Isolation and characterization of lipid rafts with different properties from RBL-2H3 (rat basophilic leukaemia) cells

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Lipid rafts are plasma-membrane microdomains that are enriched in certain lipids (sphingolipids, glycosphingolipids and cholesterol), as well as in lipid-modified proteins. Rafts appear to exist in the liquid-ordered phase, which contributes to their partitioning from the surrounding liquid-disordered glycerophospholipid environment. DRM (detergent-resistant membrane) fractions isolated from cells are believed to represent coalesced lipid rafts. We have employed extraction using two different non-ionic detergents, Brij-96 and Triton X-100, to isolate detergent-resistant lipid rafts from rat basophilic leukaemia cell line RBL-2H3, and compared their properties with each other and with plasma-membrane vesicles. DRM fractions were isolated as sealed unilamellar vesicles of similar size (135–170 nm diameter), using either sucrose-density-gradient sedimentation or gel-filtration chromatography. Lipid rafts isolated using Brij-96 and Triton X-100 differed in density, protein content and the distribution between high- and low-density fractions of the known raft constituents, Thy-1, and the non-receptor protein tyrosine kinases,

Yes and Lyn. Lyn was found in the raft microdomains in predominantly phosphorylated form. The level of enrichment of the protein constituents of the isolated lipid rafts seemed to depend on the ratio of cell lipid/protein to detergent. As indicated by reactivity with anti-Thy-1 antibodies, lipid rafts prepared using Brij-96 appeared to consist of vesicles with primarily right-side-out orientation. Both Brij-96 and Triton X-100 appear to isolate detergent-insoluble raft microdomains from the rat basophilic leukaemia cell line RBL-2H3, but the observed differences suggest that either the detergents themselves play a role in determining the physicochemical characteristics of the resulting DRM fractions, or different subsets of rafts are isolated by the two detergents.

Key words: Brij-96, detergent-resistant membrane (DRM), lipid rafts, Lyn, rat basophilic leukaemia (RBL)-2H3 cells, Thy-1, Triton X-100.

INTRODUCTION

Over the past few years, it has been recognized that one of the most important features of biological membranes is their ability to form specialized localized domains with a composition and physical properties different from the normal [1–3]. Since the proposal of their existence by Simons and Ikonen [4], lipid-raft microdomains have been the focus of intensive research [5]. Lipid rafts are dynamic assemblies within the eukaryotic cell plasma membrane, which are enriched in cholesterol, sphingolipids, glycosphingolipids and lipid-modified proteins. Sphingolipids and glycosphingolipids bear long saturated acyl chains that interact strongly with each other and with cholesterol molecules, to form densely packed structures in the outer leaflet. These microdomains segregate from glycerophospholipids with unsaturated acyl chains, which make up the balance of the plasma membrane [6]. Lipid rafts are thought to exist in the liquid-ordered phase [7], which has physical properties intermediate between those of the fluid liquid-crystalline phase and the rigid gel phase.

The protein molecules that reside within the lipid rafts are generally modified by covalent attachment of lipid molecules with long saturated acyl chains. GPI (glycosylphosphatidylinositol)-anchored proteins localize to lipid rafts on the exofacial side of the plasma membrane, whereas other proteins, such as members of the Src family of non-receptor tyrosine kinases, are inserted into the cytoplasmic leaflet of rafts by virtue of their dual acyl-chain modification [8]. Thus lipid rafts have a distinct protein composition. Until recently, the detergent-insoluble nature of

lipid-raft microdomains was the main experimental evidence in support of their existence. Extraction of intact cells with cold non-ionic detergents gives rise to a DRM (detergent-resistant membrane) fraction, which has a low buoyant density, and thus floats on sucrose gradients. This fraction is believed to represent closely the lipid-raft microdomains of the intact cells, and detergent resistance and low density have now become widely accepted hallmarks of lipid rafts. Studies with reconstituted liposomes containing GPI-anchored proteins have pointed out the importance of saturated acyl-chain interactions in determining the detergent insolubility of lipids and GPI-anchored proteins [9].

The existence of rafts was a controversial issue for several years, but an array of different techniques has now been used to verify their existence in both intact cells and model membrane systems, including FRET (fluorescence resonance energy transfer) microscopy, fluorescence microscopy and FRAP (fluorescence recovery after photobleaching), atomic force microscopy, chemical cross-linking and single-particle tracking. Nevertheless, the size, lifetime and fraction of the plasma membrane occupied by lipid rafts, and whether these properties are cell-type dependent, are still unclear, and much remains to be understood regarding the lipid-raft structure and dynamics in intact cells.

A role for lipid rafts in numerous important cellular processes has now been established, both in the normal cells and in the disease states [10,11]. Rafts appear to be part of the intracellular sorting and exocytotic trafficking machinery of the cell [12]. They are intimately involved in the entry of several enveloped viruses into the cell [13], as well as viral budding from the plasma membrane [14], and the invasion of cells by bacterial toxins and

Abbreviations used: CTB, cholera toxin B; DLS, dynamic light scattering; DRM, detergent-resistant membrane; EM, electron microscopy; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; MDCK, Madin–Darby canine kidney; NEM, *N*-ethylmaleimide; RIPA, radioimmunoprecipitation assay.

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pathological micro-organisms [15,16]. Rafts have also been shown to play a central role in transmembrane signal transduction by spatially organizing signalling components into small regions of the membrane to facilitate high efficiency and specificity [17]. Small dynamic raft structures may coalesce as a result of ligand binding during the signalling process to form larger, ordered signalling platforms. A recent quantitative proteomics approach found that large numbers of different signalling proteins, including tyrosine kinases and serine/threonine kinases, were enriched in rafts by a factor of more than 10 [18]. For the rat basophilic leukaemia cell line RBL-2H3 used in the present study, aggregation of lipid-raft components such as Thy-1 by antibody cross-linking triggers various signal-transduction events similar to those seen for mast cells activated via the high-affinity IgE receptor (F ϵ R1) [19]. These events include activation of the non-receptor tyrosine kinase, Lyn, followed by phosphorylation of membrane-bound and soluble substrates.

RBL-2H3 cells provide an excellent model system to study lipid rafts since they express high levels of the GPI-anchored protein Thy-1, a known component of glycosphingolipid-enriched membrane microdomains [20], which forms detergent-insoluble complexes with Lyn [21]. In the present study, we have investigated the molecular properties of raft microdomains isolated from RBL-2H3 cells using two different non-ionic detergents, Brij-96 and Triton X-100. We have found that the lipid rafts differ in their molecular composition depending on the type of detergent used; however, they are isolated as vesicular structures of similar size for both detergents. Surprisingly, the orientation of the isolated vesicles appeared to depend on the detergent used. As indicated by reactivity with anti-Thy-1 antibodies, lipid rafts prepared using Brij-96 appeared to form low-density vesicles of right-side-out orientation, whereas Triton X-100 lipid rafts were of higher density and formed mainly inside-out vesicles. Thus Brij-96 and Triton X-100 appear to isolate raft microdomains, which differ in density, protein composition and orientation, suggesting that either the detergents themselves may play a role in determining the physicochemical characteristics of the resulting DRM structures or the two detergents isolate different subsets of rafts that normally co-exist in the plasma membrane.

EXPERIMENTAL

Materials

The anti-Thy-1 monoclonal antibody, OX-7, was overproduced and affinity-purified using Affi-Gel Protein A-agarose as described previously [22]. Anti-Lyn, anti-Fyn, anti-Yes, anti-Lck and anti-CD71 (transferrin receptor) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Transduction Laboratories (Lexington, KY, U.S.A.) supplied the PY-20 antibody. CTB (cholera toxin B) subunit labelled with HRP (horseradish peroxidase), Protein A-agarose, Protein G-agarose, PMSF, Nonidet P40, DNase, pepstatin A and leupeptin were purchased from Sigma (St. Louis, MO, U.S.A.). HRP-labelled goat anti-rabbit and goat anti-mouse antibodies were purchased from Jackson ImmunoResearch Laboratories (Mississauga, ON, Canada). Triton X-100 was supplied by Roche Diagnostics (Laval, QC, Canada). Brij-96 was obtained from Fluka (Oakville, ON, Canada) and SDS was purchased from Fisher Scientific (Whitby, ON, Canada).

Cells

The rat basophilic leukaemia cell line RBL-2H3 was obtained from A.T.C.C. (Manassas, VA, U.S.A.). Cells were grown as

monolayers in α -minimal essential medium containing 10 % (v/v) foetal bovine serum (Hyclone, Logan, UT, U.S.A.) supplemented with 2 mM glutamine and 2 mM penicillin–streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂. Cells were harvested using 5 mM EDTA in PBS (pH 7.4).

Isolation of lipid-raft microdomains by sucrose-gradient centrifugation

Lipid rafts were isolated from either freshly harvested or frozen cells. The procedure was adapted from that of Prinetti et al. [23]. Approx. $2\text{--}5 \times 10^8$ cells (100 μ l of cell pellet) were washed twice in Tris-buffered saline (TBS: 25 mM Tris/HCl, 140 mM NaCl, pH 7.5) and then treated on ice with 1 ml of lysis buffer consisting of either 0.5 % (w/v) Brij-96 in 25 mM Tris/HCl and 140 mM NaCl (pH 7.5), or 1 % (w/v) Triton X-100 in 25 mM Tris/HCl and 140 mM NaCl (pH 8.0). The detergent concentrations were chosen based on the fact that Triton X-100 rafts are most often isolated using a 1 % concentration of this detergent, and the upper limit of the solubility of Brij-96 at 4 °C is 0.5 %. Each lysis buffer contained the protease/phosphatase inhibitors, 1 mM EDTA, 1 mM PMSF and 1 mM Na₃VO₄, plus a cocktail of protease inhibitors (Complete™, EDTA-free; Roche Diagnostics). Cells were left on ice for 20–30 min to lyse, and then centrifuged at 10 000 *g* for 5 min at 4 °C to obtain the post-nuclear fraction. The post-nuclear lysate was adjusted to 40 % (w/v) sucrose and then a 5–30 % discontinuous sucrose gradient was layered on the top. Typically, 2.2 ml of 30 % sucrose and 2.2 ml of 5 % sucrose were layered on a 0.8 ml sample in 40 % sucrose in a 5.2 ml centrifuge tube. Samples were centrifuged at 400 000 *g* for 3–4 h at 4 °C using a VTi 65.2 rotor (Beckman Coulter, Mississauga, ON, Canada). Fractions of 0.4 ml (typically 12–13 fractions in total) were collected from the top of the gradient tube using a Density Gradient Fractionator (Model 640; ISCO, Lincoln, NE, U.S.A.). Immunoblot analysis was performed to identify and confirm the fractions containing lipid-raft microdomains. Fractions containing rafts were pooled, usually fractions 2–4 when Brij-96 was used and fractions 5–7 when Triton X-100 was used. Where necessary, the pooled fractions were dialysed extensively against TBS (pH 7.5) to remove sucrose.

Isolation of lipid rafts from total cell lysates

Lipid rafts were also isolated from total cell lysates, as described above, but without the centrifugation step to recover the post-nuclear fraction. RBL-2H3 cells were lysed at a concentration of either 100 or 200 μ l of cell pellet/ml of either Brij-96 or Triton X-100 lysis buffer, as described in the previous section. For side-by-side comparison, lysates were either precleared by centrifugation at 10 000 *g* for 5 min at 4 °C or kept as the total cell lysate, then run on a 5–30 % discontinuous sucrose gradient as described above. Representative amounts of each fraction from the gradient were analysed by immunoblotting with anti-Thy-1 and anti-Lyn antibodies as outlined below.

Isolation of lipid-raft microdomains by gel-filtration chromatography

A 200–400 μ l fraction of the post-nuclear fraction from RBL-2H3 cells extracted using 1.0 % Triton X-100 was applied to a Sepharose 4B column (7 mm $\times$ 75 mm). Elution was performed using 1.0 % Triton X-100 lysis buffer, and fractions were collected. Representative amounts of each fraction were analysed by immunoblotting for the lipid-raft components Thy-1 and Lyn.

Isolation of plasma-membrane vesicles from RBL-2H3 cells

Plasma-membrane vesicles from RBL-2H3 cells were prepared by a method adapted from Holowka and Baird [24]. Semi-confluent cell monolayers from six large T175 flasks were washed twice in Hepes-buffered saline (10 mM Hepes/150 mM NaCl/2 mM CaCl₂, pH 7.4) containing 0.05 % (w/v) gelatin. Cells were then treated with 2 mM NEM (*N*-ethylmaleimide) in the same buffer for 1 h at 37 °C. Flasks were gently tapped to enhance vesicle release, the supernatant was decanted and DNase and MgCl₂ were added to final concentrations of 50 µg/ml and 2 mM respectively. The vesicle suspensions from the six flasks were combined and centrifuged at 135 *g* for 10 min at 4 °C. The supernatant was carefully removed, centrifuged at 25 000 *g* for 45 min at 4 °C, and the pelleted vesicles were then suspended in 1 ml of PBS [10 mM Na₂HPO₄ · 7H₂O/1.8 mM KH₂PO₄/150 mM NaCl/0.01 % (w/v) NaN₃, pH 7.5] using a syringe and 23-gauge needle, and subsequently, various protease inhibitors were added (1 mM PMSF/10 µg/ml leupeptin/5 µg/ml pepstatin A; all final concentrations). To remove the remaining cells, vesicles were passed through a 5 µm Nucleopore polycarbonate filter. The plasma-membrane vesicle preparation was then mixed with 60 % sucrose solution to achieve a final concentration of 40 % sucrose, and the sample was subjected to gradient centrifugation and fractionation.

Protein quantification

The protein profile of the sucrose-density fractions was determined by bicinchoninic acid protein assay [25] on aliquots of the sucrose fractions, using BSA (crystallized and freeze-dried; Sigma) as a standard.

SDS/PAGE and Western-immunoblot analysis

Representative amounts of each fraction from the sucrose gradient or the gel-filtration column were analysed by SDS/PAGE as described by Laemmli [26] on a 10 or 12 % (w/v) acrylamide gel. Separated proteins were transferred on to a nitrocellulose membrane for immunoblot analysis. Membranes were blocked in 5 % (w/v) milk in TBS-Tween [0.05 % (w/v) Tween 20 in 10 mM Tris/100 mM NaCl, pH 7.5], and then incubated with the primary antibody of choice (OX-7, anti-Lyn, anti-Yes, anti-Fyn, anti-Lck or anti-CD71). For the PY-20 antibody, membranes were blocked with 3 % (w/v) BSA. Membranes were subsequently incubated with HRP-conjugated goat anti-mouse antibody, and specific interactions were revealed using the ECL[®] (enhanced chemiluminescence) detection system (Amersham Biosciences, Baie d'Urfé, QC, Canada).

Analysis of lipid-raft-associated G_{M1}

Lipid-raft-associated G_{M1} was determined using an enzyme assay as described by Blank et al. [27]. In this procedure, the CTB-HRP conjugate was employed for detection and quantification of DRMs. Approx. 1–2 × 10⁸ cells were washed with ice-cold PBS, resuspended in 100 µl of ice-cold PBS, and then labelled with 600 m-units of CTB-HRP for 30 min on ice. Cells were then washed twice in ice-cold PBS (300 µl/wash) to remove unbound CTB-HRP. Cells were then lysed in 300 µl of either 0.5 % Brij-96 or 1.0 % Triton X-100 lysis buffer and subjected to sucrose-density centrifugation as described above. After collection of the fractions, a 50 µl aliquot of each fraction was assayed, along with a set of standards in the range 0.002–1.0 m-units of CTB-HRP, using a 96-well microplate. Standards were prepared in the appropriate lysis buffer. To all standards and samples, 50 µl of

the chromogenic peroxidase substrate, ABTS [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)], was added (ABTS tablets and buffer; Roche Diagnostics). After a dark-green colour had developed, the absorbance of the samples was read at 405 nm using a plate reader. G_{M1} in raft fractions was studied by dot-blot analysis, in which an aliquot from each fraction was spotted directly on to the nitrocellulose membrane. G_{M1} was visualized using CTB-HRP, followed by ECL[®] detection.

Cholesterol analysis

Lipids were extracted from sucrose-density fractions using chloroform/methanol (2:1, v/v). For each 200–400 µl fraction, 200–400 µl of chloroform/methanol was used, and the lower organic phase containing the lipids was transferred on to a glass tube. Extracted lipids were dried first under a stream of N₂ and subsequently in a vacuum desiccator. The ferric chloride reagent was prepared by diluting 2 ml of a stock solution of 2.5 % (w/v) FeCl₃ in 85 % H₃PO₄ to 25 ml with concentrated H₂SO₄. To each dried sample and a set of cholesterol standards, 0.75 ml of acetic acid and 0.5 ml of ferric chloride reagent were added, mixing well with a vortexer after each addition [28]. After 5–10 min, a pink/purple colour developed and the absorbance of the samples was read at 550 nm.

Vesicle lysis and immunoprecipitation

Vesicle lysis and immunoprecipitation analysis were performed as described by Iwabuchi et al. [29]. Vesicles were lysed in RIPA (radioimmunoprecipitation assay) buffer, consisting of 30 mM Hepes, 150 mM NaCl, 1 % Nonidet P40, 0.5 % (w/v) SDS, 2 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF and a cocktail of protease inhibitors (Complete[™], EDTA-free). Lipid-raft preparations, made using either 0.5 % Brij-96 or 1 % Triton X-100, were mixed with an equal volume of 2× concentrated RIPA buffer. Vesicles were incubated to allow lysis for 20–30 min on ice. Subsequently, each sample was divided into two; one half was used for immunoprecipitation analysis with anti-Lyn antibodies and the other half for immunoprecipitation with anti-Thy-1 antibodies. Immunoprecipitation was performed overnight at 4 °C with gentle rotation of the samples on a nutator. Immuno-complexes were collected using Protein-G-agarose beads, which were dissolved in Laemmli's buffer [26], and the bound proteins were separated by SDS/PAGE. Proteins of interest were detected by immunoblotting with specific antibodies and ECL[®] detection.

Tyrosine phosphorylation of Lyn

The phosphorylation status of Lyn was assessed by immunoprecipitation and Western-blot analyses as follows. Each of the fractions to be analysed was first dialysed to remove interfering sucrose. Since lipid rafts are isolated as sealed vesicles, all fractions were first lysed in RIPA lysis buffer so that access to the protein was not restricted. Subsequently, immunoprecipitation with anti-Lyn antibody was performed overnight at 4 °C with gentle mixing on a nutator, and immunocomplexes were collected on Protein G-agarose beads for 2 h at 4 °C using a nutator. Immunocomplexes were then washed with Tris/NaCl buffer, separated by SDS/PAGE and analysed by immunoblotting using PY-20 anti-phosphotyrosine antibody followed by ECL[®] detection.

EM (electron microscopy)

Membrane samples were dialysed against 200 mM Hepes buffer (pH 7.5) without NaCl to avoid undesired salting-out of the stain. Dialysed membrane vesicles were negatively stained with uranyl

acetate, and electron micrographs were obtained using an LEO 912AB instrument (LEO Elektronmikroskopie, Oberkochen, Germany) at $\times 320\,000$ magnification, with imaging software from Soft Imaging System (Munster, Germany).

DLS (dynamic light scattering) analysis

DLS was employed to assess the size of the lipid-raft vesicles. Analysis was performed for 60 min using a Brookhaven BI9000 autocorrelator with a bin time of 50 μ s. Data were analysed using DLS Software Version 1.35 (developed by Dr R. Hallett, Department of Physics, University of Guelph, ON, Canada), and results are expressed as a number distribution.

RESULTS

Isolation of DRMs by sucrose-density-gradient sedimentation and gel-filtration chromatography

Detergent-resistant microdomains were isolated from RBL-2H3 cells using extraction with cold 1% Triton X-100, followed by two different fractionation approaches, sucrose-density sedimentation and Sepharose 4B gel-filtration chromatography. The raft-containing fractions were located by Western immunoblotting of two established raft markers; the GPI-anchored protein, Thy-1 (inserted into the outer leaflet of the membrane) and the acylated non-receptor tyrosine kinase, Lyn (found on the cytoplasmic membrane face). As shown in Figure 1(A, left panels), detergent-resistant rafts were found in the low-density fractions 4–6 after sucrose-gradient sedimentation, as indicated by localization of substantial fractions of the total cellular Thy-1 and Lyn. Some of the raft marker proteins were also found in the high-density fractions, indicating that they are not exclusively located in the rafts isolated with Triton X-100. Detergent-resistant raft fractions were also isolated by Sepharose 4B gel-filtration chromatography, where they eluted close to the void volume of the column (Figure 1B), indicating the presence of large structures. In this case, the bulk of the Thy-1 was located in the early raft fractions, whereas a large portion of the Lyn kinase was located in the late-eluting non-raft fractions. To compare the behaviour of the detergent-resistant fractions with that of the entire plasma membrane, RBL-2H3 cells were treated with NEM, which induces large, unilamellar, predominantly right-side-out vesicles to pinch off from the plasma membrane [24]. The resulting preparation was subjected to the same sucrose-density-gradient separation as the Triton X-100 extract. As shown in Figure 1(A, right panel), there was no clear separation of the plasma-membrane vesicles into low- and high-density fractions (cf. Figure 1A, left panel). Fractions with densities corresponding to those of the sucrose rafts were collected for comparison with the two raft preparations.

The lipid-raft fractions obtained by each method were pooled and analysed by negative staining EM. In all cases, the raft fractions displayed a unilamellar vesicular structure regardless of the experimental approach (see Figure 1C). Sucrose-density centrifugation of non-ionic detergent-extracted RBL-2H3 cells was chosen as the means of isolation of lipid rafts for further study, due to practicality and convenience of sample handling.

Distribution of lipid-raft markers in Brij-96 and Triton X-100 rafts

It seems probable that certain detergents may differentially extract raft domains, so that the choice of detergent has an impact on the composition and properties of the resulting DRM fraction. Several researchers have suggested that the non-ionic Brij detergents

Figure 1 Comparison of RBL-2H3 plasma-membrane vesicles with detergent-resistant lipid-raft microdomains isolated using sucrose-density centrifugation and gel-filtration chromatography

(A) The post-nuclear lysate of RBL-2H3 cells extracted with 1% Triton X-100 was fractionated by sucrose-density-gradient centrifugation (sucrose rafts) and PMV (plasma-membrane vesicles) prepared by NEM treatment of intact RBL-2H3 cells were fractionated in the same way (sucrose PMV). Representative amounts of each gradient fraction were analysed by Western immunoblotting followed by ECL[®] detection for the lipid-raft protein markers Thy-1 and Lyn. (B) A 400 μ l fraction of the post-nuclear fraction from RBL-2H3 cells extracted with 1% Triton X-100 was applied to a Sepharose 4B column (7 mm $\times$ 75 mm), eluted with 1% Triton X-100 lysis buffer and 21 fractions of 160 μ l each were collected. (C) Lipid-raft fractions prepared by 1% Triton X-100 extraction of RBL-2H3 cells, fractionated by either gel-filtration chromatography (Sepharose 4B rafts; fractions 7 and 8) or sucrose-density-gradient centrifugation (sucrose rafts; fractions 4–6) were pooled, dialysed, negatively stained with uranyl acetate and visualized by EM. Plasma-membrane vesicles fractionated by sucrose-density-gradient centrifugation (sucrose PMV; fractions 5 and 6) were treated in the same way.

(see Figure 2 for detergent structures) are capable of isolating a subset of lipid rafts, which are different from Triton X-100-extracted lipid rafts [30–34]. Therefore we examined whether using these two detergents would lead to differences in the distribution of the lipid-raft constituents. RBL-2H3 cells were extracted with cold Brij-96 or cold Triton X-100, and the resulting detergent-resistant preparations were fractionated using sucrose-density-gradient sedimentation. Figure 3 shows the distribution across the gradient fractions of three protein markers of lipid

Triton X-100 **Brij-96** **Figure 2** Structures of the non-ionic detergents used in the present study

rafts (Thy-1 antigen, and the tyrosine kinases Lyn and Yes). The glycosphingolipid, G_{M1} ganglioside, was found to partition exclusively within lipid rafts and caveolae, and is therefore accepted as a sphingolipid lipid marker for these structures [35]. The integral membrane protein CD71 (transferrin receptor) was used as a marker of non-raft fractions. Thy-1 is a very abundant protein on the RBL-2H3 cell surface. As can be seen in Figure 3(A), results for the two detergents are quite different. Brij-96 rafts showed a very narrow distribution of Thy-1, with almost all of the protein recovered in the low-density raft fractions (centred around fractions 2–4). In contrast, the distribution of Thy-1 in the Triton X-100 extract was bimodal, with the smaller fraction of the protein present in the lipid rafts (centred around fractions 5–7),

and a larger fraction found in the high-density soluble fractions 10–12. Western immunoblotting of crude cell lysate revealed that, among the haematopoietic-specific Src-family tyrosine kinases (Lyn, Lck, Fyn and Yes), only Lyn and Yes were detected in the RBL-2H3 cell line (results not shown). When Brij-96 was used, Lyn and Yes showed a distribution in which the bulk of the proteins co-localized with Thy-1 in the raft fractions, with only small amounts appearing in the high-density fractions 12 and 13. In striking contrast, for Triton X-100, most of Lyn and Yes was recovered in the high-density fractions, with only a small fraction of the proteins present in the raft fractions. Thus whether the majority of the Lyn and Yes kinases are in the high- or low-density fractions depends on the type of detergent used for isolation of lipid rafts. The location of the lipid-raft-containing fraction was confirmed by G_{M1} dot-blot analysis. For each of the two different detergents used, the strongest signal for G_{M1} was detected in the raft fractions containing Thy-1 (Figure 3B), consistent with its behaviour as a raft marker. The transmembrane protein CD71 is known to be completely solubilized after cold-detergent extraction. CD71 was found only within the high-density fractions, reflecting the fact that it is not found in lipid rafts. These results confirm that the type of detergent used does not play a role in the pattern of gradient distribution seen for detergent-soluble proteins. By comparing the fractions in which the various raft markers were located, the density of the lipid rafts was found to be clearly lower for Brij-96 when compared with Triton

Figure 3 Distribution of Triton X-100 and Brij-96 lipid-raft components in fractions from the sucrose-density gradient

RBL-2H3 cells were lysed in either 0.5% Brij-96 or 1% Triton X-100. Post-nuclear lysates were fractionated by ultracentrifugation on a discontinuous sucrose gradient and 13 fractions were collected from the top of the gradient tube. (A, C) An aliquot from each fraction was run on SDS/polyacrylamide gel, and the separated proteins were transferred on to a nitrocellulose membrane and analysed by Western-immunoblotting analysis and ECL[®] detection as described in the Experimental section. (B) For G_{M1} , dot-blot analysis was performed in which an aliquot from each fraction was spotted directly on to the nitrocellulose membrane. G_{M1} was visualized using HRP-CTB subunit, followed by ECL[®] detection.

X-100; the raft markers (both protein and glycosphingolipid) were consistently found in fractions 2–4 for Brij-96 and in fractions 5–7 for Triton X-100. These observations reinforce the notion that the only membrane structures distinguished by the two detergents are the lipid rafts, and that rafts with differing physical and biochemical characteristics can be isolated, depending on the detergent used.

To rule out the possibility that the observed differences are a function of detergent concentration rather than the type of detergent used, we compared the profiles of sucrose-density fractions obtained using 0.5 % Brij-96 and 0.5 % Triton X-100 (results not shown). Lipid rafts were again detected in fractions 5–7 for Triton X-100 and in fractions 2–4 for Brij-96. The localization pattern of Thy-1, Lyn and Yes proteins remained virtually unchanged, with the only difference being that slightly more Thy-1 was distributed in the low-density fractions at a Triton X-100 concentration of 0.5 %. The total protein profile distribution remained the same for the two detergents, and the membrane protein CD71 (transferrin receptor) was completely solubilized regardless of which detergent was used. Brij-96 cannot be used at a concentration of 1 %, since this detergent precipitates at 4 °C, making it incompatible with the cell lysis procedure and all the subsequent steps of lipid-raft isolation, which are performed on ice. Since 1 % Triton X-100 is the standard concentration commonly used in procedures to isolate lipid rafts from many different cell types, we continued our studies comparing rafts isolated using 1 % Triton X-100 with those isolated using 0.5 % Brij-96.

Comparison of lipid-raft marker distribution in Brij-96 and Triton X-100 rafts from precleared and total cell lysates

Recently, Young et al. [36] showed that there is a selective loss of Lyn from lipid rafts isolated using 0.5 % Triton X-100, compared with those isolated using RIPA buffer, when the detergent lysates are precleared by centrifugation. In their study, larger amounts of Lyn were recovered in lipid rafts when total cell lysates were used with no preclearing. To ascertain whether such an effect may be biasing the observed distribution of the raft markers Thy-1 and Lyn in the present study, sucrose-gradient analysis of lipid rafts was performed using precleared and total cell lysates, for both Triton X-100 and Brij-96. Results indicated that the pattern of distribution of the lipid-raft components Thy-1 and Lyn down the sucrose-density gradient is very similar for cells lysed at a concentration of 100 μ l of cell pellet/ml of lysis buffer, regardless of whether the lysate was precleared by centrifugation or whether the total cell lysate was used (cf. Figures 4A and 4B with Figures 4E and 4F). This was true for both Brij-96 and Triton X-100. If the cells were lysed at a concentration of 200 μ l of cell pellet/ml of lysis buffer, the lipid-raft fractions (3–5 for Brij-96 and 5–7 for Triton X-100) were more enriched in Thy-1 and Lyn regardless of whether the lysates were precleared or whether total cell lysate was used (cf. Figures 4C and 4D with 4A and 4B, and Figures 4G and 4H with 4E and 4F respectively). These results suggest that the level of enrichment of the protein constituents of lipid rafts depends on the ratio of cell lipid/protein to detergent, but not on the precleared status of the lysates.

Distribution of protein, cholesterol and G_{M1} ganglioside in Brij-96 and Triton X-100 rafts

The Brij-96 and Triton X-100 sucrose-gradient fractions were analysed for total protein content (using a protein assay), cholesterol (using a colorimetric method) and the raft marker ganglioside G_{M1} (using a CTB–HRP conjugate followed by an enzyme

assay). The distribution of total protein in the gradient fractions gave similar results for both the detergents; however, some subtle differences were also evident. The Brij-96 gradient showed substantially higher levels of protein spread out over the lower-density fractions (Figure 5A). On the other hand, as shown in Figure 5(B), the Triton X-100 gradient profile showed very little protein present in the fractions corresponding to the low-density rafts; the bulk of the protein was located in high-density fractions 11–13. The distribution of cholesterol also differed significantly between the two detergents (Figures 5C and 5D). A bimodal cholesterol profile was observed for Triton X-100, with one peak corresponding to the raft fractions, and the remainder found in the higher-density fractions at the bottom of the gradient. The cholesterol distribution in the Brij-96 gradient showed that the bulk of the sterol was found in the low-density fractions at the upper end of the gradient, with very little seen in the high-density non-raft fractions. Similarly, the raft marker, G_{M1} , showed a single peak in the Brij-96 gradient (Figure 5E), indicating that virtually all of this glycosphingolipid is associated with the low-density raft fractions. On the other hand, extraction with Triton X-100 gave a bimodal G_{M1} distribution, with a large fraction of the ganglioside found in the high-density non-raft fractions. Thus the Brij-96 raft fractions showed a much 'tighter' distribution of several different raft markers, including a GPI-anchored protein (Thy-1), dually acylated tyrosine kinases (Lyn and Yes), cholesterol and a glycosphingolipid.

Size and morphology of detergent-resistant rafts

To assess the morphology of the lipid-raft domains that were isolated by Brij-96 and Triton X-100, we used EM with uranyl acetate negative staining. The electron micrographs presented in Figure 6 demonstrate the vesicular nature of the detergent-insoluble membrane microdomains. For both Brij-96 and Triton X-100, sealed, spherical vesicles were isolated, which appear to be unilamellar. Vesicular structures were observed only for the pooled low-density fractions containing lipid rafts (Figure 6A). The high-density fractions 11–13 were pooled and analysed as a negative control. In contrast with the vesicles found in the low-density fractions, only very small structures of granular appearance and irregular shape were observed in these fractions (Figure 6B). These structures probably represent macromolecular aggregates of the detergent-solubilized proteins and/or lipids present in these fractions. Thus the same type of vesicular structures were seen in the low-density raft fractions for both the detergents.

Once the vesicular nature of the isolated lipid rafts was established, we examined the size profile of these vesicles. The results of DLS analysis (Figure 7) indicate that both types of lipid-raft vesicle displayed a relatively sharp unimodal size distribution. It was found that most of the lipid-raft vesicles had diameters in the range 115–240 nm; using Brij-96, the highest percentage of vesicles had a diameter of 135 nm. The size distribution profile was skewed to slightly larger diameters for the Triton X-100 vesicles, with the size of most of the vesicles ranging from 135 to 170 nm.

Tyrosine phosphorylation of Lyn in lipid rafts

It has been suggested that lipid rafts act as signalling platforms by segregating activated components of signalling pathways into small membrane microdomains, where they are in close juxtaposition to each other. The autophosphorylation of Lyn on tyrosine residues plays a major role in its activation, which is essential for downstream signalling from various transmembrane receptors, including the T-cell receptor in immune cells and the high-affinity

Figure 4 Comparison of the distribution of Triton X-100 and Brij-96 lipid-raft components in fractions from the sucrose-density gradient of precleared and total cell lysates

RBL-2H3 cells were lysed in either Brij-96 lysis buffer (A–D) or Triton X-100 lysis buffer (E–H) at a concentration of 100 μ l (A, B, E, F) or 200 μ l (C, D, G, H) of cell pellet/ml of lysis buffer. Lysates were either precleared by centrifugation at 10000 g for 5 min at 4 °C or kept as the total cell lysate, then run on a 5–30% discontinuous sucrose gradient as described in the Experimental section. An aliquot from each of the 13 fractions was run on SDS/polyacrylamide gel, and the separated proteins were transferred on to a nitrocellulose membrane and analysed for Thy-1 and Lyn by Western immunoblotting and ECL[®] detection as described in the Experimental section.

IgE receptor in mast cells. Phosphorylation of Lyn at Tyr³⁹⁷, within the active site of the enzyme, leads to a 17-fold enhancement of its specific activity [37]. Immunoprecipitation of Lyn from the sucrose-gradient fractions, followed by Western immunoblotting with an anti-phosphotyrosine antibody, revealed that the Lyn protein localized in the Brij-96 lipid rafts (fractions 2–4) was tyrosine-phosphorylated (Figure 8). Lyn protein found in the

Triton X-100 rafts was also phosphorylated (results not shown). The fact that phosphorylated Lyn was found in the lipid rafts is in accordance with the proposed role of these microdomains in signalling processes. However, phosphorylation of Lyn can also take place at a C-terminal negative regulatory site (Tyr⁵⁰⁸), and determination of its kinase activity would confirm that it is indeed activated.

Figure 5 Distribution of total protein, cholesterol and G_{M1} ganglioside in sucrose-density-gradient fractions from lipid rafts isolated using Brij-96 and Triton X-100

Brij-96 extracts (**A, C, E**) and Triton X-100 extracts (**B, D, F**) of RBL-2H3 cells were run on sucrose gradients, and the separated gradient fractions were assayed for the distribution of total protein (**A, B**), cholesterol (**C, D**) and G_{M1} ganglioside (**E, F**), as described in the Experimental section. (**A, B**) The protein content is shown for a 20 µl aliquot of each gradient fraction from $2-5 \times 10^8$ cells lysed in 1 ml of buffer. (**C, D**) The cholesterol content is shown for the entire gradient fraction from $2-5 \times 10^8$ cells lysed in 300 µl of buffer. (**E, F**) The activity of CTB-HRP in a 50 µl aliquot of each gradient fraction from $1-2 \times 10^8$ cells.

Orientation of lipid-raft vesicles isolated using Brij-96 and Triton X-100

To investigate further the vesicular nature of the isolated lipid-raft structures, a biochemical approach was undertaken. We hypothesized that vesicles formed after lipid-raft isolation would seal after re-orientation of the membrane leaflet in either direction, resulting in either right-side-out or inside-out raft vesicles. In the first case, Thy-1 would be distributed on the outer side of the vesicle, whereas Lyn would be located on the inside (Figure 9A). As a result, if we attempt to isolate a native intact raft vesicle by immunoprecipitation, we would be able to do so only if anti-Thy antibody is used, since the Lyn kinase will not be accessible under these conditions. However, if the intact nature of the raft vesicles is disrupted by detergent lysis, then each of the proteins will be immunoprecipitated by its respective antibody, either alone or in a complex with the other molecule, depending on the strength of their interaction under the experimental conditions. The relative recoveries of Thy-1 and Lyn with the respective immunoprecipitation antibodies also depend on the extent of their co-localization within the rafts, as indicated in Figure 9(A). For example, for immunoprecipitation performed with anti-Thy-1, the bulk of the Lyn is expected to be recovered with Thy-1, since Lyn is localized primarily within the lipid rafts with Thy-1. How-

Figure 6 EM of lipid-raft structures isolated using Brij-96 and Triton X-100

Electron micrographs of lipid-raft fractions (**A**) and high-density fractions (**B**) isolated from RBL-2H3 cells using either Brij-96 (left panels) or Triton X-100 (right panels). Post-nuclear lysates were fractionated by ultracentrifugation on a sucrose gradient and fractions containing lipid rafts (as indicated by raft markers, see Figures 3 and 4) were pooled and dialysed against Hepes-buffered saline. High-density fractions were treated in exactly the same way as a negative control. All samples were negatively stained with uranyl acetate.

ever, for immunoprecipitation with anti-Lyn, only a fraction of the Thy-1 is expected to be recovered with Lyn, since Thy-1 is in excess when compared with Lyn.

Results from such an experiment are presented in Figures 9(B) and 9(C). Vesicles isolated using either Brij-96 or Triton X-100 were immunoprecipitated using anti-Thy-1 antibody, and immunocomplexes were then analysed for the presence of Thy-1 and Lyn proteins (Figure 9B). It is noticeable that the amount of Thy-1 in the native (unlysed) Triton X-100 rafts (Figure 9B, upper panel, lane 3) is severalfold lower than the amount of Thy-1 detected in native Brij-96 isolated rafts (Figure 9B, upper panel, lane 1). However, if the raft vesicles are lysed using RIPA buffer and then immunoprecipitation of Thy-1 is performed, the levels of Thy-1 recovered are the same, regardless of the detergent used for vesicle isolation (cf. Figure 9B, upper panel, lanes 2 and 4). The

Figure 7 Size of the lipid-raft vesicle populations isolated using Brij-96 and Triton X-100

DRM fractions containing lipid rafts prepared with either Brij-96 (A) or Triton X-100 (B) and isolated using sucrose-density-gradient ultracentrifugation were pooled, dialysed extensively, and subjected to DLS analysis. Scattering intensity data were used to calculate the number distribution profile of the total vesicle population. (A) ●, fractions 2–5 were pooled as the Brij-96 raft fractions, and ○, fractions 11–13 were pooled as representative high-density non-raft (soluble) fractions (see Figures 3 and 4). The samples contained equivalent amounts of protein in each case. (B) Fractions 5–7 were pooled as the Triton X-100 raft fractions (see Figures 3 and 4). The number of vesicles with different diameters present is expressed as a percentage of the total vesicle population.

Figure 8 Phosphorylation of Lyn in lipid rafts isolated using Brij-96

Lipid-raft fractions were isolated using Brij-96, followed by sucrose-gradient ultracentrifugation, dialysis and lysis in RIPA buffer. Each treated fraction was subjected to IP (immunoprecipitation) with anti-Lyn, followed by immunoblotting with either anti-phosphotyrosine antibody (PY-20, upper panel) or anti-Lyn (lower panel), followed by ECL[®] detection. WCE, whole cell extract.

increase in the amount of Thy-1 immunoprecipitated from lysed Triton X-100 rafts (Figure 9B, upper panel, lane 4) compared with native Triton X-100 rafts (Figure 9B, upper panel, lane 3) is significant. This large difference is not observed for Brij-96-isolated raft vesicles (Figure 9B, upper panel, lanes 1 and 2). These differences can be explained by the sidedness of the vesicles. We propose that for rafts isolated using Triton X-100, the plasma membrane inverts, so that only a small fraction of the vesicles have Thy-1 located on the outside (right-side-out orientation), whereas the bulk of vesicles have Thy-1 located on the inside (inside-out orientation). As a result, when Thy-1 is immunoprecipitated from native Triton X-100 raft vesicles, the level of Thy-1 is quite low, since under these conditions, the inside-out vesicles do not immunoprecipitate. However, when the vesicles are lysed, anti-Thy-1 antibody can immunoprecipitate Thy-1 from the right-side-out, as well as from the inside-out, vesicles, since all the Thy-1 molecules are now accessible. The Brij-96 raft vesicles appear to

be primarily right-side-out, since Thy-1 is largely accessible to anti-Thy-1 antibody in the native (unlysed state).

It was interesting to determine the results when rafts are immunoprecipitated using anti-Lyn antibody. The anti-Lyn immunocomplexes from Triton X-100 native raft vesicles contained a high level of Lyn, and also a high level of Thy-1 (Figure 9C, upper and lower panels, lane 3), 2–3-fold greater than the amount detected in immunocomplexes when anti-Thy-1 antibody is used. This is in contrast with the Brij-96 native raft vesicles (Figure 9C, upper and lower panels, lane 1), where little Thy-1 is detected. Again, these results can be explained if the orientation of Triton X-100 rafts is inside-out, and that of the Brij-96 vesicles is right-side-out. The amount of Thy-1 in the anti-Lyn immunocomplexes formed from the inside-out Triton X-100 vesicles (Figure 9C, upper panel, lane 3) makes up the difference between the Thy-1 in the native (inside-out) vesicle population (Figure 9B, upper panel, lane 3) and the total Thy-1 in the lysed vesicles (Figure 9B, upper panel, lane 4). When Brij-96 raft vesicles were lysed, the amount of immunoprecipitated Lyn increased compared with non-lysed vesicles (cf. lanes 2 and 1 in Figure 9C, lower panel), which is expected for right-side-out vesicles. The low level of detection of both proteins in the Triton X-100 raft vesicles after lysis with RIPA buffer may arise from the combined effects of Triton X-100, Nonidet P40 and SDS on immunoreactivity of the anti-Lyn antibody with its epitope. In contrast, the interaction of anti-Thy-1 antibody with its epitope appears more robust, and it behaved consistently in the presence of RIPA buffer with both Triton X-100 and Brij-96 rafts.

DISCUSSION

Treatment of intact cells with cold non-ionic detergents leads to solubilization of many cellular proteins; however, regions that were originally present as raft microdomains appear to coalesce to form DRMs. In the present study, we have compared the DRMs resulting from two detergents with different chemical structures (see Figure 2); Triton X-100 (which has traditionally been used for such extractions) and Brij-96. We show that detergent-resistant DRMs can be isolated by either gel-filtration chromatography, which relies on the relatively large size of these structures, or sucrose-density-gradient sedimentation, which separates the DRMs from the remaining cellular material based on their low buoyant density. Raft markers used in the present study include the GPI-anchored protein, Thy-1, located exclusively on the extracellular leaflet of the membrane, the glycosphingolipid G_{M1} ganglioside, also located on the extracellular leaflet, and the non-receptor tyrosine kinase, Lyn, situated exclusively on the cytoplasmic leaflet. All these membrane components have been shown previously to associate with lipid rafts. For Triton X-100, the pattern of raft marker protein distribution is somewhat different when the DRM preparation is fractionated by gel-filtration chromatography (which separates by Stokes radius) compared with sucrose-density-gradient sedimentation (which separates by buoyant density). Specifically, the bulk of the Thy-1 elutes from Sepharose 4B in the early fractions (i.e. large vesicular structures), whereas most of the Lyn elutes in the later fractions (small vesicles and soluble complexes). When the DRMs are fractionated using a density gradient, more Thy-1 appears in the low-density non-raft fractions, whereas a greater fraction of the Lyn protein appears in the high-density raft fractions. The use of total cell lysates, which were not precleared by centrifugation for isolation of either Triton X-100 or Brij-96 rafts, did not substantially alter the pattern of protein distribution down the sucrose gradient, indicating that loss of Lyn during the centrifugation step [36] is not responsible

Figure 9 Immunoprecipitation of lipid-raft vesicles with right-side-out orientation

(A) Expected outcome in the IP (immunoprecipitation) analysis of native (non-lysed) or lysed lipid-raft vesicles for right-side-out vesicle orientation. The number of + in the Table indicates the expected relative abundance of the two raft marker proteins Thy-1 and Lyn. The expected absence of a protein is indicated by —. For IP performed with anti-Thy-1, the bulk of the Lyn is expected to be recovered with Thy-1, since Lyn is localized primarily within the lipid rafts, whereas for IP with Lyn, only a fraction of the Thy-1 is expected to be recovered with Lyn, since the amount of surface Thy-1 is in large excess over Lyn. (B, C) Cells were lysed in either Brij-96 or TX-100 (Triton X-100). Post-nuclear lysate was fractionated by ultracentrifugation on a sucrose-density gradient, and fractions containing lipid rafts were pooled and dialysed against TBS. After dialysis each sample was divided into two: one half was kept as native vesicles, whereas the other half was lysed in RIPA buffer. Immunoprecipitation was subsequently performed using either (B) anti-Thy-1 (OX-7) or (C) anti-Lyn, and immunocomplexes were collected using Protein G-agarose beads. Bound proteins were separated by SDS/PAGE and analysed by Western-immunoblotting analysis using either OX-7 or anti-Lyn, followed by ECL[®] detection.

for the observed differences between the two detergents. The detergent/lipid ratio did, however, appear to alter the pattern of protein distribution, which suggests that more attention should be paid to standardizing and controlling this parameter in studies of lipid rafts, as suggested by Edidin [3].

The use of non-ionic detergent appears to be important for isolation of lipid-raft fractions from RBL-2H3 cells. In the present study, fractionation of plasma-membrane vesicles isolated from cells under detergent-free conditions resulted in no apparent separation into high- and low-density fractions, but instead, a continuum of raft marker distribution down the gradient was observed. In contrast, Luria et al. [38] reported that the detergent-free fractionation by sucrose-density-gradient sedimentation of plasma membrane from *Xenopus laevis* produced a heavy fraction, and a light fraction resembling lipid rafts. Fractionation of mouse brain synaptosomes in the absence of detergent also produced a low-density fraction, but this contained much lower levels of raft markers when compared with the equivalent fractions prepared using the detergent [39]. The role of the detergent is probably to promote coalescence of smaller preexisting lipid rafts into larger structures (see below) [40], which can then bud off to form bilayer vesicles.

In the present study, we present evidence that the lipid rafts isolated from RBL-2H3 cells using either Brij-96 or Triton X-100 form sealed, spherical unilamellar bilayer vesicles, as demonstrated by negative staining EM. The existence of relatively large vesicular structures is also in accordance with elution of the DRM fractions at the void volume of a Sepharose 4B gel-filtration column. For both Triton X-100 and Brij-96, vesicular structures were only observed in the pooled low-density lipid-raft fractions and not in the pooled high-density fractions. The diameter of the vesicles was in the range 115–240 nm as measured by light scattering. However, whether a single DRM vesicle represents a single raft microdomain or several microdomains coalesced together, is difficult to determine. The size of raft microdomains in intact cells is still a controversial issue. Depending on the techniques used, and the cell system under study, different values on a submicron scale have been reported. Immunofluorescence co-localization experiments performed on detergent-treated RBL-2H3 cells estimated the size of the putative raft domains containing both Thy-1 and Lyn to be approx. 100 nm [20], but these may have resulted

from detergent-induced coalescence of smaller structures. In C3H-10T1/10T2 cells, Thy-1 and G_{M1} were found to be transiently confined to regions of 230 nm average diameter as determined by single-particle tracking [41]. Another study using fluorescence energy transfer experiments in Chinese-hamster ovary cells transfected with a GPI-anchored folate receptor concluded that lipid-raft microdomains are considerably smaller, < 70 nm in diameter [42]. Pralle et al. [43] used a laser-trap approach to estimate that the average raft in BHK (baby hamster kidney)-21 cells transfected with GPI-anchored placental alkaline phosphatase had a diameter of 26 ± 13 nm, and a lifetime of many minutes. Translating the surface area of a microdomain on a flat surface to a spherical vesicle, the upper end of the size range estimate of 200 nm would be compatible with a single raft in the plasma membrane forming a single 100 nm DRM vesicle; however, more than 60 rafts of 25 nm diameter would be needed to form a 100 nm bilayer vesicle.

The restricted location of the raft markers allowed the sidedness of the raft/DRM vesicles to be explored. Even though both detergents yielded lipid-raft vesicles of similar size, the nature and orientation of the vesicles were different. When cells were extracted with Brij-96 and analysed using anti-Thy-1 antibody, the isolated lipid-raft vesicles behaved in a fashion consistent with a primarily right-side-out orientation, with Thy-1 located on the external leaflet (accessible to binding of antibody) and Lyn on the inward-facing leaflet. In contrast, Triton X-100 led to the isolation of a vesicle population, which appeared to be predominantly inside out, with a large fraction of Lyn being accessible to antibody on the external vesicle surface. It should be noted that the immunoreactivity of the two antibodies towards their epitopes may be affected differently by Triton X-100 treatment and the RIPA buffer (which contains both Nonidet P40 and SDS) used to lyse the vesicles, which may in turn influence the immunoprecipitation step and subsequent detection of the two proteins. Thus the results of the present study suggest, but do not prove, that Brij-96 raft vesicles are primarily right-side-out, whereas Triton X-100 raft vesicles are mainly inside out. Attempts to repeat the orientation experiment using raft fractions from total cell lysates rather than from precleared lysates were unsuccessful, owing to the crude nature of the former and the low levels of Lyn in these fractions.

It is not yet clear whether native lipid rafts in intact cells include both bilayer leaflets; however, liquid-ordered microdomains visualized in vesicular model systems certainly span the lipid bilayer [44]. It has been suggested that rafts isolated by detergent extraction are derived from selective extraction of the exofacial leaflet of the membrane [5]. However, the fact that the DRM fractions comprised of bilayer vesicles with a defined orientation argues against this. It seems more probable that the detergent induces coalescence and aggregation of small rafts to form larger aggregates or 'barges' [45], which can then pinch off and seal to form bilayer vesicles of right-side-out orientation, or, if membrane inversion takes place, inside-out orientation. Indeed, recent *in vitro* studies using Triton X-100 with lipid bilayers comprising raft lipids concluded that partitioning of this detergent into the membrane bilayer could drive liquid-ordered domains to grow in size and number under certain conditions, especially low temperatures [40]. The chemical nature of the detergent may direct the extent of membrane inversion during vesicle formation, possibly by modulating the size and curvature of the aggregated raft domains, so that different sidedness is observed for DRM vesicles isolated using different detergents.

The DRM fractions produced by Triton X-100 and Brij-96 also differed in their physicochemical characteristics. The lipid-raft fractions isolated using the two different detergents displayed differences in their density properties: those isolated using Brij-96 were consistently of lower buoyant density. Other researchers reported different density behaviour of Thy-1 membrane microdomains isolated from RBL-2H3 cells when different detergents were used [46]. The results presented in Figure 3 also demonstrate that Brij-96 rafts were substantially more enriched in the raft markers, Thy-1, Lyn and Yes kinase, when compared with Triton X-100 lipid-raft fractions. The Brij-96 raft fractions also showed a much 'tighter' distribution of the raft proteins, Thy-1, Lyn and Yes, suggesting that this detergent is a better choice when compared with Triton X-100 for isolation of rafts from RBL-2H3 cells. Use of Triton X-100 led to the appearance of substantial portions of Thy-1, Lyn and Yes in the non-raft fractions, and a bimodal distribution for lipid-raft markers, ganglioside G_{M1} and cholesterol. A bimodal distribution of G_{M1} was also reported by Blank et al. [27] for extraction of Jurkat cells using 1% Triton X-100. The Triton X-100 raft fractions also contained much lower amounts of protein than those isolated using Brij-96. Recently, Schuck et al. [47] investigated the use of several different detergents to isolate DRM fractions from MDCK (Madin–Darby canine kidney) cells. On the basis of their results, they proposed that the detergents differed in their 'selectivity' for extracting lipid rafts. Highly selective (strong) detergents, such as Triton X-100, isolated DRM fractions with a lower protein content by disrupting lipid–protein interactions in the rafts to a greater extent when compared with poorly selective (weaker) detergents, such as Tween 20, which isolated DRM fractions with a much higher protein content. The selectivity of different detergents appeared to vary with cell type: Brij-96 appeared to have good selectivity (although not as good as Triton X-100) in MDCK cells, but was poorly selective in Jurkat cells. Our results are in general agreement with theirs: rafts isolated with Brij-96 had a higher protein content when compared with those isolated using Triton X-100, suggesting that it is a less 'selective' detergent than Triton X-100 for RBL-2H3 cell rafts.

Schuck et al. [47] suggested that, in general, the use of a very strong detergent is preferable for raft isolation. Although Brij-96 appears to be a less selective detergent when compared with Triton X-100 in RBL-2H3 cells, it appears to preserve better the lipid–protein interactions of the raft structure. The fact that all of the cellular Thy-1 is found associated with Brij-96 rafts (none

is present in the soluble fractions) suggests that this detergent isolates the entire lipid-raft pool in RBL-2H3 cells. Our results suggest that the use of too strong a detergent, such as Triton X-100, results in loss of raft constituents into the soluble fractions, giving a false impression of the level of association of these components with microdomains. Madore et al. [30] reported that Triton X-100 was unsuitable for isolation of rafts from neuronal cells, since it promoted intermingling of non-raft protein components with DRMs. Thus the optimal detergent for isolation of rafts may have to be worked out empirically for each cell line and tissue type.

The fact that Brij-96 and Triton X-100 appear to isolate lipid-raft microdomains with different characteristics can also be explained if lipid rafts are viewed, not as homogeneous structures, but rather as a heterogeneous collection of microdomains with slightly different biophysical and biochemical characteristics, while nevertheless sharing common properties associated with rafts. Thus Brij-96 and Triton X-100 may be isolating different subsets of rafts that normally co-exist in the plasma membrane. Since rafts are known to be highly dynamic structures, these domain subsets are probably constantly exchanging components with each other. Lipid rafts are known to be associated with the actin cytoskeleton in many cell types, so it is also possible that the two detergents differentially disrupt cytoskeleton–raft interactions to generate discrete subsets of rafts. There is considerable evidence for the existence of distinct subsets of rafts in membranes of several different cell types. Taylor et al. [34] extracted myelin membrane with four different detergents (including Triton X-100 and Brij-96), and on the basis of the observed large difference in raft composition, proposed that detergents of different solubilization stringency isolate different subsets of rafts. This stringency appears to vary with the cell type being extracted. The epidermal growth factor receptor appears to be localized in non-caveolar rafts that are soluble in Triton X-100, but insoluble in Brij-58 [32], as was the HIV-1 viral core protein, Pr55^{gag} [48]. Prominin, a pentaspan integral protein, was found to be soluble in Triton X-100, but insoluble in Lubrol WX, and appeared to be located in a novel raft subpopulation that is targeted to the microvilli [49]. Distinct raft subdomains have also been reported for neuronal cells [30] and Chinese-hamster ovary cells [50]. These raft subsets may play important physiological roles; for example, initiation of signalling via liganding of the T-cell receptor appears to take place within distinct membrane raft domains [31]. Further study will be required to establish the mechanism(s) for stably maintaining these different raft subsets on the cell surface.

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