lipid compositions even when using identical isolation procedures (26), suggesting that raft composition is cell-specific. Stimulation of THP-1 monocytes to macrophage differentiation is associated with changes in the level of expression of many proteins related to lipid metabolism, including caveolin-1. THP-1 monocytes express very little caveolin-1, whereas PMA-induced differentiation stimulates caveolin-1 expression (37, 44). Therefore, we compared the composition of nondetergent lipid rafts and 0.2% Triton DRMs in THP-1 monocytes (**Table 3**) with those from differentiated macrophages (Tables 1, 2).

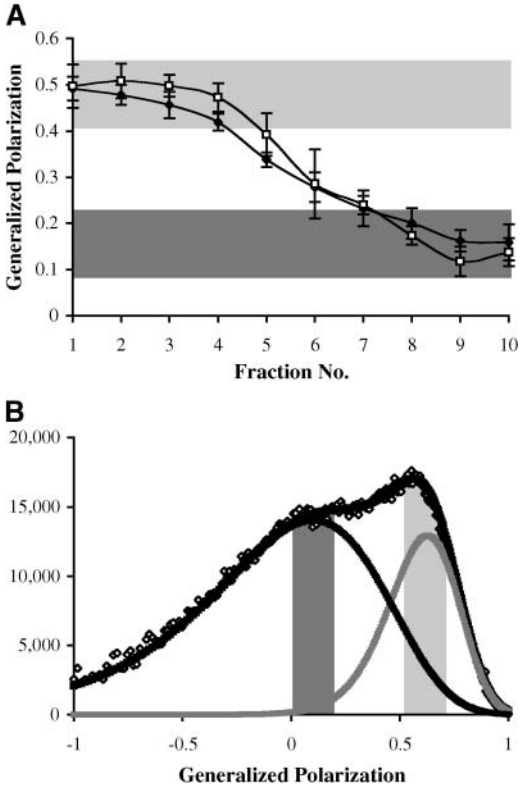

Fig. 4. Lipid order of isolated lipid rafts/DRMs and intact cells. Lipid order was determined as generalized polarization (GP) of Laurdan-labeled membranes as described in Materials and Methods. A: GP was calculated for density gradient fractions after fractionation of THP-1 macrophage plasma membranes treated with 0.2% Triton X-100 (open squares) or sonication (closed diamonds). Error bars represent standard deviatins derived from three independent experiments. B: GP distribution of live, intact THP-1 macrophages was calculated as two Gaussian populations with mean GPs of 0.103 (nonraft population; dark gray) and 0.628 (raft population; light gray). The area under each curve equates to coverage as a percentage of all Laurdan-labeled membranes; raft coverage was 38.4% and nonraft coverage was 61.6%. All Laurdan measurements were conducted at 22°C.

TABLE 3. Lipid composition of THP-1 monocyte membranes

Lipid	Plasma Membrane	Sonication		0.2% Triton	
		LR	NR	LR	NR
CH	55.5 ± 5.5	18.1 ± 1.0 (32.5%)	28.2 ± 3.3 (50.8%)	25.7 ± 4.3 (46.4%)	24.0 ± 4.0 (43.2%)
PL	95.8 ± 9.1	24.1 ± 4.6 (25.2%)	51.3 ± 6.1 (53.5%)	43.4 ± 2.3 (45.3%)	39.8 ± 8.1 (41.5%)
CH/PL	37:63	43:57	35:65	37:63	38:62
PG	1.7 ± 0.4	3.0 ± 1.4	4.1 ± 1.9	10.2 ± 9.8	5.8 ± 2.5
PE	33.4 ± 4.0	27.0 ± 2.9	34.6 ± 3.1	26.2 ± 3.2	36.8 ± 5.6
PI	6.0 ± 1.1	6.2 ± 5.4	7.3 ± 0.5	6.6 ± 0.5	10.2 ± 3.0
PS	1.5 ± 0.6	6.6 ± 5.8	1.2 ± 0.3	1.1 ± 0.4	1.5 ± 1.3
PC	51.9 ± 2.5	38.4 ± 1.5[a]	49.3 ± 3.9	44.6 ± 2.7[a]	42.5 ± 6.7
SM	5.4 ± 3.3	12.7 ± 2.9[a]	3.6 ± 3.2	17.6 ± 1.4[a]	3.2 ± 2.5

Plasma membrane (1.13 ± 0.14 mg protein/ml; n = 3) was isolated from THP-1 monocytes as described in Materials and Methods, and raft and nonraft membranes were isolated from 1 ml samples by sonication or using 0.2% Triton. Cholesterol (CH) and phospholipid (PL) are expressed as nmol in rafts and nonrafts. Values in parentheses are percentages of plasma membrane lipids in raft (LR; 2–4) and nonraft (NR; 8–10) fractions. CH/PL represents the molar ratio of cholesterol to total phospholipid. Phospholipid subclasses are given in molar ratios (mol%) as described for Table 2. Data are means ± SD of three independent experiments.

[a] Statistically significant difference ($P < 0.05$) compared with plasma membrane.

Overall, the lipid content and cholesterol-phospholipid ratio (CH/PL ratio) of THP-1 monocyte plasma membrane were similar to those of the differentiated macrophages. However, monocytes contained significantly less SM ($P < 0.05$) and more PE ($P < 0.05$) and PC than THP-1 macrophages (Tables 2, 3). Like the mature cells, rafts/DRMs prepared by sonication or 0.2% Triton were enriched in SM and depleted of PC relative to whole plasma membrane, consistent with previous studies (37). Rafts isolated from monocytes by sonication contained a lesser proportion of plasma membrane cholesterol than rafts from THP-1 macrophages (~33% and ~43%, respectively) and less plasma membrane phospholipid (~25% and ~33%, respectively) but maintained a similar CH/PL ratio. This is consistent with the monocyte plasma membrane containing fewer rafts than macrophages. In contrast, 0.2% Triton DRMs from plasma membranes of THP-1 monocytes and macrophages were similar. This indicates that monocyte plasma membranes have a subtle but significant differential sensitivity to disruption by sonication versus detergent, unlike THP-1 macrophages, in which a striking similarity between rafts generated by these preparation methods was observed (Tables 1, 2). Despite the difference in lipid composition between light membranes and DRMs in monocytes, protein distribution was similar (Fig. 4C for 0.2% Triton; sonication data not shown) and no difference in flotillin-1 and TfR distribution was found between monocytes and mature macrophages (Fig. 1 vs. Fig. 2B). These data indicate that similarities in protein distribution (between cell types or isolation methods) do not necessarily lead to similarities in lipid composition.

Manipulation of lipid rafts with mβCD or SMase

As lipid rafts/DRMs are relatively enriched in cholesterol and SM, a common tool for probing the association of proteins with lipid rafts is to demonstrate the sensitivity of their distribution to depletion of cholesterol (45) using cyclodextrin (46, 47) or depletion of SM using SMase (26). Few studies have directly compared the effects of these depletion methods on residual raft composition or their effects on recovery of proteins in rafts/DRMs prepared by different methods. We treated whole cell homogenates with either mβCD or SMase (26), isolated the plasma membranes, and prepared lipid rafts/DRMs either by sonication or 0.2% Triton. The data are shown in **Table 4** and **Fig. 5**. Cyclodextrin treatment removed ~42% of plasma membrane cholesterol (changing the plasma membrane CH/PL ratio from 1:2 to 1:5) without altering its phospholipid content or the proportions of individual phospholipid species. Cyclodextrin depletion removed 70–86% of raft cholesterol and 44–47% of nonraft cholesterol, indicating preferential but nonselective depletion of cholesterol from raft domains. Remarkably, even under conditions in which the majority of raft cholesterol was removed, total phospholipid content and the distribution of phospholipid species within these domains were unchanged, relative to untreated control membranes. One exception to this was a modest but reproducible 30% decrease in SM content of rafts prepared by sonication from cyclodextrin-pretreated membranes. Therefore, in most respects, the changes in lipid content of raft/DRM caused by cyclodextrin depletion of cholesterol were similar, independent of the method of raft isolation. However, this was not the case for raft marker proteins (Fig. 5). After cholesterol depletion, DRMs prepared with 0.2% Triton still contained the majority of plasma membrane caveolin-1 and flotillin-1. This is consistent with other studies (26). In contrast, when sonication was used to fractionate cholesterol-depleted membranes, essentially all caveolin-1 or flotillin-1 was lost from rafts to the nonraft fractions.

SMase pretreatment removed 55% of total plasma membrane SM and ~20% of total phospholipids without altering cholesterol content (Table 4). Raft/DRM SM was decreased by 55–77% and total raft phospholipid was decreased by ~35%. The cholesterol content of DRMs prepared using 0.2% Triton was unaffected by SMase pretreatment, whereas in rafts isolated by sonication, cholesterol was reduced by 35%. Significantly, depletion of SM affected raft marker protein distribution in the same way as cholesterol depletion (Fig. 5). Caveolin-1 and flotillin-1 in Triton-resistant DRMs were unaffected by SMase pretreatment, but these raft marker proteins were completely lost from rafts prepared using the detergent-free sonication method.

In intact, live cells, mβCD treatment results in a decrease in raft surface coverage and an increase in raft fluidity, suggesting that raft abundance within the plasma membrane is decreased and their structure is altered (15). The material generated by sonication reflects this change induced by cholesterol depletion, whereas DRMs prepared using Triton do not reflect the decrease in raft abundance caused by mβCD treatment. One possible explanation for the discrepancy is that the detergent treatment during solubilization of nonraft membrane domains

TABLE 4. Effects of cholesterol and SM depletion on THP-1 macrophage membrane domains

Lipid	Control					mβCD					SMase				
	Plasma Membrane	Sonication		0.2% Triton		Plasma Membrane	Sonication		0.2% Triton		Plasma Membrane	Sonication		0.2% Triton	
		LR	NR	LR	NR		LR	NR	LR	NR		LR	NR	LR	NR
CH	58.1 ± 6.7	23.9 ± 3.3 (41.1)	20.1 ± 4.2 (34.6)	25.8 ± 3.0 (44.4)	25.2 ± 1.9 (43.7)	33.8 ± 4.1[a]	3.3 ± 1.7[a] (9.7)	11.3 ± 1.9[a] (33.4)	7.6 ± 1.5[a] (22.5)	13.4 ± 1.6[a] (39.6)	52.3 ± 6.0	15.4 ± 2.6[a] (29.4)	18.2 ± 2.2 (34.8)	23.7 ± 2.3 (45.3)	22.1 ± 2.2 (42.2)
PL	114.7 ± 4.0	36.9 ± 4.2 (32.2)	64.9 ± 5.7 (56.6)	44.3 ± 5.0 (38.6)	57.2 ± 6.1 (49.9)	128.9 ± 10.5	39.9 ± 4.8 (30.9)	65.0 ± 6.9 (50.4)	45.1 ± 4.6 (35.0)	59.2 ± 2.6 (45.9)	93.5 ± 5.4[a]	24.6 ± 1.7[a] (26.3)	60.4 ± 3.4 (64.6)	32.0 ± 3.2 (34.2)	50.1 ± 3.7 (53.6)
CH/PL	34:66	42:58	24:76	45:55	32:68	20:80	10:90	18:82	15:85	17:83	36:64	38:62	26:74	50:50	31:69
PG	8.1 ± 2.4	8.3 ± 1.3	7.6 ± 0.6	7.0 ± 2.2	10.7 ± 1.2	4.6 ± 2.9	8.4 ± 3.1	7.7 ± 2.5	6.0 ± 2.4	9.4 ± 2.1	4.4 ± 1.5	10.8 ± 2.6	7.9 ± 2.3	9.1 ± 2.1	10.3 ± 2.3
PE	15.4 ± 2.8	19.2 ± 2.0	15.2 ± 2.6	20.9 ± 3.4	12.3 ± 1.8	18.1 ± 1.9	22.8 ± 2.3	13.3 ± 3.4	20.0 ± 1.6	16.8 ± 4.6	16.6 ± 2.5	20.6 ± 0.8	14.6 ± 3.1	20.2 ± 2.3	15.0 ± 3.7
PI	6.7 ± 1.3	3.5 ± 1.2	10.4 ± 3.0	4.0 ± 1.5	9.3 ± 1.1	5.4 ± 1.4	6.8 ± 2.4	7.6 1.8	5.3 2.2	9.5 ± 3.2	7.01 ± 0.7	4.6 ± 1.1	8.0 ± 0.8	4.2 ± 1.5	6.0 ± 2.9
PS	5.2 ± 1.1	2.4 ± 1.2	7.0 ± 1.9	5.8 ± 2.6	8.8 ± 2.1	3.8 ± 1.2	5.9 ± 2.6	4.5 ± 3.8	4.1 ± 1.9	6.4 ± 2.6	4.4 ± 1.2	5.9 ± 2.9	7.2 ± 0.2	3.2 ± 1.2	5.9 ± 1.5
PC	45.6 ± 6.9	26.1 ± 4.6	47.1 ± 2.6	29.3 ± 2.0	49.8 ± 6.2	51.2 ± 6.9	28.8 ± 3.9	52.3 ± 3.8	33.6 ± 3.6	48.0 4.9	58.9 ± 4.5[a]	48.6 ± 6.7[a]	57.2 ± 2.3[a]	48.2 ± 3.8[a]	55.7 ± 5.1
SM	19.0 ± 1.7	40.4 ± 6.8	12.8 ± 1.6	33.0 ± 6.9	9.2 ± 3.8	16.9 ± 4.2	27.4 ± 3.5[a]	14.6 ± 1.1	31.1 ± 1.3	9.9 ± 0.6	8.6 ± 2.4[a]	9.4 ± 1.9[a]	5.1 ± 1.6[a]	15.1 ± 1.9[a]	7.2 ± 1.8

THP-1 macrophage cell homogenates were incubated with 10 mM methyl-β-cyclodextrin (mβCD) or 0.5 U/ml sphingomyelinase (SMase) for 60 min at 37°C as described in Materials and Methods. Plasma membranes were then isolated [1.33 ± 0.15 mg/ml protein (n = 3); no statistically significant difference between pretreatment conditions], and lipid rafts/detergent-resistant membranes were prepared by sonication or 0.2% Triton extraction. Cholesterol (CH) and phospholipid (PL) are expressed as nmol in rafts and nonrafts. Values in parentheses are proportions of plasma membrane lipid present in raft (LR; 2–4) and nonraft (NR; 8–10) fractions. Phospholipid subclasses are given in molar ratios (mol%). The data are means ± SD of three independent experiments.

[a] Statistically significant difference ($P < 0.05$) compared with control plasma membrane, control raft, or nonraft membrane, respectively.

Fig. 5. Effects of cholesterol or SM depletion on the distribution of raft and nonraft proteins. Whole cell homogenates of THP-1 macrophages were incubated with 10 mM methyl β-cyclodextrin (mβCD) or 0.5 U/ml sphingomyelinase (SMase) for 60 min at 37°C as described in Materials and Methods. Plasma membranes were isolated, fractionated by sonication or treated with 0.2% Triton X-100, and separated on 5–45% sucrose gradients. Equal volumes of pooled lipid raft (LR; fractions 2–4) and nonraft (NR; fractions 8–10) regions of the sucrose gradients were loaded onto a SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed for caveolin-1 (CAV-1), flotillin-1 (FLO-1), or TfR. The blots are representative of three independent experiments.

actually generates new DRMs from these nonraft lipids (13, 48). To test this hypothesis, we first isolated nonraft material of control or cholesterol-depleted membranes using the detergent-free sonication method. This material was then incubated with 0.2% Triton and reisolated on a second 5–45% sucrose gradient. If Triton does induce DRM formation from nonraft membrane lipids, these "new" domains would float as buoyant membranes, similar to other DRMs/rafts. The results are shown in **Fig. 6**. When nonraft membranes were reisolated without any other treatment, >95% of the lipids remained in the nonraft density range. However, if the membranes were incubated with 0.2% Triton (Fig. 6), 10–15% of cholesterol or phospholipid from control and 22–38% of lipids from cholesterol-depleted nonraft membranes appeared as DRMs. These data suggest that 0.2% Triton can rearrange membrane lipids to induce the formation of DRMs of similar density to preexisting rafts. These effects were greatest in cholesterol-depleted membranes and may account for the differences in lipid recovery seen between DRMs and

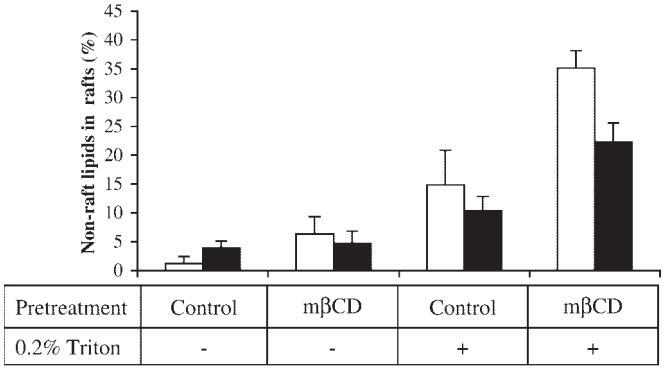

Fig. 6. Triton-induced redistribution of lipids. Nonraft membranes were prepared from control or cholesterol-depleted (mβCD) plasma membranes by sonication. The lipid composition of these membranes is given in Table 4 (control and mβCD, sonication, NR). Subsequently, these membranes were treated with or without 0.2% Triton for 30 min on ice and then separated on a second 5–45% sucrose gradient. The *y* axis gives the percentage of cholesterol (open bars) and phospholipids (closed bars) redistributed from nonraft membranes to float as rafts/DRMs in fractions 2–4. Data shown are averages ± range of two independent experiments.

light (raft) membranes prepared by sonication (Table 4). Similarly, proteins that favor rafts, such as flotillin-1 and caveolin-1, may also redistribute to these newly formed DRMs, affecting protein distribution, as observed in Fig. 5.

Lipid raft composition in primary human macrophages

We also examined the lipid and protein composition of rafts isolated from primary HMDMs. These cells express flotillin-1 and TfR but no detectable caveolin-1 (Fig. 4B). Nevertheless, the distribution of raft and nonraft marker proteins prepared by sonication (data not shown) or Triton extraction (Fig. 4B) was similar to that of THP-1 macrophages (Fig. 1). HMDM (**Table 5**) and THP-1 macrophage (Table 1) plasma membranes contained similar amounts of phospholipid (~80 nmol/mg protein), but there was less cholesterol in HMDM membranes (28 vs. 42 nmol/mg protein). Rafts isolated from HMDM and THP-1 cells were similar in lipid composition (Tables 1, 2, 5). Both contained 43–44% plasma membrane cholesterol, were enriched in SM and PE, and were depleted of anionic phospholipid relative to the plasma membrane, although overall phospholipid levels (27% vs. 39% plasma membrane phospholipids) and plasma membrane PC levels (20 mol% vs. 38 mol%) were lower in HMDM relative to THP-1. As HMDMs do not express caveolin-1, it is clear that this structural protein in not required for the maintenance of rafts/DRMs in macrophages, as found in other cell types (42).

TABLE 5. Lipid composition of primary human monocyte-derived macrophage membranes

Lipid	Plasma Membrane	LR	NR
CH	25.4 ± 6.3	10.8 ± 0.9 (42.6%)	7.9 ± 1.1 (31.2%)
PL	76.9 ± 10.5	20.7 ± 3.6 (27.0%)	35.9 ± 3.4 (46.7%)
CH/PL	25:75	34:66	18:82
PG	8.9 ± 2.1	6.6 ± 2.9	9.9 ± 4.4
PE	12.3 ± 1.6	17.5 ± 2.0[a]	10.7 ± 5.3
PI	8.9 ± 1.8	3.8 ± 1.6[a]	5.8 ± 2.3
PS	8.1 ± 2.7	1.9 ± 2.1[a]	3.0 ± 2.8
PC	41.9 ± 3.8	20.2 ± 4.6[a]	54.5 ± 6.2[a]
SM	19.9 ± 4.5	50.2 ± 5.0[a]	16.1 ± 4.5[a]

Plasma membranes (0.88 ± 0.25 mg protein/ml) were isolated from primary human monocyte-derived macrophages, and raft (LR) and nonraft (NR) membranes were isolated from 1 ml samples by the detergent-free method. Cholesterol (CH) and phospholipid (PL) are expressed as nmol in rafts and nonrafts. Values in parentheses are percentages of plasma membrane lipid in rafts and nonrafts. CH/PL gives the ratio of cholesterol to phospholipid for the membranes. Phospholipid subclasses are given in molar ratios (mol%) as described for Table 2. The data are means ± SD of three independent experiments.

[a] Statistically significant difference ($P < 0.05$) compared with plasma membrane.

DISCUSSION

There are relatively few studies of macrophage lipid raft composition. Others have shown that caveolin-1 is expressed and associated with lipid rafts in mouse macrophages (15, 49) and differentiated human THP-1 macrophages (37) but that caveolin-1 is undetectable in undifferentiated THP-1 "monocytes" (37) and primary HMDMs (12). However, the absence of caveolin-1 does not preclude the existence of lipid rafts in such cells (12, 36), and the present study clearly shows that the proportion of plasma membrane cholesterol present in lipid rafts is the same in all types of human macrophages, irrespective of their level of caveolin-1 expression. There were more subtle differences in lipid content between the different types of macrophages. For example, THP-1 monocyte rafts contained a lower proportion of SM (~15% phospholipid) than differentiated THP-1 macrophages (~30%), whereas primary HMDMs contained more (~50%). These differences are unlikely to be attributable solely to the expression of caveolin-1. This is consistent with another report that the expression of caveolin-1 in otherwise caveolin-1-negative cells did not alter the phospholipid composition of lipid rafts (42). But our data indicate that macrophage lipid raft composition and abundance can vary significantly under different conditions and suggest that raft-associated functions in macrophages may be similarly modified.

The functions associated with lipid rafts in macrophages have received relatively little attention. Recent proteomic analyses of Triton DRMs from THP-1 monocytes identified >70 raft-associated proteins (38, 50, 51). Besides conventional raft markers (flotillin-1 and -2), a broad array of proteins were identified, including many implicated in phagosome formation, vesicle trafficking and fusion, and cytoskeletal assembly, suggesting a role for rafts in macrophage motility and phagocytosis. We have shown previously that liquid-ordered domains are enriched on areas of the plasma membrane associated with underlying cytoskeletal structure, such as filipodia and adhesion points (15). Several macrophage membrane proteins involved in immune function, such as CD14, Toll-

like receptor 4, and class II MHC molecules, are also located in rafts either constitutively or after activation (40, 52, 53). We examined the distribution of a range of proteins known to be associated with the macrophage plasma membrane and found CD14, syntaxin 4, SR-BI, and Rac1 at least partially associated with lipid rafts (Fig. 2A). CDC42, Ras, and Munc18 were found predominantly, and dynamin II, SNAP23, and ATPase were found exclusively, in nonraft membranes in nonstimulated cells.

The current controversy concerning the validity of methods for the isolation of lipid rafts from cellular membranes led us to compare the protein and lipid composition of raft/DRM fractions prepared by different detergent-dependent and -independent methods from macrophage membranes. Our rationale was that if significant lipid reorganization occurs during raft isolation, as reported previously for Triton X-100 and model membranes (24, 25), then the lipid and protein composition of DRMs would be expected to vary, depending on the type and concentration of detergent used, and to differ greatly from those of rafts prepared by a detergent-free isolation method (27). On the other hand, if the composition of rafts/DRMs generated by different methods have similar structure and content, it would be more likely that they bear a relationship to undisrupted rafts in intact cells.

In fact, we found a remarkable initial similarity between the protein and lipid composition of 0.2% Triton DRMs and rafts prepared by a detergent-free sonication method. Both raft/DRM preparations contained similar amounts of cholesterol and SM. There were some differences in PC content, accounting for the differences in total phospholipid content between 0.2% Triton DRMs and detergent-free rafts. Hence, our data confirm previously reported enrichments of SM and PE and the relative greater depletion of PC from rafts prepared by sonication compared with detergent methods (42). In addition, the lipid molar ratios of isolated macrophage raft/DRM (CH/PL ratio of ~40:60; SM content, ~31 mol%; PE, ~19 mol%; PC, 25–38 mol%) agree well with those of rafts isolated from other cell types (26, 42). The similarities in lipid composition between detergent-free rafts and 0.2% Triton DRMs were also reflected in their structure, probed with Laurdan fluorescence. Their GP values, as a measure of lipid order, were comparable and agreed well with data obtained for condensed membranes on living cells (Fig. 4) (15). Others have reported that DRMs are more highly enriched with saturated phospholipids than detergent-free rafts (42, 54). We did not measure this in the present study but must conclude that if such differences exist between DRM and rafts from macrophage membranes, they do not have a significant effect on lipid order. On the basis of this similarity between 0.2% Triton DRMs and nondetergent rafts in protein distribution, lipid composition, and lipid order, it appeared possible that these domains were an accurate reflection of lipid rafts in intact membranes.

Differences between DRMs prepared using different detergents have been reported extensively (26, 35, 36, 42, 55). Clearly, weaker detergents solubilize less membrane than stronger detergents (26). It is also evident (22, 56) that the detergent concentration (i.e., the detergent-to-membrane lipid ratio) critically affects the composition of the insoluble membranes.

We could not adjust the Lubrol concentration to yield DRMs similar to those prepared with 0.2% Triton. It required a high concentration (>1%) (26) of Lubrol to solubilize any nonraft transmembrane proteins (TfR in Fig. 1D) (57), yet at 1%, Lubrol solubilized ~50% of plasma membrane phospholipids but only 27% of plasma membrane cholesterol and no SM (Tables 1, 2). The complete DRM sequestration of SM strongly suggests that 1% Lubrol induces artifactual enrichment of cholesterol and SM into DRMs. Similar to the high Lubrol concentration, 1% Triton also solubilized only minor amounts of SM (~4%; Table 2), consistent with the differential solubilization of phospholipids other than SM at high detergent concentration (58). Although with 1% Triton hardly any proteins were associated with DRM (~1% of plasma membrane proteins), a significant amount of membrane proteins were found in "Lubrol rafts" (~8% compared with ~3% with 0.2% Triton). As a protein-"scrambling" effect at high detergent concentrations, similar to the differential solubilization of lipids, cannot be excluded, the existence of raft subclasses in a single cell type cannot be shown by the use of Lubrol. In addition, we reiterate that detergent concentrations are critical for the isolation of DRMs (26).

In several previous studies, DRM-associated proteins were shown to be resistant to cholesterol or SM depletion (26, 59, 60). It was suggested that sufficient lipid remains, or is redistributed from other membrane(s), to preserve the structure and lipid-protein interactions of DRM. Our results do not support this line of evidence. When we depleted membranes of cholesterol or SM and then subjected identical samples to either detergent extraction or detergent-free raft preparation, we obtained different results. There was a complete transfer of both raft marker proteins into the nonraft fractions of membranes disrupted by the detergent-free method, whereas these proteins remained largely resistant to detergent extraction in identical samples of SMase- or mβCD-treated membranes (Fig. 5). This suggests that the raft proteins in the depleted membranes are affected differently by the different raft isolation methods. One possibility is that the depletion of cholesterol or SM severely alters lipid raft structure (reflected in a loss of raft cholesterol in SMase membrane and vice versa; Table 4), causing the dispersal of raft-associated proteins into the nonraft domains of the plasma membrane. This is consistent with the pattern of protein distribution measured in depleted membranes fractionated by the nondetergent method.

We suggest that when similar depleted membranes are exposed to detergent extraction, both the requisite reduction in temperature and the propensity of Triton to artifactually drive the formation of ordered domains may generate "membranes" into which the raft marker proteins once again partition and that are isolated as DRMs, as observed previously (24, 48). The depletion of membrane lipids (42% plasma membrane cholesterol or 63%

plasma membrane SM) might be sufficient to shift the detergent-membrane ratio beyond a nonscrambling range. Certainly, the failure to generate similar raft fractions from a single type of membrane by two different isolation methods indicates that at least one of these protocols generates domains that do not reflect their structure in the whole membrane. With the possibility that Triton scrambles lipids (24, 48), as seen with DRMs generated from nonraft material prepared by sonication, we currently place more confidence in the material generated by the detergent-free sonication protocol. However, we recommend that raft isolation always be accompanied by alternative experimental approaches when the study of raft function is undertaken.

The possibility of different subclasses of rafts on a single cell type remains. For example, caveolae, which are ~50 nm flask-shaped membrane invaginations (61, 62), contain caveolin-1 and are also detergent-insoluble and associated with the light membranes of a detergent-free isolation; therefore, caveolae are currently regarded as specialized lipid rafts. Anderson and Jacobson (63) proposed that some proteins contain a lipid shell of <100 individual lipids, so that they float as light membranes on sucrose gradients. Röper, Corbeil, and Huttner (35) suggest that different subdomains of the apical plasma membrane resist the solubilization of either Triton or Lubrol, and such selective solubilization may contribute to the lack of protein redistribution we observed with 0.2% Triton (Fig. 5). Although the methods tested here are not sufficiently specific to allow differentiation between raft subclasses, improvements in raft isolation methods have been suggested (55) that might be able to differentiate between raft subclasses. As the protein cargo of rafts could vary with cell type and raft subclass, we envisage that the lipid composition could vary similarly. To obtain a more complete picture of raft heterogeneity, a combination of lipid quantification of isolated membranes and in vivo studies is required.

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