Biochemical characterization of detergent-resistant membranes: a systematic approach

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Lateral segregation of cholesterol- and sphingomyelin-rich rafts and glycerophospholipid-containing non-raft microdomains has been proposed to play a role in a variety of biological processes. The most compelling evidence for membrane segregation is based on the observation that extraction with non-ionic detergents leads to solubilization of a subset of membrane components only. However, one decade later, a large body of inconsistent detergent-extraction data is threatening the very concept of membrane segregation. We have assessed the validity of the existing paradigms and we show the following. (i) The localization of a membrane component within a particular fraction of a sucrose gradient cannot be taken as a yardstick for its solubility; a variable localization of the DRMs (detergent-resistant membranes) in sucrose gradients is the result of complex associations between the membrane skeleton and the lipid bilayer. (ii) DRMs of variable composition can be generated by using a single detergent, the increasing

concentration of which gradually extracts one protein/lipid after another. Therefore any extraction pattern obtained by a single concentration experiment is bound to be 'investigator-specific'. It follows that comparison of DRMs obtained by different detergents in a single concentration experiment is prone to misinterpretations. (iii) Depletion of cholesterol has a graded effect on membrane solubility. (iv) Differences in detergent solubility of the members of the annexin protein family arise from their association with chemically different membrane compartments; however, these cannot be attributed to the 'brick-like' raft-building blocks of fixed size and chemical composition. Our findings demonstrate a need for critical re-evaluation of the accumulated detergent-extraction data.

Key words: annexin, cholesterol, detergent-resistant membrane (DRM), membrane segregation, raft.

INTRODUCTION

Individual signalling events are processed in distinct spatially segregated domains of the plasma membrane. The principles underlying the organization of the plasma membrane into macrodomains are well established. These include protein–protein interactions between the components of the extracellular matrix, lipid bilayer and subplasmalemmal cytoskeleton. Recent studies suggest that the plasma membrane is also laterally segregated into cholesterol- and sphingomyelin-rich rafts and glycerophospholipid-containing non-raft microdomains. Lateral segregation is thought to play a role in signal transduction, membrane trafficking and cytoskeletal organization [1,2]. The most compelling evidence of its existence is based on the observation that a subset of membrane components is more tightly packed than the bulk of the plasma membrane and, consequently, resistant to solubilization with non-ionic detergents at low temperatures [3]. In combination with sucrose density-gradient ultracentrifugation, detergent extraction allows the separation of DRMs (detergent-resistant membranes) from detergent-soluble material of non-raft regions.

Initially, DRMs were considered to represent a biochemical equivalent of membrane rafts. However, one decade later, a multitude of incoherent 'investigator-dependent' DRMs have raised concerns about the suitability of DRM methodology and the validity of the raft hypothesis itself (summarized in [4–7]). We have undertaken the present study in order to identify the reasons for the perceived inconsistency of detergent-extraction methodologies and to assess the validity of the existing raft hypothesis.

Smooth muscle is uniquely suited for this investigation for two reasons. First, its myocytes display an exceptionally precise structural organization of the plasma membrane: firm regions of

adherens junctions alternate with flexible caveolae-rich zones [8]. These regions are characterized by a specific subset of membrane proteins, embedded in their particular and distinct lipid environment: the caveolae are located within a cholesterol- and sphingomyelin-rich microenvironment, whereas the adherens junctions are surrounded by glycerophospholipids [9]. Secondly, smooth muscle sarcolemma contains high amounts of different annexins, members of a family of Ca^{2+} -dependent membrane-associated proteins [9,10]. The high degree of their structural similarity minimizes the effects of protein-dependent solubilization, which might arise from the differences in their individual chemical properties rather than from their spatial lipid-based distribution. Moreover, the ability of the annexins to interact with the plasma membrane in a reversible Ca^{2+} -dependent manner [10] allows us to separate membrane components in the absence of detergent, which is not possible for other irreversibly associated proteins. It is precisely this reversibility which renders the annexins an invaluable tool for the investigation of membrane organization.

In the present study we provide an extensive analysis of the DRMs obtained in flotation experiments after extraction of smooth muscle microsomes with non-ionic detergents.

EXPERIMENTAL

Antibodies

Monoclonal antibodies against annexins 1, 4 and 6, and a polyclonal antibody against caveolin, were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal antibodies against RhoA, vinculin and transferrin receptor were from Santa Cruz Biotechnology, Sigma and Zymed respectively.

Abbreviations used: DRM, detergent-resistant membrane; MCD, methyl- β -cyclodextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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Isolation of smooth muscle microsomes

Unless otherwise stated, all procedures were performed at 4 °C or on ice. Smooth muscle microsomal membranes were isolated from porcine stomach smooth muscle according to a protocol described in [10]. Minced muscle (100 g) was routinely extracted in 300 ml of buffer A (60 mM KCl, 2 mM MgCl₂, 0.2 mM CaCl₂ and 20 mM imidazole, pH 7.0). After low-speed centrifugation at 12 000 *g* for 30 min, the supernatant was filtered through glass wool and then subjected to high-speed centrifugation at 50 000 *g* for 90 min. The pellets thereby obtained were washed three times (with intervening centrifugations at 10 000 *g* for 30 min) in 10 vol. of buffer B (120 mM KCl, 0.2 mM CaCl₂ and 20 mM imidazole, pH 7.0) and finally resuspended in 10 ml of the same buffer.

Detergent extraction, detergent-free membrane separation and sucrose density-gradient ultracentrifugation

Smooth muscle microsomes (0.5 ml) were incubated at 4 °C or 37 °C for 30 min with either Triton X-100 or Tween 20 (protein and detergent concentrations for each experiment are given in the Figure legends).

Smooth muscle microsomes (0.25 ml; 4 mg/ml of protein) were diluted 2-fold with 1 M Na₂CO₃ (pH 11) and incubated on ice for 30 min, followed by sonication (three 20 s bursts) at 80 % power in a Bandelin Sonoplus HD 2070 sonicator (Bandelin Electronic, Berlin, Germany). Detergent- and high-pH-treated samples were diluted 2-fold in buffer B containing 80 % sucrose and overlaid with a discontinuous gradient of 30 % (3.5 ml)–5 % (0.5 ml) sucrose. The gradients were subjected to ultracentrifugation at 35 000 rev./min for 16 h at 4 °C in a Beckman SW50.1 swing-out rotor. Ten fractions of 0.5 ml were collected starting from the top of a centrifugation tube. The ultracentrifugation pellet was resuspended in 0.5 ml of buffer B (fraction 11). Where indicated, an additional low-speed centrifugation step was performed: a 100 µl aliquot of each ultracentrifugation fraction was diluted 4-fold with buffer B and centrifuged for 30 min at 12 000 *g*. A 400 µl volume of the supernatant was collected; the pellet was resuspended in 400 µl of buffer B. The lipid and protein composition of the ultracentrifugation gradient fractions, as well as supernatants and pellets obtained after the second centrifugation step, were analysed by TLC and Western blotting. Image analysis was performed using a PowerLook 1120 scanner and ImageQuant TL (v2003) from Amersham Biosciences.

Other procedures

TLC, SDS/PAGE and Western blotting were performed as described previously [10]. All the data shown are representative of at least three independent experiments and were highly reproducible.

RESULTS AND DISCUSSION

An association with actin is a key factor for the assignment of membranes to distinct fractions within sucrose gradients

The comparison of results from different studies is nearly impossible when even basics such as the position of the DRMs within the gradient are in dispute. In addition to their 'classic' localization within the low-density sucrose, their appearance in the pellets and even in the 40 % sucrose ('soluble') fractions of the gradients has been reported [11,12]. Interactions with the cytoskeleton and variations in the size of the vesicles are held responsible for an aberrant localization of DRMs [11,12].

As an initial step, our study addresses the influence of variations under basic experimental conditions (detergent and membrane concentrations) on the localization of DRMs within the sucrose

gradients. We analysed the distribution of typical DRM lipid- (sphingomyelin) and protein- (caveolin) markers in sucrose density-gradient fractions of smooth muscle microsomes after their extraction with various concentrations of Triton X-100.

Under the detergent-free conditions of a control experiment (Figure 1; 0 % Triton X-100) the sarcolemmal lipids (including sphingomyelin) and proteins (including caveolin) co-distribute within two peaks; the first corresponding to fraction 2 (interface between 5 % and 30 % sucrose) and the second comprising fractions 10 and 11 (40 % sucrose and the pellet). Despite its partial localization in 40 % sucrose (fraction 10; routinely attributed to the soluble material), the second peak contains insoluble material as confirmed by a second centrifugation step: after dilution (to reduce the amount of sucrose) and low-speed centrifugation, lipids and proteins from both peaks are found exclusively in the pellet (results not shown). Therefore both peaks contain insoluble membranes which differ in their intrinsic density.

The first peak at the interface of 5 % and 30 % sucrose is the position where low-density lipid-rich membranes are expected to reside. The localization of lipids and membrane-associated proteins in the second peak can be explained by their tight association with the submembranous actin-containing cytoskeleton. To address this assumption, we have investigated the distribution of α -smooth muscle actin within the fractions of the sucrose gradients. Actin is present in both peaks, but in contrast with other proteins and lipids localizes preferentially to the second one (Figures 1A and 1B; 0 % Triton X-100). It follows that the ratio of low-density lipids to high-density actin filaments (and thus the ability to float in the gradients) is higher in the membranes of the first than in the membranes of the second peak.

The actin-attachment sites within the adherens junctions, which are discernible by their marker protein vinculin [8] are the primary target for detergent extraction: vinculin is effectively solubilized already at a low concentration of Triton X-100 [note its shift from fraction 2 at 0 % Triton X-100 to fractions 9 and 10 at 0.125 % Triton X-100 (Figures 1A and 1B) and its presence in the supernatant after the second centrifugation (Figure 1B; pell/sup)]. As a result, the high-density membranes (Figures 1A and 1B, fractions 9–11 at 0 % Triton X-100) partially lose their actin, acquire lower density and shift to fractions 6–8 of the 0.125 % Triton X-100 gradient (Figures 1A and 1B). The progressive loss of actin at higher concentrations of detergent converts all DRMs into conventional low-density membranes: the second peak of high-density DRMs disappears (Figure 1A, 0.25–1 % Triton X-100; Figure 1B; 1 % Triton X-100).

A dramatic change in the sucrose-gradient profile is observed at 2–2.5 % Triton X-100 (Figures 1A and 1B): under these conditions, caveolin and sphingomyelin redistribute into the 'soluble' fractions of 40 % sucrose, together with most of the actin. However, the additional low-speed centrifugation demonstrates that the greater part of sphingomyelin remains insoluble, as does the majority of the caveolin (Figure 1B; 2 % Triton X-100; pell/sup). When the concentration of detergent reaches 3–4 %, they accumulate in the pellet; again, together with actin (Figures 1A and 1B). Responsible for this mobility shift, this time in the opposite direction, is again the interaction between the lipid bilayer and the actin-based submembranous cytoskeleton. Solubilization of cholesterol itself and the onset of sphingomyelin extraction (Figures 1A and 1B) leads to a partial solubilization of the DRMs' lipid bilayer; their lipid/actin ratio decreases, while their density increases, which makes the DRMs unable to float in the gradients.

An accumulation of the DRMs within the pellet is observed even at a detergent concentration of 1 % when a high detergent/membrane ratio favours the efficient extraction of the membranes

(Figure 2A; 2 and 0.8 mg/ml protein). This implies that other investigators who, as a rule, use 1% Triton X-100 but various amounts of starting material are confronted with a similar phenomenon.

In order to demonstrate that the localization of the DRMs within the high-density sucrose/pellet fractions depends on their direct association with actin, we compared the distribution of their lipid markers in the fractions of the sucrose gradients performed after Triton X-100 extraction of cultured smooth muscle cells pre-incubated in the presence or absence of the actin-destabilizing agent latrunculin A. Pre-treatment with latrunculin A significantly reduced the amount of sphingomyelin and cholesterol in the pellet, suggesting that the association between filamentous actin and the membranes is responsible for the appearance of the DRMs in the pellet (Figure 2B).

Our results show that DRMs localize to different fractions of the sucrose gradients depending on the degree of membrane extraction. Hence the localization of a membrane component within a particular fraction of a sucrose gradient cannot be taken as standard for its solubility. A variable localization of the DRMs within the sucrose gradients is the result of complex associations between the membrane skeleton and the lipid bilayer and has significant bearing on the interpretation of any detergent-extraction experiment. Often, a second centrifugation step is essential for avoiding erroneous conclusions.

Recently, Gaus et al. [13] demonstrated that Triton X-100 extraction and re-fractionation of high-density (presumably non-raft) membranes prepared using a detergent-free protocol resulted in the accumulation of membrane material in the low-density fractions of sucrose gradients. They concluded that Triton rearranges membrane lipids to induce formation of new artificial rafts from initial non-raft material. Our results provide an alternative explanation since the high-density fractions contain not only non-raft material but also rafts that are tightly associated with actin filaments. The impairment of membrane-actin association at high concentrations of detergent converts these pre-existing high-density DRMs into conventional low-density DRMs without rearrangement of the membrane lipids (Figure 1).

The spatial organization of the cytoskeleton also needs to be taken into account: since the cytoskeleton forms a three-dimensional lattice across the plasma membrane connecting it to the intracellular membranes, sarcolemmal DRMs might be contaminated by components co-localizing with filamentous and membranous structures of the intra- or extra-cellular compartments [14].

The graded extraction of membrane components by detergent leads to the formation of DRMs which vary in lipid and protein composition

Experimental conditions do have a bearing not only on the localization of DRMs within the gradient, but also on their lipid/protein composition. A low concentration of Triton X-100 (0.125%) preferentially solubilizes vinculin and the transferrin receptor (Figures 1A and 1B). The selective extraction of a small quantity of PE (phosphatidylethanolamine) and PC (phosphatidylcholine) is also noticeable (Figure 1B; 0.125% Triton; pell/sup).

At concentrations between 0.25% and 0.5% Triton X-100, RhoA is being increasingly extracted; this coincides with a marked increase in the solubilization of the glycerophospholipids (Figure 1A). Under these experimental conditions, the annexins, caveolin, cholesterol and sphingomyelin remain insoluble (Figure 1A). Higher concentrations of Triton X-100 (up to 1%) lead

to additional partial solubilization of annexin 1, which coincides with the onset of cholesterol extraction (Figures 1A and 1B). Annexins 4 and 6, caveolin and sphingomyelin remain largely insoluble. DRM localization changes drastically at concentrations of 2–2.5% Triton X-100 (Figures 1A and 1B). Here, a second centrifugation step is critical in order to distinguish the distinct solubilization pattern of a particular membrane component: the first centrifugation step shows identical sucrose gradient profiles for annexin 4, annexin 6 and caveolin at 2% Triton X-100 (Figures 1A and 1B). Yet, only after a second centrifugation step does it become obvious that annexin 4 is already partially soluble, while annexin 6 and caveolin are insoluble (Figure 1B). At this concentration of Triton X-100, vinculin, RhoA and annexin 1 are completely, and cholesterol is partially, extracted (Figure 1B). Thus the following pattern of solubility emerges: glycerophospholipids are more soluble than cholesterol, which in turn is more soluble than sphingomyelin. In consequence, the distinct extractability of the individual lipids leads to their differential enrichment within the DRMs created under different extraction conditions.

Our results are consistent with previous reports. The decreased detergent solubility of cholesterol and sphingolipids compared with glycerophospholipids is generally accepted and the preferential solubilization of cholesterol versus sphingomyelin has been reported [3,13]. However, in contrast with previous findings [13,15], we do not observe any differences in detergent solubility between PE and PC. One possible explanation is the difference in the structural organization of smooth muscle sarcolemma (used in our study) and the plasma membrane of human epidermal carcinoma cells/macrophages [13,15]. Undergoing constant changes in length and charged with the efficient transmission of force, smooth muscle cells display a high degree of structural organization, with their sarcolemma being heavily reinforced by interactions with the cytoskeleton and extracellular matrix components [8]. The mechanical stability of smooth muscle sarcolemma might protect its lipid bilayer from artificial loss of lipids during experimental manipulations, which might occur in the less stable membranes of other cell types. In analogy to the graded solubility pattern observed for different lipids, a similar one can be drawn for the membrane-associated proteins: vinculin = transferrin receptor > RhoA > annexin 1 > annexin 4 > annexin 6 = caveolin (Figure 1).

Since a graded extraction pattern might be the artefact of diverse detergent concentrations, we next varied the concentration of the microsomes while the detergent was kept constant at 1%. A combination of high protein concentration (5 mg/ml protein) with 1% Triton X-100 extracts neither annexins nor caveolin (Figure 1). Lowering the concentration of microsomes (2 mg/ml protein) leads to complete solubilization of annexin 1 and partial solubilization of annexin 4, while annexin 6 and caveolin remain insoluble (Figure 2A). A further decrease in microsomal concentration results in complete solubilization of annexin 4 and partial extraction of annexin 6 and caveolin (Figure 2A; 0.8 mg/ml protein). Extraction at an even higher detergent/membrane ratio leads to complete solubilization of annexin 6, caveolin and sphingomyelin (Figure 2A; 0.32 mg/ml protein). The solubility pattern which emerges from this experiment (annexin 1 > annexin 4 > annexin 6 = caveolin) is identical with the previous one. This suggests that the graded extraction pattern is not an artefact due to a variation in detergent concentration and implies that other investigators who, as a rule, use 1% Triton X-100 but various amounts of starting material are confronted with a similar phenomenon.

The graded extraction of membrane-associated proteins might be based on their individual chemical properties rather than their

Figure 1 For legend see facing page

Figure 1 Localization and composition of DRMs are dependent on the extraction conditions

Smooth muscle microsomes (5 mg/ml) were extracted with the indicated concentrations of Triton X-100 and subjected to sucrose density-gradient ultracentrifugation. The lipid and protein composition of the gradient fractions were analysed by TLC and Western blotting (**B**) and quantified (**A**). (**A**) Relative amounts of individual proteins and lipids in the fractions of the sucrose gradients are presented as percentages of their total amount. Sm, sphingomyelin; Cav, caveolin; Vinc, vinculin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Ch, cholesterol; Anx, annexin. (**B**) Fractions of some sucrose gradients (0.125, 1, 2 and 4% Triton X-100) were diluted 4-fold with buffer B and subjected to additional low-speed centrifugation. Initial gradients, as well as pellets (pell) and supernatants (sup) of the second centrifugation step, were analysed by TLC and Western blotting. TrfR, transferrin receptor.

Figure 3 The graded extraction profile of annexins 1, 4 and 6 reflects the extraction of their binding sites within the lipid bilayer

Smooth muscle microsomes (0.5 ml; 2 mg/ml of protein) were incubated for 10 min with 1 mM EGTA at 4°C. After low-speed centrifugation, the soluble annexins were recovered in the supernatant. The microsomes were pelleted. The annexin-containing supernatant was clarified by an additional low-speed centrifugation step. The pellet was washed extensively in buffer B additionally containing 1 mM EGTA, resuspended in 0.5 ml of the same buffer and extracted with 1% Triton X-100. After extensive washes in buffer B/1 mM EGTA to remove detergent, the pellet, now lacking annexins, was recombined with the annexin-containing supernatant in a final volume of 0.5 ml (No detergent). To initiate annexin-membrane binding, the samples were supplemented with 1 mM CaCl_2 . In a control experiment, the microsomes were extracted with 1% Triton X-100 omitting the EGTA-induced separation of annexins from the membrane (Control). The samples were subjected to sucrose density-gradient ultracentrifugation, and fractions were analysed by Western blotting.

differential spatial distribution within raft or non-raft microdomains. The annexin family is an invaluable tool to address this question. These proteins are conditionally associated with membranes and can be detached by decreasing the Ca^{2+} concentration in the solution. Therefore the microsomes were treated with 1 mM EGTA and centrifuged to separate the insoluble membranes from the now soluble annexins (Figure 3). In the following step the microsomes were treated with 1% Triton X-100. After extensive washing to remove the detergent, the extracted annexin-free microsomes [10] were recombined with the annexins in the presence of Ca^{2+} . Provided that their respective membrane-binding sites are still preserved and not extracted by detergent, the annexins will re-associate with the membrane. Comparison of the sucrose gradients revealed almost identical extraction profiles for the annexins in this and in the routine extraction experiments. In both experiments, annexin 1 was completely, and annexin 4 partially, soluble, while annexin 6 remained completely insoluble in 1% Triton X-100. Since in

Figure 2 Association with actin is a key factor for the assignment of DRMs to high-density fractions within sucrose gradients

(A) Smooth muscle microsomes at the indicated concentrations of total protein were extracted with 1% Triton X-100 and subjected to sucrose density-gradient ultracentrifugation. The lipid and protein composition of the initial gradient fractions and the pellets (pell) and supernatants (sup) obtained after the second centrifugation were analysed by TLC and Western blotting. (B) Cultured human myometrial cells (passages 3–8) [32] were grown to dense confluency on plastic Petri dishes. Cells were scraped in 5 ml Na^+ /Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose and 10 mM HEPES; pH 7.4), divided into two 2.5 ml portions and incubated in the presence (Lat) or absence (Control) of the actin-destabilizing agent latrunculin A (5 μM) for 2 h at 37°C. For each sample, the cells were pelleted at 20 g, resuspended in 500 μl of buffer B (2 mg/ml total protein), and then subjected to extraction (0.5% Triton X-100) and sucrose density-gradient centrifugation. The lipid composition of the gradient fractions was analysed using TLC. Sm, sphingomyelin; Cav, caveolin; Vinc, vinculin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Ch, cholesterol; Anx, annexin.

the experiment described in Figure 3, the annexins have never been exposed to detergent, it can be ruled out that they were physically removed from the membranes by the detergent. It is the graded extraction of their binding sites within the lipid bilayer that is responsible for the graded extraction of the annexins by a detergent.

Our results show that DRMs of variable composition can be generated from the same starting material (smooth muscle microsomes) by using one single non-ionic detergent: its increasing concentrations gradually extract one protein/lipid after the other until the DRMs are completely solubilized. Moreover, previous studies have shown that the DRM composition critically depends on the cell type, the starting material and the isolation protocols [4]. It is therefore likely that any extraction pattern obtained by a single detergent-concentration experiment in a particular biological system is bound to be 'investigator'-specific.

Current models suggest that multiple membrane microdomains of different lipid and protein composition co-exist within the plasma membrane [4,11,12,16]. It is therefore all the more surprising that so far the concept has not entered experimental practice. In nine out of ten recent publications randomly selected from PubMed (late 2005–early 2006; <http://www.pubmed.gov>) single concentration detergent-extraction experiments are used to analyse putative rafts. Such an experimental approach would be sensible if the amount of microdomains in the plasma membrane were limited to merely two: one soluble and one insoluble. However, this approach does not take into account that more than two microdomains of different detergent solubility might co-exist within the plasma membrane. The graded 'one-by-one' extraction pattern for individual proteins and lipid classes which we observed in our study is not compatible with the predicted 'all-or-nothing' extraction pattern expected for membranes composed of only two spatially separated components.

Detergent-specific DRMs

One of the most intriguing questions remains the existence of detergent-specific DRMs. In detail discussed by several investigators, they have been reported to differ in their protein and lipid composition depending on the type of detergent used for their preparation [4,11,12]. These results have been interpreted in terms of the existence of spatially and chemically different rafts (Lubrol rafts, Triton rafts etc.). In addition, they prompted the notion that different detergents do not reflect the same aspects of the membrane organization [4] and suggested that the detergent-extraction approach was not applicable to raft research. Our results (see Figures 1 and 2) imply that the Triton X-100 DRM of fixed composition does not exist: protein and lipid composition varies depending on the extent of membrane solubilization. It is highly likely that this mechanism applies to any detergent.

In our attempts to investigate the detergent-specificity of rafts, we compared DRMs generated by different concentrations of two detergents. This approach demonstrates that Tween 20, the least potent detergent [4], shows an extraction profile which is very similar to that obtained with one of the most potent detergents, Triton X-100. The only difference is that higher concentrations of Tween 20 than Triton X-100 are required to obtain DRMs of the same composition. DRMs generated using 0.5 % Tween 20 resemble DRMs generated using 0.125 % Triton X-100 and DRMs generated using 5 % Tween 20 are similar to those generated by 1 % Triton X-100 (compare Figure 1B and Figure 4). Thus, when the solubilization activities of two detergents are matched by adjusting their relative concentrations, they produce DRMs of the same composition.

Figure 4 Graded extraction of smooth muscle microsomes by Tween 20

Smooth muscle microsomes (5 mg/ml) were extracted with the indicated concentrations of Tween 20, subjected to sucrose density-gradient ultracentrifugation and subsequently to low-speed centrifugation (pel, pellet; sup, supernatant). The lipid and protein composition of gradient fractions were analysed by TLC and Western blotting. Sm, sphingomyelin; Cav, caveolin; Vinc, vinculin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Ch, cholesterol; Anx, annexin; TrfR, transferrin receptor.

Detergent-free protocol

With the reliability of the detergent-extraction protocols in question, a number of detergent-free approaches have been developed, and significant differences in the composition of DRMs and detergent-free rafts were reported [13,17,18]. In general, the rafts isolated by detergent-free methodologies contain more protein and lipid components than DRMs.

We have followed a Na_2CO_3 protocol (pH 11) to investigate whether a detergent-free methodology can be used as an alternative to detergent extraction in assessing membrane segregation. Surprisingly, a non-specific segregation between the bulk of membrane lipids and the bulk of membrane proteins occurs at high pH in smooth muscle microsomes (Figure 5A). The majority of lipids float to the low-density fractions of the gradient, whereas the majority of proteins (including caveolin) localize to the

Figure 5 Detergent-free membrane separation: the effects of cholesterol-depletion and temperature on the extractability of smooth muscle microsomes

(A) Smooth muscle microsomes (0.25 ml; 4 mg/ml protein) were diluted 2-fold with 1 M Na_2CO_3 (pH 11) and incubated on ice for 30 min, followed by sonication. (B and C) Smooth muscle microsomes (0.5 ml; 2 mg/ml protein) were incubated at 37°C for 60 min without or with the indicated concentrations of MCD (methyl- β -cyclodextrin). After low-speed centrifugation, the pellet was resuspended in 0.5 ml of buffer B and extracted with the indicated concentrations of Triton X-100 on ice. (D) Smooth muscle microsomes (2 mg/ml protein) were extracted with 1% Triton X-100 at 37°C for 30 min. The samples were subjected to sucrose density-gradient ultracentrifugation and low-speed centrifugation (pel, pellet; sup, supernatant). Fractions were analysed by TLC, SDS/PAGE (Coomassie Blue staining) and Western blotting. Sm, sphingomyelin; Cav, caveolin; Vinc, vinculin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Ch, cholesterol; Anx, annexin; TrfR, transferrin receptor.

high-density fractions. The proteins remain largely insoluble (Figure 5A; pel/sup) presumably due to protein–protein interactions within the submembranous cytoskeleton. No segregation of either raft compared with non-raft lipids (sphingomyelin/cholesterol compared with glycerophospholipids) or proteins (caveolin compared with vinculin) could be detected.

Recent studies demonstrated that a successful separation of membrane components by detergent-free protocols is critically dependent on the experimental conditions [13]. Although our study does not aim to undertake an extensive characterization of detergent-free approaches, our results imply that a detergent-free approach used in a single-condition experiment is at least as prone to misinterpretation as a single-concentration detergent-extraction experiment.

The quality of detergent-free protocols is frequently measured by their success in producing cholesterol-enriched membranes. Some studies monitor the ratio of cholesterol to total protein [18,19]. In the present study we demonstrate that, although the

low-density fractions of the detergent-free gradient have a higher ratio of cholesterol to protein than the high-density fractions (Figure 5A), this occurs due to a non-selective separation between the bulk of the lipids and the bulk of the proteins. Therefore the use of the cholesterol/protein ratio in characterizing lateral membrane segregation is misleading; the only valid criterion is the evaluation of raft (cholesterol/sphingolipids) compared with non-raft lipids (glycerophospholipids).

Cholesterol depletion affects the extractability of the remaining plasma membrane components

Cholesterol is thought to stabilize membrane rafts [20]. Therefore its selective depletion by MCD (methyl- β -cyclodextrin), in combination with Triton X-100 extraction, is frequently used to validate a putative association of various membrane components with membrane rafts.

Figure 6 Interdependent but not identical gradients of individual lipids exist within the membrane bilayer

The lipid moieties are responsible for targeting proteins to their respective places within the membrane. Interactions between the transmembrane and peripheral membrane proteins add new dimensions to the lipid-dependent segregation [33]. The segregation is stabilized further via an interaction of the membrane with the submembranous cytoskeleton. Anx, annexin; Vinc, vinculin.

To investigate the effects of cholesterol extraction on the solubility of DRMs, we depleted smooth muscle microsomes of cholesterol using standard conditions (2 % MCD for 1 h at 37 °C), followed by our routine Triton X-100 (1 %) extraction protocol (Figure 5B). In this particular experiment, the depletion of cholesterol was incomplete (Figure 5B); and we assume that this is likely to be the case in many other studies, as the amount of residual cholesterol is not frequently monitored. Our experiment demonstrates that a partial depletion of cholesterol does not automatically lead to complete DRM elimination [compare sphingomyelin, annexin 6 and caveolin of the MCD pre-extracted and control, Triton X-100-only gradients (Figure 5B)]. However, it did significantly increase the solubilization of annexin 4 (Figure 5B). Cholesterol depletion is more efficient at 5 % MCD (Figure 5B). When followed by a routine 1 % Triton X-100 extraction protocol, these conditions result in the disappearance of the low-density DRMs. However, the high-density DRMs survive, as indicated by a selective accumulation of sphingomyelin, annexin 6 and caveolin in the pellet (Figure 5B).

As expected by the membranes' graded response to non-ionic detergents, the composition of cholesterol-depleted DRMs does not depend exclusively on the MCD concentration, but also on the Triton X-100 concentration. Cholesterol depletion performed at the same MCD concentration (2 %) but subsequently subjected to extraction by two different Triton X-100 concentrations shows DRMs which differ in their annexin profile. After extraction with 0.25 % Triton X-100, cholesterol depletion affects annexin 1 only, without consequences for annexin 4, annexin 6 and caveolin (Figure 5C), in contrast with 1 % Triton X-100, when cholesterol depletion facilitates extraction of annexin 4 (Figure 5B; under these extraction conditions annexin 1 is soluble, irrespective of cholesterol depletion).

Our results suggest that the depletion of cholesterol also has a graded effect on membrane solubility: depending on the extent of cholesterol extraction, individual membrane regions become more sensitive to the non-ionic detergent.

Lipid segregation of biological membranes is not a temperature-induced artefact

Lateral membrane segregation has been considered to be a temperature-induced artefact [6]. We have addressed this concern by comparing the sucrose gradient profiles of the membrane com-

ponents extracted by Triton X-100 at 4 °C and at 37 °C. Typical cholesterol-, sphingomyelin- and caveolin-rich DRMs formed at either temperature (Figures 5B and 5D) suggesting that the lipid segregation of biological membranes is not a temperature-induced artefact.

The lipid gradient model

The account of mobile lipid–protein microdomains prompted the emergence of novel membrane models. However, the lipid and protein composition of these microdomains has not yet been determined nor has their final size been agreed upon [21]. Various attempts have been made to classify them into caveolar and non-caveolar types, Triton-insoluble, Triton-soluble, Lubrol-insoluble, cholesterol-dependent and cholesterol-independent [22–24]. In contrast with small mobile rafts floating within a sea of glycerophospholipids, the concept of a superraft has emerged [25,26]. In another important development, the actin cytoskeleton has been proposed to constitute a physical barrier which impedes unhindered movement of lipid–protein complexes across the cell surface [27].

To encompass these findings, a concept of multiple membrane domains has been proposed ([11,12], and reviewed in 16]). Our extraction data are consistent with the idea of multiple membrane domains. However, in our case, the existence of at least six distinct domains for the eight proteins we have investigated so far, and, following this trend, hundreds of separate domains for all membrane-associated proteins, must be inferred. Since the existence of hundreds of separate membrane domains is highly unlikely, we propose to modify the current multiple domain model by suggesting that continuous overlapping but not identical gradients of individual lipids exist within the membrane bilayer (Figure 6). Depending on the overall lipid composition, and influenced by associated proteins, each distinct membrane stretch possesses its own characteristic solubility in non-ionic detergents which gradually decreases with the increase in the ratio of sphingomyelin to cholesterol to glycerophospholipid.

Conclusions

The plasma membrane is thought to contain microdomains of liquid-ordered membrane rafts, which are characterized by their resistance to extraction by non-ionic detergents [1,28,29]. These

assemblies of cholesterol and glycosphingolipids form dynamic units and are interspersed with less ordered and more fluid glycerophospholipid-enriched regions. Their ability to segregate proteins provides a mechanism for the compartmentalization of signalling components within the plasma membrane [1,2].

New membrane concepts have been welcomed enthusiastically by some [30], sceptically by others [6]. The scepticism is fuelled by many inconsistent and often contradictory results, obtained by imaging and separation approaches used to characterize membrane segregation [4,6,21,31]. In the present study we demonstrate that the considerable number of contradictory results might have their origin in methodological problems. The interaction of the lipid bilayer with the membrane cytoskeleton as well as the graded solubilization of the membrane components can be held accountable for the complex biochemical extraction pattern of DRMs.

We have also shown that:

(i) The differences in detergent solubility at least for the members of the annexin protein family arise from their association with chemically different membrane microdomains and not from variations in their interactions with the detergent (Figure 3);

(ii) The extraction profile of PC, which preferentially localizes to the outer leaflet of the lipid bilayer is identical with that of PE, which is largely situated on the inner leaflet; their extraction profile differs from that of cholesterol and sphingomyelin (Figure 1). This suggests that PE and PC are extracted simultaneously from the same structural entity which spans both membrane leaflets.

(iii) DRMs are formed at 4°C and at 37°C, suggesting that temperature is not a dominant factor in inducing membrane segregation (Figure 5).

Our findings support the membrane segregation paradigm; however, they also imply that the membrane segregation cannot be attributed to the brick-like raft-building blocks of fixed size and chemical composition. This explains not only the persistent controversy of the detergent-extraction experiments but also has a bearing on the interpretation of imaging data: in contrast with the spatially separated microdomains, the overlapping gradients of different lipids/proteins might not be readily distinguishable.

We acknowledge financial support from the Swiss National Science Foundation [grants 31-66963.01, 7UKPJ62216 (SCOPES, Scientific Co-operation between Eastern Europe and Switzerland) and 405340-104679/1].

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Received 9 January 2006/13 March 2006; accepted 12 April 2006

Published as BJ Immediate Publication 12 April 2006, doi:10.1042/BJ20060056