Detergent resistance as a tool in membrane research

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The biological membrane is a complicated matrix wherein different lipid environments are thought to exist. The more ordered or raft environment has been perceived biochemically accessible via its relative resistance to detergent. This paper outlines the protocols developed in our laboratory for the analysis of such detergent-resistant membranes (DRMs). We stress the fact that DRMs are artifactual in nature and should not be equivocated to lipid rafts, their usefulness being limited to assigning raft-association potential most convincingly when changes in DRM composition are induced by biochemically/physiologically relevant events. These protocols are completed in 1–2 d.

INTRODUCTION

Lipid rafts provide a means for cell membranes to form dynamic platforms within the bilayer that function in membrane trafficking, signal transduction and cell polarization[1]. They are currently defined as dynamic sterol–sphingolipid assemblies that associate and dissociate on a sub-second time scale[2,3]. Protein localization to rafts is primarily mediated by a glycosylphosphatidylinositol (GPI) anchor, by acylation or by certain transmembrane domains[4]. The metastable raft resting state can be activated to coalesce into larger and more stable raft domains by selective protein–protein and protein–lipid interactions[5]. Raft clustering can also lead to inclusion of proteins partitioning only weakly to rafts, for example, by scaffolding or by oligomerization[5–7]. Rafts in the clustered state are thought to constitute a liquid-ordered phase wherein lipid acyl chains are longer and more saturated. In combination with tight cholesterol interdigitation, this results in a more condensed assemblage in a phase that is separate from the surrounding liquid-disordered unsaturated glycerophospholipids[7,8].

Fluorescence recovery after bleaching and antibody crosslinking experiments indicate that the raft phase can become dominating when the mole fraction of sphingolipids and cholesterol increase, as is the case in the apical membrane of epithelial cells[9]. In any event, recent evidence suggests that large-scale phase separation can be induced when the cell membrane is released from the underlying cytoskeletal constraint and left undisturbed by exocytic/endocytic events[10].

Detergent-resistant membranes

Brown and Rose[11] suggested 'detergent-insoluble microdomain' as a working description of lipid rafts when they observed that sphingolipids and GPI-anchored proteins were insoluble in Triton X-100 (TX100) at 4 °C and floated to a characteristic density following equilibrium density gradient centrifugation. Coupled with the observation that this detergent insolubility was cholesterol related[12], these preparations (broadly defined as detergent-resistant membranes or DRMs) quickly became the ruling method for assigning lipid and protein raft affinity. However, it was clear from the start[13] that DRMs do not reflect membrane organization at steady state and therefore cannot be directly equated with lipid rafts[14,15].

Solubilization by detergent

When detergent is applied in increasing amounts to membranes, solubilization occurs by three successive stages[16]: detergent incorporation, lamellar–micellar phase transition (formation of mixed micelles) and total solubilization (separation of protein and lipid mixed micelles). The insoluble residue, remaining after progression through these events, is enriched in sphingolipids, cholesterol and raft proteins. The lower density and larger size of these DRMs allow for their separation from the remaining solubilized membrane components via equilibrium density gradient centrifugation (or 'flotation'). Localization to the DRM fraction in itself cannot, however, be an accurate criterion for assigning raft residency; DRMs are the product of an artificial aggregation process in which the chemicophysical parameters are neither fully understood nor accounted for.

While there is evidence from model membranes that lipids in the liquid-ordered phase resist incorporation of detergent into the membrane[17–20], there is also evidence that detergent itself can induce phase separation in uniform lipid mixtures[21,22]. Furthermore, using the standard TX100 preparation, DRMs from animal cells can be obtained at 4 °C but not at 37 °C, indicating that these Triton-insoluble complexes are not isolations of pre-existing membrane structures[15]. The solubilization of lipids and proteins of the liquid-disordered state by the detergent leads to artifactual coalescence of raft constituents into DRMs. Less efficient nonionic detergents, such as the Brij series, Lubrol WX and Tween-20, do yield distinct, less temperature-sensitive and less cholesterol-dependent DRM preparations, leading to the hypothesis that these detergents are able to isolate different subsets of rafts[23–26]. However, what these preparations reflect at the membrane level is not clear. There are a number of concerns; chiefly, the discrimination is based on detergent efficiency. Weaker detergents solubilize less effectively and therefore less selectively[27] meaning that apparent membrane resistance can be due merely to inadequate membrane solubilization[15]. Similarly, decreasing the concentration of an efficient detergent like TX100 decreases its selectivity and increases the proportion of DRM components[28]. Such a result is not evidence for a sub-raft population, but rather the consequence of a manipulation of the effective detergent/cellular lipid/protein mole ratio required for solubilization (this parameter rather than detergent concentration alone always needs to be taken into account[29]; for TX100 a mass ratio of greater than 5:1 is necessary for maximum solubilization[30]). On the other hand, efficient solubilization may disrupt weak interactions of proteins with raft domains[31]. In any event, DRM compositional differences are cell type dependent[27], which means that general rules for comparison cannot be drawn.

Usefulness

Despite the shortcomings of DRMs, it is important to realize that they remain the only biochemical means of assessing potential raft affinity. Lichtenberg *et al.*[14] wrote, "DRM extraction characterizes the properties of a molecule in terms of its differential affinity towards various (artificial) environments". It is possible to work within the confines of this artifactual system via an analysis of 'paired' differences. Detergent solubility/insolubility are not strict criteria in themselves; however, many of the major breakthroughs in the raft field originated from the observations of changes in DRM association upon induction of physiologically relevant stimuli. Paramount is the raft connection to transmembrane signaling; a concept largely spear-headed by a DRM-based analysis of the allergic immune response. Upon binding its multivalent ligand IgE, the crosslinked FcεRI receptor *becomes* TX100 insoluble along with its downstream effector Lyn kinase[32]. While subsequently supported as a raft-dependent phenomenon[33–35], it was the initial DRM observation that laid the conceptual framework for lipid-based amplification of protein-mediated signal transduction. Such analyses of DRM partitioning changes have served as major work-horses for the evaluation of many raft-related phenomena, including assessing roles in disease pathogenesis[36], membrane trafficking[11] and identifying 'raftophilic' peptide moieties[15].

Experimental design

The protocols described herein were developed by Schuck *et al.*[27,29]. To perform the DRM analysis, two alternative approaches are covered in this protocol. The OptiPrep gradient analysis approach, detailed in **Box 1**, provides the most rapid and convenient assessment. However, the sucrose gradient analysis, detailed in the main body of the PROCEDURE, should be favored for use in lipidomic mass spectrometric studies. Iodixanol can remain with lipids after extraction from DRMs[29], thus adversely affecting the reliability and precision of mass spectrometry data. The choice of a step versus linear gradient is largely one of personal preference. The flotation behavior of proteins and lipids is better resolved on a linear gradient; however, this does not necessarily translate to increased information when monitoring for changes in DRM partitioning.

DRM analyses often involve the investigation of ectopically expressed proteins. In such studies, it is important to consider that detergent resistance is conditional on the membrane composition in which the protein resides. This was first demonstrated by Brown and Rose[11], when they showed that the DRM resistance of GPI-anchored proteins was *acquired* upon transport to the Golgi complex during biosynthetic trafficking. Since DRM association is not typically seen for raft proteins in the endoplasmic reticulum (ER), the trafficking behavior of ectopically expressed proteins must be accounted for before DRM analysis. If not checked microscopically, failure to traffic from the ER may be mistaken for apparent detergent solubility.

It has been shown that incorporation of detergent into the density gradient enables isolation of DRMs devoid of weakly associated proteins[37]. While this provides a more stringent test of DRM association, we feel that this stringency does not increase the usefulness of DRMs, that is, the capacity to detect changes in DRM partitioning upon induction of a physiologically relevant event. If anything, it could weaken such a capacity since more efficient solubilization may disrupt weak interactions of proteins with raft domains[31].

BOX 1 | DETERGENT-RESISTANT MEMBRANE ANALYSIS WITH THE OPTIPREP GRADIENT SYSTEM

MATERIALS

REAGENTS

- Minimal essential medium (MEM) with Earle's salts (Invitrogen, cat. no. 21090-022)
- Gln (Invitrogen, cat. no. 25030-024)
- Penicillin–streptomycin (Invitrogen, cat. no. 15140-122)
- FCS (PAA Laboratories, cat. no. A15-042)
- 35-mm plastic tissue dish (Nunc, cat. no. 153066)
- Chymostatin (Sigma, cat. no. C7268)
- Leupeptin (Sigma, cat. no. L2884)
- Antipain (Sigma, cat. no. A6191)
- Pepstatin (Sigma, cat. no. P5318)
- Methyl-β-cyclodextrin (Sigma, cat. no. C4555)
- OptiPrep [60% (wt/vol) iodixanol; Progen Biotechnik, cat. no. 1030061]
- 10% (wt/vol) Triton X-100 (Surfact-Amps X-100; Pierce, cat. no. 28314)
- Antibodies (see **Table 1**).

EQUIPMENT

- Beckman Optima MAX ultracentrifuge
- Beckman TLS55 rotor
- Ultraclear centrifuge tubes for a Beckman TLS55 rotor (11 × 34 mm^2; Beckman, cat. no. 347356)
- 1.5-ml microfuge tubes
- Cell scrapers
- 25-gauge (25-G) Needles
- 1-ml plastic syringes

BOX 1 | DETERGENT-RESISTANT MEMBRANE ANALYSIS WITH THE OPTIPREP GRADIENT SYSTEM (CONTINUED)

1. Grow MDCK cells (strain II) to confluency (MEM, 5% FCS/2 mM Gln/100 U/ml penicillin/streptomycin) in 3.5-cm plastic tissue dishes.
2. Wash once with 1 ml PBS and once with 1 ml TNE. Scrape cells in 1 ml TNE and centrifuge at 380*g* for 5 min at 4 °C.
▲ CRITICAL STEP: Unless stated otherwise, all extraction and subsequent isolation steps are rigorously maintained at 4 °C. This is best implemented in a refrigerated room.
3. Resuspend cell pellet in 200 µl 1× TNE/CLAP (chymostatin, leupeptin, aprotinin, and pepstatin) and homogenize with a 25-G needle (25 strokes).
? TROUBLESHOOTING
Problem: CLAP stock is solidified.
Possible reason: DMSO freezes at 4 °C.
Solution: Allow a CLAP aliquot from the stock to warm up to room temperature.
4. (Optional) Cholesterol extraction of cell homogenate. Detergent-resistant membrane (DRM) flotation is typically cholesterol dependent. Even though cholesterol-dependent flotation does not define raft association in itself, it sheds light on the contribution of lipids in relation to the conformation of detergent resistance. To extract cholesterol, incubate 180 µl of the cell homogenate with 20 µl of 100 mM methyl-β-cyclodextrin (in TNE) at 37 °C for 30 min. Then cool the sample to 4 °C and add 23 µl of 10% Triton X-100 (TX100) + 7 µl TNE and incubate for 30 min.
▲ CRITICAL STEP: Do not overhandle sample during detergent extraction, as this can lead to changes in sample temperature.
? TROUBLESHOOTING
Problem: A large insoluble flocculent material appears during detergent extraction.
Possible reason: Sample is overloaded and/or nuclei have been damaged during homogenization.
Solution: Homogenization treatment should break greater than 90% of cells without damaging their nuclei (this can be checked microscopically). Ignore and continue with protocol or pellet the flocculent precipitate by centrifugation 10,000*g* at 4 °C and continue with the postnuclear supernatant. Both solutions should yield the same result.
5. (Optional) Adjust the buffer concentration to 40% (w/v) iodixanol by adding 460 µl OptiPrep (60% iodixanol). Take 600 µl of the resulting mixture and continue to Step 8.
6. If optional Steps 4 and 5 have not been implemented, transfer 180 µl of the cell homogenate to a new microfuge tube and add 20 µl of 10× detergent (e.g., 10% TX100). Gently invert the sample to mix and incubate for 30 min.
▲ CRITICAL STEP: Do not overhandle sample during detergent extraction, as this can lead to changes in sample temperature.
? TROUBLESHOOTING (See ? TROUBLESHOOTING details listed in Step 4.)
7. Adjust sample composition to 40% iodixanol (wt/vol) by mixing with 400 µl OptiPrep (60% iodixanol).
8. Transfer the 600 µl mixture from Step 7 (or optional Step 5) to a TLS55 centrifuge tube and overlay it with 1.2 ml of 30% iodixanol and then 0.2 ml TNE. Centrifuge at 55,000 r.p.m. (259,000*g*) with a TLS55 rotor for 2 h.
9. Separate into two 1-ml fractions. The upper fraction contains the DRMs. See **Table 1** for DRM marker screening.
? TROUBLESHOOTING
Problem: DRM markers are not floating.
Possible reason: DRM isolation is a procedure with inherent variability in terms of results.
Solution: Maintain 4 °C throughout detergent extraction and flotation (Steps 3–8 in this box); minimize handling of samples during detergent extraction (Step 4 and/or Step 6 in this box); use fresh highest available grade TX100.
10. It is crucial that DRMs are well separated from detergent molecules and solubilized lipids. The separation between DRMs and detergent micelles is based largely on differences in size[29], and should be well separated under the centrifugation conditions presented. The distribution of TX100 in the gradient can be measured by its absorption at 275 nm (absorbance for a 1% TX100 solution, $E^{1\%}_{1cm} = 21$)[39]; thus it has a percent extinction coefficient ($\varepsilon_{percent}$) of 21 (g/100 ml)$^{-1}$ cm^{-1}. While proteins do absorb at 280 nm [e.g., for a 1% BSA solution $\varepsilon_{percent} = 6.6$ (g per 100 ml^{-1} cm^{-1})][40], their concentration in the gradient will be relatively minor in comparison with detergent. Consequently, absorption at 275 nm will mostly reflect the distribution of TX100, which should peak in the soluble fractions. For studies using detergents other than TX100, their distribution can be determined semiquantitatively by thin layer chromatography.
11. (Optional) If gradient fractions prove too dilute for subsequent analysis, DRMs can be concentrated. For this purpose, dilute 1:4 (in TNE) and transfer to a new SW40 centrifuge tube. Centrifuge for 2 h at 24,000 r.p.m. (100,000*g*) with an SW40 rotor; DRMs can be recovered from the pellet.
● TIMING
Step 1, time required for cell confluency; Step 2, 15 min; Step 3, 30 min; Steps 4 and 5 (optional), 1 h 20 min; Step 6, 30 min; Step 7, 5 min; Step 8, 15 min + 2 h (centrifugation); Step 9, 5 min; Step 10, 30 min; Step 11 (optional), 2 h

A common feature of all DRM analyses is variation in results, even between replicates of the same experiment. This variability appears inherent to this type of membrane analysis, underscoring yet another example of weakness in the DRM approach. Questioned as a tool as early as 1995 (ref. 13), we must reiterate this fundamental point: sterol/sphingolipid-dependent insolubility in itself is only a starting point for analyzing raft association; DRMs are most useful in assessing raft potential when differences in DRM composition are induced by a meaningful event. For example, a weak DRM association does not provide much information with regard to raft affinity. However, if this protein was further drawn into the DRM fraction by a biological phenomenon, then an enhanced preference to lipid rafts could be assigned. In this light, we recommend using the more convenient step gradients as an initial screen, following which time repeatable differences can be examined in more detail using the more sensitive linear gradient.

MATERIALS

REAGENTS

- Madin–Darby canine kidney epithelial cells (MDCK, strain II)[38]
- Potassium phosphate monobasic (KH_2PO_4; Sigma, cat. no. P5655)
- Sodium phosphate dibasic heptahydrate (Na_2HPO_4 $7H_2O$; Sigma, cat. no. 431478)
- EDTA (Sigma, cat. no. 46081) (see REAGENT SETUP)
- Hydrochloric acid (HCl; Sigma, cat. no. H1758)
- Sodium hydroxide (NaOH; Sigma, S8045)
- Minimal essential medium (MEM) with Earle's salts (Invitrogen, cat. no. 21090-022)
- Gln (Invitrogen, cat. no. 25030-024)
- Penicillin–streptomycin (Invitrogen, cat. no. 15140-122)
- Chymostatin (Sigma, cat. no. C7268)
- Leupeptin (Sigma, cat. no. L2884)
- Antipain (Sigma, cat. no. A6191)
- Pepstatin (Sigma, cat. no. P5318)
- Methyl-β-cyclodextrin (Sigma, cat. no. C4555) (see REAGENT SETUP)
- OptiPrep [60% (wt/vol) iodixanol; Progen Biotechnik, cat. no. 1030061]
- Sucrose (USB, cat. no. 21938) (see REAGENT SETUP)
- 10% (wt/vol) TX100 (Surfact-Amps X-100; Pierce, cat. no. 28314)
- Antibodies (see **Table 1**)
- PBS (see REAGENT SETUP)
- TNE (see REAGENT SETUP)
- CLAP (chymostatin, leupeptin, aprotinin, and pepstatin) (see REAGENT SETUP)
- Iodixanol (see REAGENT SETUP)

EQUIPMENT

- Beckman SW40 Ti rotor
- Ultraclear centrifuge tubes for a Beckman SW40 Ti rotor (14 × 95 mm^2; Beckman, cat. no. 344060)
- Beckman TLS55 rotor
- Ultraclear centrifuge tubes for a Beckman TLS55 rotor (11 × 34 mm^2; Beckman, cat. no. 347356)
- Beckman Optima XL-100K ultracentrifuge (Beckman)
- Beckman Optima MAX ultracentrifuge (Beckman)
- Gradient maker
- 1.5-ml Microfuge tubes
- 100-mm Plastic tissue dish (Nunc, cat. no. 150350)
- 35-mm Plastic tissue dish (Nunc, cat. no. 153066)
- Cell scrapers
- 25-gauge (25-G) Needles
- 1-ml Plastic syringes

REAGENT SETUP

PBS (155 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM Na_2HPO_4, pH 7.2) Dissolve 0.21 g KH_2PO_4, 0.73 g $Na_2HPO_4 \cdot 7H_2O$ and 9 g NaCl in double-distilled water and bring to 1 l.

EDTA stock (1 M EDTA) Dissolve 372.2 g EDTA in double-distilled water, adjust to pH 8 with NaOH and bring to 1 l.

5× TNE (750 mM NaCl, 10 mM EDTA, 250 mM Tris–HCl, pH 7.4) Dissolve 43.8 g NaCl and 30.3 g Tris in double-distilled water, add 10 ml of 1 M EDTA stock, adjust to pH 7.4 with HCl and bring to 1 l.

1,000× CLAP Dissolve 25 mg each of chymostatin, leupeptin, antipain and pepstatin in 1 ml DMSO; store at −20 °C.

56% Sucrose (wt/wt) Dissolve 70.75 g sucrose/100 ml 1× TNE; refractive index 1.4329.

35% Sucrose (wt/wt) Dissolve 40.29 g sucrose/100 ml 1× TNE; refractive index 1.3902.

5% Sucrose (wt/wt) Dissolve 5.1 g sucrose/100 ml 1× TNE; refractive index 1.3403.

30% Iodixanol Dilute OptiPrep 1:2; final buffer concentration is 1× TNE.

Methyl-β-cyclodextrin in TNE stock (100 mM) Just before use, dissolve 15 mg methyl-β-cyclodextrin in 100 µl 1× TNE.

PROCEDURE

Cell culture ● TIMING Time required for cells to reach confluency + 15 min to collect cells

1| Grow cells (MDCK II strain) to confluency (MEM, 5% FCS/2 mM Gln/100 U/ml penicillin/streptomycin) in a 10-cm tissue culture plate (approximately 1.5 mg protein).

2| Wash once with 1.5 ml PBS and once with 1.5 ml TNE. Scrape cells in 1.5 ml TNE and centrifuge at 380*g* for 5 min at 4 °C. ▲ CRITICAL STEP Unless stated otherwise, all extraction and subsequent isolation steps are rigorously maintained at 4 °C. This is best implemented in a refrigerated room.

Cell homogenization and detergent extraction

3| In this step two options are presented. The investigator can decide between isolation of unmodified DRM material (option A) or DRMs arising from a cellular homogenate, which has been cholesterol depleted (option B). DRM flotation is typically cholesterol-dependent. Even though cholesterol-dependent flotation does not define raft association in itself, it sheds light on the contribution of lipids in relation to the conformation of detergent resistance.

TABLE 1 | Markers used in the analysis of mammalian detergent-resistant membranes (standard Triton X-100 preparation).

Relation to detergent-resistant membrane (DRM)	Antigen	Antibody name	Source
Strongly DRM associated	Caveolin-1	Rabbit anti-human caveolin-1	Santa Cruz
	Annexin II	Mouse anti-annexin II	BD Biosciences Pharmingen
	Placental alkaline phosphatase (PLAP)	Mouse anti-human PLAP	Dako
	Flotillin-1	Mouse anti-flotillin-1	BD Biosciences Pharmingen
Predominantly not DRM associated	Transferrin receptor	Mouse anti-human transferrin receptor	Zymed
	Calnexin	Mouse anti-human calnexin	BD Transduction laboratories
	Gp135 (podocalyxin)	Mouse anti-podocalyxin	R&D Systems

(A) Standard detergent extraction ● TIMING 1 h

(i) Resuspend pellet in 550 µl TNE supplemented with CLAP (dilute stock 1:1,000) and shear the cells with a 25-G needle (25 strokes).

? TROUBLESHOOTING

(ii) Transfer 500 µl of homogenate to a new tube and add 500 µl of 1× TNE/CLAP containing 2× detergent [e.g., 2% (wt/vol) TX100]. Gently invert the sample to mix and incubate for 30 min. Continue to Step 4.

▲ CRITICAL STEP Do not overhandle sample during detergent extraction, as this can lead to changes in sample temperature.

? TROUBLESHOOTING

(B) Detergent extraction after cholesterol depletion of cell homogenate ● TIMING 1 h 20 min

(i) Resuspend pellet in 500 µl TNE supplemented with CLAP (dilute stock 1:1,000) and shear the cells with a 25-G needle (25 strokes).

? TROUBLESHOOTING

(ii) To extract cholesterol, incubate 450 µl cell homogenate with 50 µl of 100 mM methyl-β-cyclodextrin (in TNE) at 37 °C for 30 min.

(iii) Cool the sample to 4 °C and add 500 µl of 2% TX100 (in 1× TNE/CLAP) and incubate for 30 min. Continue to Step 4.

▲ CRITICAL STEP Do not overhandle sample during detergent extraction, as this can lead to changes in sample temperature.

? TROUBLESHOOTING

Flotation; DRM separation from soluble material

4| To achieve the separation of the DRMs from the solubilized material (flotation), one can employ either the step sucrose gradient approach (see option A below) or the linear sucrose gradient approach (option B). In order to make an informed choice between the two procedures, please be aware that the flotation behavior of proteins and lipids are better resolved on a linear versus step gradient, but the step gradient approach is easier and more straightforward to implement.

(A) Flotation with a step sucrose gradient ● TIMING 30 min + 18 h centrifugation + 5 min fractionation

(i) Adjust sample composition to 40% (wt/wt) sucrose by adding 2 ml 56% sucrose.

(ii) Transfer to a SW40 centrifuge tube and carefully overlay it with 8.5 ml of 35% sucrose and then 0.5 ml 5% sucrose.

■ PAUSE POINT Using the SW40 rotor centrifuge at 39,000 r.p.m. (271,000g) for 18 h.

(iii) Collect 2.5 ml from the top as the floating (DRM) fraction. See **Table 1** for DRM marker screening.

? TROUBLESHOOTING

(B) Flotation with a linear sucrose gradient ● TIMING 1 h + 18 h centrifugation + 20 min fractionation

(i) Adjust sample composition to 40% (wt/wt) sucrose by adding 2 ml of 56% sucrose, and transfer to SW40 centrifuge tube.

(ii) Using a gradient maker, overlay the sample with a 5–35% sucrose gradient (in TNE). This gradient is made by pumping 4.5 ml of 5% sucrose through 4.5 ml of 35% sucrose in the gradient maker mixing chamber.

■ PAUSE POINT Centrifuge at 39,000 r.p.m. (271,000*g*) for 18 h in a SW40 rotor.

? TROUBLESHOOTING

(iii) Draw in succession from the top layer of the gradient 12 1-ml fractions. Detergent-soluble material is found in fractions 9–12. See **Table 1** and **Figure 1** for DRM marker screening.

? TROUBLESHOOTING

Detergent micellar check ● TIMING 20 min

5| Check for location of detergent micelles in the gradient. It is crucial that DRMs are well separated from detergent molecules and solubilized lipids. The separation between DRMs and detergent micelles is based largely on differences in size[29], and should be well separated under the centrifugation conditions presented. The distribution of TX100 in the gradient can be measured by its absorption at 275 nm (absorbance for a 1% TX100 solution, $E^{1\%}_{1cm} = 21$)[39]; thus it has a percent extinction coefficient ($\varepsilon_{percent}$) of 21 (g/100 ml)$^{-1}$ cm^{-1}. While proteins do absorb at 280 nm [e.g., for a 1% BSA solution $\varepsilon_{percent} = 6.6$ (g/100 ml)$^{-1}$ cm^{-1}][40] their concentration in the gradient will be relatively minor in comparison with detergent. Consequently, absorption at 275 nm will mostly reflect the distribution of TX100, which should peak in the soluble fractions. For studies using detergents other than TX100, their distribution can be determined semiquantitatively by thin layer chromatography.

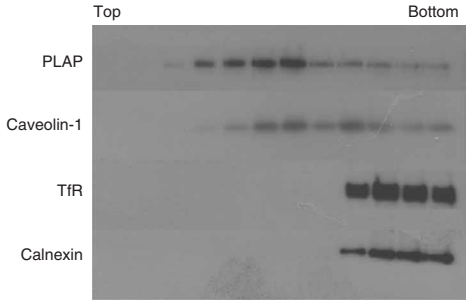

Figure 1 | Triton X-100 (TX100) detergent-resistant membranes (DRMs) isolated from MDCK II cell homogenates using a linear sucrose gradient. Detergent resistance is seen by the floating DRM markers, placental alkaline phosphatase (PLAP) and caveolin-1. Transferrin receptor (TfR) and calnexin are markers for the detergent-soluble material, and are restricted to the heavy-density fractions. (Data are reproduced from ref. 27).

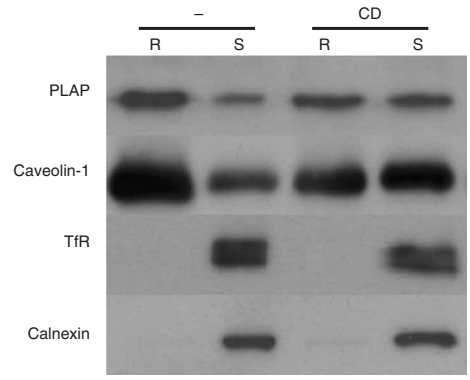

Figure 2 | Triton X-100 (TX100) detergent-resistant membranes (DRMs) isolated from methyl-β-cyclodextrin treated (CD) and unmodified (–) MDCK II cell homogenates using a single-step OptiPrep gradient. The DRM markers placental alkaline phosphatase (PLAP) and caveolin-1 are recovered predominately from the top [resistant (R)] fraction, while the detergent soluble (S) markers reside in the bottom fraction. (Data are from ref. 27 and Schuck, unpublished data.)

DRM concentration (optional) ● TIMING 2 h

6| If gradient fractions prove too dilute for subsequent analysis, DRMs can be concentrated. For this purpose, dilute 1:4 (in TNE) and transfer to a new SW40 centrifuge tube. Centrifuge for 2 h at 24,000 r.p.m. (100,000*g*) with an SW40 rotor; DRMs can be recovered from the pellet.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
3A(i) and/or 3B(i)	CLAP (chymostatin, leupeptin, aprotinin, and pepstatin) stock is solidified	DMSO freezes at 4 °C	Allow a CLAP aliquot from the stock to warm up to room temperature (18–23 °C)
3A(ii) and/or 3B(iii)	A large insoluble flocculent material appears during detergent extraction	Sample is overloaded and/or nuclei have been damaged during homogenization	Homogenization treatment should break greater than 90% of cells without damaging their nuclei (this can be checked microscopically). Ignore and continue with the protocol or pellet the flocculent precipitate by centrifugation 10,000*g* at 4 °C and continue with the postnuclear supernatant. Both solutions should yield the same result
4B(ii)	Sample in 40% sucrose disrupted upon overlaying with linear sucrose gradient (i.e., gradient is overmixed and ruined)	Force of gradient maker pump is too strong and therefore prevents a clean separation between sample and overlayed gradient	Layer the gradient first and then carefully underlay the sample in 40% sucrose using a Pasteur pipette
4A(iii) and 4B(iii)	Detergent-resistant membrane (DRM) markers are not floating	DRM isolation is a procedure with inherent variability in terms of results	Maintain 4 °C throughout detergent extraction and flotation (Steps 3 and 4); minimize handling of samples during detergent extraction [Step 3A(ii) and/or Step 3B(iii)]; use fresh highest available grade Triton X-100

ANTICIPATED RESULTS

DRM isolation is fairly reproducible with the protocols presented; however, failure of DRM lipids and proteins to float are not uncommon and are likely due to the variability inherent to this type of membrane analysis (see discussion under EXPERIMENTAL DESIGN). Markers for the detergent-resistant and soluble membrane fractions (**Table 1**) must therefore be run in parallel with all DRM experiments.

Successful DRM preparations, using both the sucrose and OptiPrep systems, are presented in **Figures 1** and **2**, respectively. With a linear sucrose gradient, we find that the soluble markers [calnexin and transferrin receptor (TfR)] are restricted to fractions 9–12 (from the top). By contrast, the DRM markers placental alkaline phosphatase (PLAP) and caveolin-1 are enriched in lighter fractions. A similar picture is seen in the OptiPrep system; PLAP and caveolin-1 are enriched in the top fraction, while TfR and calnexin reside in the bottom fraction. Enrichment in the DRM fraction is offset to some degree by methyl-β-cyclodextrin treatment. It should be noted that sensitivity to detergent is considerably lower in MDCK cells in comparison to other cell types, a phenomenon perhaps attributable to the extraordinary sturdiness of the apical membrane in polarized epithelia[27].

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1. Rajendran, L. & Simons, K. Lipid rafts and membrane dynamics. *J. Cell. Sci.* **118**, 1099–1102 (2005).

2. Pike, L.J. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J. Lipid Res.* **47**, 1597–1598 (2006).
3. Hancock, J.F. Lipid rafts: contentious only from simplistic standpoints. *Nat. Rev. Mol. Cell Biol.* **7**, 456–462 (2006).
4. Lucero, H.A. & Robbins, P.W. Lipid rafts-protein association and the regulation of protein activity. *Arch. Biochem. Biophys.* **426**, 208–224 (2004).
5. Meder, D. & Simons, K. Lipid rafts, caveolae, and membrane traffic. In *Lipid Rafts and Caveolae. From Membrane Biophysics to Cell Biology* (ed. Fielding, C.J.) 1.1–1.23 (Wiley-VGH Verlag GmbH & Co., Wienheim, Germany, 2006).
6. Schuck, S. & Simons, K. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J. Cell Sci.* **117**, 5955–5964 (2004).
7. Simons, K. & Vaz, W.L. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 269–295 (2004).
8. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
9. Meder, D., Moreno, M.J., Verkade, P., Vaz, W.L. & Simons, K. Phase coexistence and connectivity in the apical membrane of polarized epithelial cells. *Proc. Natl. Acad. Sci. USA* **103**, 329–334 (2006).
10. Baumgart, T. *et al.* Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci. USA* **104**, 3165–3170 (2007).
11. Brown, D.A. & Rose, K.J. Sorting of GPI-anchored proteins to glycolipid enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544 (1992).
12. Schroeder, R., London, E. & Brown, D. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. USA* **91**, 12130–12134 (1994).
13. Kurzchalia, T.V., Hartmann, E. & Dupree, P. Guilty by insolubility—does a protein's detergent insolubility reflect caveolar location? *Trends Cell Biol* **5**, 187–189 (1995).
14. Lichtenberg, D., Goñi, F.M. & Heerklotz, H. Detergent-resistant membranes should not be identified with membrane rafts. *Trends Biochem. Sci.* **30**, 430–436 (2005).
15. Brown, D.A. Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology* **21**, 430–439 (2006).
16. Helenius, A. & Simons, K. Solubilization of membranes by detergents. *Biochim. Biophys. Acta* **415**, 29–79 (1975).
17. Ahmed, S.N., Brown, D.A. & London, E. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered phase in model membranes. *Biochemistry* **36**, 10944–10953 (1997).
18. Sáez-Cirión, A. *et al.* Equilibrium and kinetic studies of the solubilization of phospholipid-cholesterol bilayers by C12E8. The influence of the lipid phase structure. *Langmuir* **16**, 1960–1968 (2000).
19. Xu, X. *et al.* Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. *J. Biol. Chem.* **276**, 33540–33546 (2001).
20. Dietrich, C. *et al.* Lipid rafts reconstituted in model membranes. *Biophys. J.* **80**, 1417–1428 (2001).
21. Heerklotz, H. Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* **83**, 2693–2701 (2002).
22. Heerklotz, H., Szadkowska, H., Anderson, T. & Seelig, J. The sensitivity of lipid domains to small perturbations demonstrated by the effect of Triton. *J. Mol. Biol.* **329**, 793–799 (2003).
23. Montixi, C. *et al.* Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* **17**, 5334–5348 (1998).
24. Röper, K., Corbeil, D. & Huttner, WB. Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. *Nat. Cell. Biol.* **2**, 582–592 (2000).
25. Radeva, G. & Sharom, F.J. Isolation and characterization of lipid rafts with different properties from RBL-2H3 (rat basophilic leukaemia) cells. *Biochem. J.* **380**, 219–230 (2004).
26. Alfalah, M. *et al.* A novel type of detergent-resistant membranes may contribute to an early protein sorting event in epithelial cells. *J. Biol. Chem.* **280**, 42636–42643 (2005).
27. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A. & Simons, K. Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. USA* **100**, 5795–5800 (2003).
28. Arni, S., Keilbaugh, S.A., Ostermeyer, A.G. & Brown, D.A. Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J. Biol. Chem.* **273**, 28478–28485 (1998).
29. Schuck, S., Honsho, M. & Simons, K. Detergent-resistant membranes and the use of cholesterol depletion. In *Cell Biology: A Laboratory Handbook* Vol. 2 (ed. Celis, J.E.) 1.5–1.9 (Elsevier Science, USA, 2006).
30. Ostermeyer, A.G., Beckrich, B.T., Ivarson, K.A., Grove, K.E. & Brown, D.A. Glycosphingolipids are not essential for formation of detergent-resistant membrane rafts in melanoma cells. Methyl-beta-cyclodextrin does not affect cell surface transport of a GPI-anchored protein. *J. Biol. Chem.* **274**, 34459–34466 (1999).
31. Shvartsman, D.E., Kotler, M., Tall, R.D., Roth, M.G. & Henis, Y.I. Differently anchored influenza hemagglutinin mutants display distinct interaction dynamics with mutual rafts. *J. Cell Biol.* **163**, 879–888 (2003).
32. Field, K.A., Holowka, D. & Baird, B. Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl. Acad. Sci. USA* **92**, 9201–9205 (1995).
33. Stauffer, T.P. & Meyer, T. Compartmentalized IgE receptor-mediated signal transduction in living cells. *J. Cell Biol.* **139**, 1447–1454 (1997).
34. Holowka, D., Sheets, E.D. & Baird, B. Interactions between FcεRI and lipid raft components are regulated by the actin cytoskeleton. *J. Cell Sci.* **113**, 1009–1019 (2000).
35. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39 (2000).
36. Simons, K. & Ehehalt, R. Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* **110**, 597–603 (2002).
37. Korzeniowski, M., Kwiatkowska, K. & Sobota, A. Insights into the association of FcγRII and TCR with detergent-resistant membrane domains: isolation of the domains in detergent-free density gradients facilitates membrane fragment reconstitution. *Biochemistry* **42**, 5358–5367 (2003).
38. Louvard, D. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA* **77**, 4132–4136 (1980).
39. Holloway, P.W. A simple procedure for removal of Triton X-100 from protein samples. *Anal. Biochem.* **53**, 304–308 (1973).
40. Fasman, D.G. (ed.) *Practical Handbook of Biochemistry and Molecular Biology* (CRC Press, Boston, Massachusetts, 1992).