Triton X-100 promotes a cholesterol-dependent condensation of the plasma membrane

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The molecular components of membrane rafts are frequently defined by their biochemical partitioning into detergent-resistant membranes. In the present study, we used a combination of epifluorescence and two-photon microscopy to visualize and quantify whether this insolubility in detergent reflects a pre-existing organization of the plasma membrane. We found that the treatment of cells with cold TX (Triton X-100) promotes a profound remodelling of the plasma membrane, including a rapid rearrangement of the glycosphingolipid GM1 and cholesterol into newly formed structures, only partial solubilization of fluid domains and the formation of condensed domains that cover 51% of the remaining membrane. TX does not appear to induce the coalescence of pre-existing domains; instead, the domains that remain after TX treatment seem to be newly formed with a higher degree of condensation than those observed in native membranes. However, when cholesterol was complexed physically by treatment with a second detergent, such as saponin, cholesterol did not separate into the newly formed structures, condensation of the domains was unaltered, and the relative area corresponding to ordered domains increased to occupy 62% of the remaining membrane.

Our results suggest that detergent can be used to enrich ordered domains for biochemical analysis, but that TX treatment alone may be insufficient to alter the organization of the PM.

Key words: caveolin (CAV), cholesterol, detergent-resistant membrane (DRM), Laurdan, membrane raft, Triton X-100 (TX).

INTRODUCTION

The lipid-raft hypothesis proposes that cholesterol and sphingolipids segregate from glycosphospholipids and associate dynamically to form distinct liquid-microdomains in a lipid bilayer [1,2]. Once formed, membrane rafts could provide a temporal and spatial compartment for selected lipids and proteins and thus introduce specificity into processes such as signal transduction, cell migration and the dynamic sorting of membrane components [3–7]. Biologically, raft-mediated membrane sorting offers the cell two unique properties to overcome the limitations that are demonstrated by coat-protein-mediated sorting: first, it drives the dynamic sorting of selected lipids into newly formed domains and vesicles; and, secondly, it provides a mechanism for the segregation of non-raft molecules away from specific trafficking pathways.

Probably owing to their small size and transient nature, membrane rafts have been difficult to characterize in living cells. An important part of the current knowledge of their existence and function has been obtained by biophysical means or indirect techniques, such as cold-detergent extraction or acute cholesterol depletion. It is generally assumed that membrane rafts could be preferentially isolated by taking advantage of their unique insolubility in cold detergents such as TX (Triton X-100) and consistently low buoyant density in sucrose gradients. Previous electron-microscopy observations of DRMs (detergent-resistant membranes) described these membrane fragments as a heterogeneous population of vesicles ranging from 0.1 to 1 μm in diameter that contain GPI (glycosylphosphatidylinositol)-anchored proteins [8]. Since then, it has been difficult to prove such PM (plasma membrane) organization in living cells. Recently, the poorly understood interaction between surfactants and the components of the membrane bilayer has led to questioning of the extent to which DRMs reflect the organization of the intact cell membrane [9–11]. For example, it was observed that raft composition differs substantially when isolated in the presence or absence of detergents [12] and depends on which detergents are used [13]. This apparent contradiction may be explained by the fact that treatment of liposomes with TX is sufficient to promote the formation of liquid-ordered domains, suggesting that detergent solubilization could involve the formation of non-physiological structures [14–17]. However, results obtained from artificial membranes are difficult to extrapolate to living cells, as the PM is composed of a complex mixture of lipids and proteins that combine to form the asymmetrical bilayer. Although DRMs may not reflect PM organization, the insolubility of lipids and proteins often establishes a preliminary link with membrane rafts. Indeed, DRMs are still used to define raft affinity or preferential partitioning, most convincingly when changes in DRM composition can be promoted by physiological events [9].

Therefore a detailed characterization of the interaction between surfactants and the asymmetrical bilayer remains fundamental to the study of PM organization. In this study, we have combined epifluorescence and two-photon microscopy to visualize and quantify the remodelling of the PM of living cells by detergents in order to (i) understand the action of TX on the cell membrane, and (ii) further the establishment of a detergent protocol

Abbreviations used: CAV1, caveolin 1; CTB, cholera-toxin B subunit; DRM, detergent-resistant membrane; GP, generalized polarization; GP, GP-index; GPI, glycosylphosphatidylinositol; PFA, paraformaldehyde; PM, plasma membrane; TX, Triton X-100; TX/sap, a combination of 1% (v/v) TX and 0.05% (v/v) saponin.

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that enriches components more accurately in pre-existing raft domains.

EXPERIMENTAL

Cell culture and reagents

COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin sulphate (Biological Industries). Filipin, TX and saponin were purchased from Sigma–Aldrich. CTB (cholera-toxin B subunit) conjugated to Alexa fluorophores, were obtained from Molecular Probes. Rabbit polyclonal anti-CAV1 (caveolin 1) antibody was purchased from Transduction Laboratories. Protein was quantified using the Lowry method [18]. Cholesterol in cell lysates was quantified as described previously [19].

Detergent solubilization

Cells (2.5 × 10⁶) were plated (18 h before the experiment) in a 10-cm dish (60 cm²) that contained glass coverslips 1 cm in diameter (0.79 cm²). At the time of the experiment, each dish contained approx. 1140 μg of protein and 9.12 μg of cholesterol, and thus each coverslip contained approx. 15 μg of protein and 0.12 μg of cholesterol. Next, the coverslips were inverted and washed for 10 s in a cold Tris-based buffer [5 mM Tris/HCl (pH 7.5), 150 mM NaCl and 5 mM EDTA], then incubated at 4 °C in a 30 μl drop of the Tris-based buffer that, where indicated, contained 1% (v/v) TX or TX/sap [a combination of 1% (v/v) TX and 0.05% (v/v) saponin] (freshly prepared prior to use). After various incubation periods (ranging from 10 s to 30 min), the coverslips were fixed for 10 min in 50 μl of 4% (v/v) PFA (paraformaldehyde) at 4 °C and for an additional 50 min in 50 μl of 4% (v/v) PFA at room temperature (24 °C). In other experiments, cells were plated at low density (7.5 × 10³ cells) and treated as described above.

Laurdan, filipin, CTB and CAV1 staining and imaging

The filipin staining of fixed cells was performed as described previously [20]. Briefly, after fixation with 4% (v/v) PFA, the coverslips were washed in PBS and incubated in 100 μl of PBS containing 5 μg/ml filipin [freshly prepared from a 5 mg/ml stock solution (in DMSO) stored at −80 °C] at room temperature for 30 min. For detection of the glycosphingolipid GM1, the cells were treated at 4 °C for 30 min with CO₂-independent medium (GIBCO) containing 2 μg/ml Alexa-594-conjugated CTB [from a 2 mg/ml stock solution (in water) stored at −20°C]. Next, the coverslips were treated with detergents as described above, fixed in 4% (v/v) PFA and labelled with filipin. CAV1 immunolabelling was performed as described previously [20]. In the present experiments, 5 μg/ml filipin was added to the blocking solution and to the buffers of the primary and secondary antibodies. Finally, the coverslips were mounted in Mowiol, prepared according to the manufacturer’s instructions (Calbiochem). Cells were observed using an oil-immersion Plan-Apo63x/1.4 objective in an Axioplan or Axiosvert 200M microscope (Zeiss). In the Axioskop microscope, the images were captured with an AxioCam HRc camera and were digitally manipulated with AxioVision 3.1 software (Zeiss). In the 200M microscope, the images were captured with a Photometrics CoolSNAP HQ camera controlled by Slide-Book 3.0.10.5 software (Intelligent Imaging Innovations). Image analysis was performed with Adobe Photoshop 5.5 software (Adobe). Labelling of live and fixed cells with the fluorescent probe Laurdan (6-acetyl-2-dimethylaminonaphthalene) and two-photon microscopy was performed using a DM IRE2 microscope (Leica) as described previously [21]. In brief, Laurdan was excited at 800 nm, and emission intensities were recorded simultaneously in the ranges 400–460 nm and 470–530 nm. Intensity images for each pixel were converted into GP (generalized polarization) images (WIT Software), with GP (the GP-index) calculated as follows (eqn 1):

\[
GP = \frac{I_{(400–460)} - I_{(470–530)}}{I_{(400–460)} + I_{(470–530)}}
\]

where \(I\) is the emission intensity.

GP distributions were obtained from the histograms of the GP images, normalized (sum = 100) and fitted to Gaussian distributions using the non-linear fitting algorithm Solver (Microsoft Excel). Microscope calibrations were carried out as previously described [21–23]. The GP histograms were calculated from approx. 80 cells for each treatment.

RESULTS

TX promotes the formation of new ordered domains in the PM

We analysed the lipid structure of the cell surface of COS cells by means of two-photon microscopy. The fluorescent probe Laurdan has been used to characterize phase separation in model membranes [24,25] and visualize ordered domains on the surface of living cells [21]. Laurdan does not partition preferentially into either lipid phase but undergoes a shift in its peak-emission wavelength from 500 nm in fluid membranes to 440 nm in condensed membranes. We simultaneously recorded the Laurdan fluorescence intensity in two channels: 400–460 nm (condensed membranes) and 470–530 nm (fluid membranes). Then, for each pixel, a normalized ratio of the two emission regions was given by the GP-value. Next, GP histograms were obtained from approx. 80 cells for each treatment and each histogram was fitted to two Gaussian populations, providing us with the mean GP-value of each population and their relative abundance or coverage. GP-values range from −1 (very fluid) to +1 (very condensed), and measure membrane fluidity with fluid domains in the approx. range −0.05–0.25 and ordered domains in the range 0.25–0.55. Throughout this paper, we will refer to lipid rafts as the hypothesized entity and to domains as the areas within the cell membrane that were examined microscopically. Given the spatial resolution of light microscopy (~200 nm), it is therefore likely that lipid rafts are substantially smaller than the experimentally observed domains.

COS-7 cells on coverslips (equivalent to 15 mg of protein and 0.12 mg of cholesterol per coverslip) were labelled with Laurdan. In Figure 1(A)–1(D), the resulting GP images are pseudo-coloured to show fluid membranes in green (GP < 0.3) and ordered membranes in red to yellow (GP > 0.3). At the cell surface, we found condensed domains (Figure 1A) (with a mean GP of 0.48±0.49 ± 0.06) that occupied 22.7±5.5% of the PM (shown by the black circles in Figure 1E for the Gaussian distribution of the GP-values; see also Figure 1G for the mean GP, and the relative coverage between fluid and condensed populations). After incubating the cells for 10 min (Figure 1B, white circles in Figure 1E, and Figure 1G) or 20 min (Figure 1C, black circles in Figure 1F, and Figure 1H) in a cold Tris/HCl (pH 7.5) buffer, the abundances of ordered domains were similar (23.2% or 20.1% coverage respectively). Identical results for GP and relative coverage were obtained in fixed and living cells (results
Triton X-100 condenses the plasma membrane

Figure 1 Laurdan labelling of TX-solubilized cells

(A–D) COS cells were labelled with Laurdan (A) and imaged for an additional 10 min (B) and 20 min (C) in ice-cold Tris/HCl buffer, pH 7.5 (see the Experimental section). After 20 min (C), the cells were treated additionally with 1 % (v/v) TX for 10 min and imaged for the final time (D). GP images were calculated from intensity images (see the Experimental section) and were pseudocoloured, with blue to yellow representing low to high GP-values respectively (see the colour scale between panels C and D). (E–G) COS cells (black lines in E and F) were incubated for 10 min in ice-cold buffer (white circles in E) or for 10 min in 1 % (v/v) TX (white circles in F), labelled with Laurdan and the GP-value calculated. Pixel intensities were normalized and fitted to Gaussian populations (E and F). The corresponding mean GP-values of ~80 cells per treatment, and the relative coverage between fluid and condensed populations, were quantified (G and H). The average S.D. of mean GP-values for fluid and condensed populations are 0.06 and 0.05 respectively. The average S.D. of coverage is 5.5 %. For (A–D), scale bars, 20 μm (low magnification) and 5 μm (high magnification).

not shown). Next, in order to quantify whether the insolubility of the PM in non-ionic detergents correlates with its organization into ordered/fluid domains, 1 % (v/v) TX was added to the cells and the GP analysed after 10 min. Interestingly, the addition of TX induced a dramatic shift in the mean GP of the ordered membrane population to 0.64 ± 0.05 %, and condensed domains now covered 51.4 ± 5.5 % of the cell membrane (Figure 1D, white circles in Figure 1F, and Figure 1H). Therefore TX promoted two different processes: (i) it partially solubilized fluid domains of the PM (48.6 % of the cell membrane remained in a fluid state with a GP of 0.079); and (ii) it promoted the formation of highly ordered domains that are distinctly different from those in native membranes. The latter is demonstrated by the increase in the mean GP from 0.493 to 0.639. Thus it is likely that condensed regions are formed de novo rather than being the result of the coalescence of pre-existing domains. Whether these apparently new highly ordered domains are created by the coalescence of pre-existing membrane rafts that are below the resolution of the microscope cannot be resolved with the present approach. In model membranes, GP-values of > 0.6 typically signify gel phases rather than ordered phases. Thus it is unlikely that pre-existing rafts are responsible for such a dramatic membrane condensation. It is, however, remarkable that highly ordered domains were generally formed in regions in which the cell membrane already presented a relatively high degree of condensation (compare Figures 1C and 1D), which suggests the possibility that lipids and/or proteins redistribute dynamically into already condensed regions of the membrane (see the Discussion).
Figure 2 Filipin staining reveals cholesterol-enriched domains in the PM of COS cells

(A–C) COS cells were fixed in PFA and incubated with filipin to detect free cholesterol. Note that although filipin is visible in the UV channel of the microscope, in the present Figures it is shown in white or green to provide better visual resolution. Cholesterol is distributed widely in the PM, but accumulates in a peripheral cortex (arrowheads) and at points of contact between neighbouring cells (arrows). In addition, numerous rounded, filipin-enriched domains are apparent within the membrane. 

(D and E) For the detection of GM1, cells were incubated with CTB–Alexa 594, fixed in PFA and labelled with filipin. CTB (red) appears widely distributed in the PM. Although there is a clear overlap with cholesterol (arrowheads), GM1 shows a more diffuse distribution, and GM1 is not located exclusively to filipin-stained domains (arrows in E). 

(F–G) Cells were fixed and labelled with filipin and with an anti-CAV1 antibody. Filipin (green) and CAV1 (red) partially co-localize (arrows). For (A, D, F), scale bar, 20 μm; for (B, C, E, G), scale bar, 5 μm.

Visualization of cholesterol-enriched domains at the cell surface of COS cells

Highly ordered domains are characterized by an increased concentration of cholesterol when compared with the bulk of the PM. Cholesterol-enriched domains have proven difficult to visualize in the PM of living cells [26]. However, by conventional fluorescence microscopy of chemically fixed cells, non-esterified free cholesterol is conveniently detected using filipin as a fluorescent marker [27–29]. In COS cells, filipin labelling was found in the Golgi-recycling-endosomal area, PM and scattered intracellular vesicles [30]. In the PM, cholesterol, although widely distributed, accumulated in regions of contact between neighbouring membranes (arrows in Figures 2A–2C), similarly to ordered domains detected by Laurdan. In addition, strong labelling was detected close to the edge of the cell,
forming a peripheral cortex of approx. 1.5–2 μm (arrowheads in Figures 2A–2C). An identical distribution of filipin was observed when the cells were fixed with a combination of 4 % (v/v) PFA and 0.1 % (v/v) glutaraldehyde for 10 min at 4 °C and for an additional 50 min at 24 °C, or when cells were fixed in 4 % (v/v) PFA for 1 h at 24 °C to reduce the possibility of clustering and/or extraction of lipids and proteins during the fixation protocol (results not shown). At high magnification, numerous filipin-enriched domains were prominent within the membrane (Figures 2B and 2C). Thus taking into account the optical resolution and the limitations of filipin for the detection of free cholesterol, the discontinuous distribution of filipin labelling may be consistent with the existence of distinct cholesterol-enriched domains in the PM. The filipin-enriched domains were generally round in shape, with an apparent average diameter of 253.3 ± 44.5 nm and occupied 28.63 ± 13.3 % of the cell surface. This is similar to the coverage of ordered domains shown by Laurdan labelling (22.7 %).

To validate the use of filipin for the overall visualization of free cholesterol at the cell surface, we investigated whether filipin colocalizes with two well-defined markers of membrane rafts: the glycosphingolipid GM1 and the protein CAV1. CTB, which binds to GM1 in the PM, is hypothesized to be enriched in membrane rafts [31–33]. When cells were incubated with CTB at 4 °C, the toxin appeared widely distributed within the PM (Figure 2D; see Figure 2E for a high magnification of selected areas). Although an overlap with filipin was evident (arrows in Figures 2D and 2E), GM1 showed a more diffuse distribution and was not located exclusively to filipin-stained domains. Next, we analysed whether filipin colocalizes with CAV1. In COS cells, CAV1 was detected in the Golgi complex and in a punctate pattern in the PM (Figures 2F and 2G). In contrast with filipin, CAV1 did not accumulate at the edge of the cells and showed a less homogeneous distribution. Although some of the filipin-enriched domains could be seen to colocalize with CAV1 at high magnification (arrows in Figure 2G), most did not, suggesting the existence of different subpopulations of cholesterol-enriched domains.

**TX promotes the reorganization of cholesterol in the PM**

As shown above by means of Laurdan imaging, TX promotes the formation of highly ordered domains. To determine whether a similar redistribution of cholesterol occurs upon TX treatment, COS cells solubilized with 1 % (v/v) TX at 4 °C were labelled with filipin. After a very short treatment (30 s), the pattern of cholesterol-rich domains lost its homogeneous distribution to form a network of tubular aggregates that occupied the entire membrane (compare Figures 3A and 3B). After 10 min, the membrane appeared fenestrated (Figure 3C), with numerous holes surrounded by a ring of cholesterol (arrowheads in Figure 3C). In the remaining membrane, cholesterol (arrows in Figure 3C) formed discrete ring-like, tubular and rounded structures that co-existed with filipin-negative domains. No significant additional changes were observed with longer incubations. Other authors have described similar TX-induced ‘Swiss cheese’-like morphologies of putative raft proteins [34,35]. Under the present experimental conditions, 26.2 ± 3.3 % of the total cellular protein was resistant to TX extraction. In contrast, 70.2 ± 6.6 % of the cellular cholesterol was resistant to the detergent. When fewer cells (equivalent to approx. 5 μg of protein and 0.04 μg of cholesterol per coverslip) were incubated with the same volume of detergent, cholesterol was not observed in discrete aggregates, but instead formed a homogeneous fenestrated sheet (Figures 3D and 3D'), indicating the importance of the molar ratio between detergents and membranes during the extraction. In addition, it is possible that changes in the concentration of proteins within the solubilized membranes modify significantly the effective detergent/lipid molar ratio required for micellization. In conclusion, these results demonstrate that TX promotes the reorganization and aggregation of cholesterol into newly formed structures of the PM, rather than a simple solubilization of specific domains.

**TX promotes the reorganization of GM1, but not CAV1, in the PM**

Glycosphingolipids and sphingomyelin segregate from glycerophospholipids independently of other lipids or cholesterol [36]. To elucidate whether sphingolipids co-segregate with cholesterol in response to TX, we studied the effect of the detergent on GM1. After incubation of the cells with TX for 10 min, GM1 and cholesterol were detected in the same domains of the PM (Figure 4A; see Figure 4B for a high magnification). This is in contrast with untreated cells, where only a partial colocalization was observed. After TX treatment, the overlap was visible in the majority of the membrane and, at high magnification, in the tubular aggregates of cholesterol (arrows in Figure 4B). The labelling of GM1 appeared more diffuse when compared with filipin, probably reflecting the fact that whereas filipin intercalates within cholesterol-enriched membranes, CTB binds to GM1 on the PM. The increased colocalization between cholesterol and
GM1 was confirmed and quantified by the Pearson correlation coefficient [36a], which shifts from $0.36 \pm 0.1$ in untreated cells to $0.80 \pm 0.05$ in the cells treated with TX (average values calculated from the analysis of 65 cells).

In contrast with cholesterol and GM1, CAV1 distribution in the PM was almost unaffected by treatment with TX (compare Figure 2F with Figure 4C). Although some CAV1 was found in the newly formed cholesterol domains, at high magnification very little colocalization was observed, and instead CAV1 accumulated at the edges of the holes (arrows in Figure 4D). It is generally accepted that the pool of CAV1 at the cell surface is relatively immobile unless the cells are perturbed experimentally [37–40], making CAV1 a good marker for stable PM-associated cholesterol-enriched domains. Thus the results shown in the present study may indicate that a preformed lipid domain, although unaffected by extraction with detergent, is displaced during the remodelling of the PM with TX. In contrast with CAV1 and GM1, under the experimental conditions used in the present study, a non-raft protein (the transferrin receptor) was completely solubilized by the detergent (results not shown).

Role of cholesterol in the TX-induced reorganization of the PM

So far, we have demonstrated that TX promotes the formation of ordered domains, and the clustering of cholesterol and GM1, in the PM without affecting the distribution of CAV1. This prompted us to identify a biochemical method which would prevent such reorganization without affecting preformed ordered domains. It has been described previously that some GPI-anchored proteins are almost, but not completely, soluble when cells are treated with saponin, to complex cholesterol, prior to extraction with TX [41,42]. In contrast, CAV1 is highly resistant to the extraction with saponin (results not shown). Taking these precedents into account, we hypothesized that saponin could complex cholesterol and, consequently, prevent its clustering in response to TX treatment.

Therefore COS cells were treated for 10 min with TX/sap, and the distribution of cholesterol, GM1 and CAV1 was analysed. The combination of detergents did not induce formation of the characteristic holes in the PM that were observed after TX solubilization (Figures 5A–C). Cholesterol was restricted to discrete rounded structures, frequently connected by tubular elements, which coexisted with filipin-negative domains. The shape and size of the filipin-enriched domains resembled those described in untreated cells (compare Figures 5C and 2C). In addition, some of the filipin-enriched domains overlapped with CTB (Figures 5D and 5E). The Pearson correlation analysis confirmed the increased colocalization between cholesterol and GM1 in response to TX/sap treatment ($0.66 \pm 0.6$, in contrast with $0.36 \pm 0.1$ in untreated cells), although it was markedly lower than the colocalization shown after solubilization with TX ($0.80 \pm 0.05$).
Triton X-100 condenses the plasma membrane

**Figure 5** The presence of saponin inhibits the TX-induced aggregation of cholesterol and GM1

(A–C) Cells were solubilized with TX/sap for 10 min, fixed in PFA and incubated with filipin. Cholesterol aggregates in discrete tubulo-vesicular structures that resemble in shape and size those described in untreated cells (compare A, B, and C with Figures 1A, 1B and 1C). (D, E) Cells were incubated with CTB, solubilized for 10 min with TX/sap, fixed with PFA and labelled with filipin. CTB colocalizes with some of the tubulo-vesicular filipin-enriched structures (arrows in D and E). (F, G) TX/sap-treated and fixed cells were incubated with filipin and labelled with anti-CAV1 antibodies. CAV1 partially colocalizes with filipin labelling (arrows in G). (H) COS cells (black circles) were incubated for 10 min in TX/sap (white circles) then labelled with Laurdan, and the GP was calculated as described in the legend to Figure 1. The addition of TX/sap to the cells did not modify the mean GP of condensed domains (0.47), but increased its relative enrichment at the cell membrane from 22.1 to 61.9%. The average S.D.s of mean GP-values for fluid and condensed populations are both 0.06, and the average S.D. of coverage is 5.5%. For (A–D, F), scale bar, 20 μm; for (E, G), scale bar, 5 μm.
In addition, in contrast with the results of TX treatment, CAV1 was not excluded from the filipin-enriched domains (Figures 5F and 5G). Under these experimental conditions, 29 ± 7.5% of the protein and 59.7 ± 9.5% of cellular cholesterol (compared with 70.2% in TX-treated cells) was resistant to extraction with TX/sap. Finally, we studied the TX/sap-mediated solubilization of cells by means of two-photon microscopy. Interestingly, the addition of TX/sap for 10 min to the cells (white circles in Figure 5H) did not modify the mean GP of condensed domains (0.47), but increased considerably the relative enrichment of condensed domains at the cell membrane (to 61.9%) (table in Figure 5H). Therefore, in contrast with the solubilization mediated by TX, the mixture of detergents does not alter the condensation of ordered domains, although it efficiently solubilizes fluid domains.

**DISCUSSION**

The essence of the raft concept proposes that there is a lipid-driven segregation of membrane components to form distinct domains. Raft lipids, particularly glycosphingolipids and cholesterol, may pack together tightly and promote an active separation of other lipids, such as glycerophospholipids. In the present study, we have visualized how cold TX promotes a similar, although artificial, lateral segregation of domains in the PM of COS cells.

Insolubility in cold TX has been often used as the biochemical criterion to define the existence and function of lipid raft components. Indeed, DRMs were considered to be the isolated cellular fraction that is equivalent to membrane rafts. Although it is clear that DRMs do not reflect the organization of the PM, the insolubility of lipids and proteins in cold detergents identifies a potential link of these molecules with membrane rafts. Indeed, a non-raft protein, such as the transferrin receptor, is completely solubilized by TX. However, in the present study, we have observed that TX induces a profound remodelling of the PM. In response to TX treatment, cholesterol and GM1 were relocated and clustered into new structures, inducing the formation of holes at the cell surface. We demonstrated that TX partially solubilizes fluid domains (48.6% of the cell membrane remains in a fluid state, with a GP of 0.079) and promotes the formation of new, highly ordered domains (with a new GP of 0.64), which are absent in native membranes.

These results clearly suggest that molecules with raft-affinity redistribute dynamically in response to the detergent to condense domains. After incubation with TX, GM1 and cholesterol showed a high degree of colocalization, which was not observed in untreated cells. Whether these apparently new, highly ordered domains are created by the coalescence of pre-existing membrane rafts that are below the resolution of the microscope cannot be resolved. However, it seems unlikely that pre-existing rafts are responsible for the dramatic membrane condensation seen here. Heerklotz et al. [16] have shown that sphingomyelin-rich domains that are present in model membranes containing phosphatidylycholine/sphingomyelin/cholesterol (1:1:1, molar ratio) are only marginally stable. The slight perturbation of the membrane (by 0.2 kJ/mol) that is caused by TX addition induces substantial domain formation in a previously homogeneous membrane, but in addition it may change the composition of pre-existing domains. Interestingly, the degree of solubilization also depends on the cell/detergent ratio, possibly reflecting the fact that the saturating critical ratio (at which solubilization begins and detergent-rich micelles form) is determined by lipids and by the presence of proteins within the solubilized membranes. It is remarkable that in response to TX, highly ordered domains generally formed in regions where the cell membrane already presented a relatively high degree of condensation. As the PM is composed of a complex mixture of lipids and proteins, it is possible to speculate on the presence of naturally occurring raft stabilizers/raft promoters that may favour TX-induced domain formation in specific regions of the asymmetrical bilayer. In this context, the role of the highly insoluble cortical actin as a determinant for raft ‘nucleation sites’ is an interesting concept that deserves further attention. In fact, the distribution of CAV1 at the cell surface was unchanged by TX, suggesting that caveolae are apparently unaffected by extraction with the detergent. However, a more detailed biochemical analysis will be required to characterize how detergents promote further condensation or modify the lipid/protein composition of caveolae.

When cholesterol is physically complexed by saponin treatment, the results of the present study show that lipid clustering and hole formation is inhibited, cholesterol accumulates in structures that resemble its original domains in location, shape and size, and CAV1 colocalizes with the remaining filipin-enriched domains. In addition, the combination of TX and saponin does not promote the formation of new condensed domains, but efficiently solubilizes fluid domains at the cell surface. In contrast to TX-extracted cells (70%), only 59.7% of the cellular free cholesterol was insoluble by TX/sap treatment. Accordingly, we established that saponin treatment inhibits the TX-induced remodelling of the PM [34]. In agreement with this, the degree of condensation of the insoluble domains was unaffected by the mixture of detergents (GP = 0.47), but the relative area corresponding to condensed domains increased from 22% to 62%. Consequently, it is clear that the ability to partition into insoluble domains in the presence of TX reflects a potential affinity for ordered domains. In addition, the ability to partition into insoluble domains in the presence of TX/sap may provide a supplementary biochemical criterion to define stable and ordered domains at the PM. For example, as commented above, it has been described that some GPI-anchored proteins are almost, but not completely, soluble when cells are treated with saponin, in order to complex cholesterol, prior to extraction with TX [41, 42]. Further investigation will be necessary to determine whether the molecules that possess the affinity to associate with DRMs maintain the capability to aggregate into the newly formed domains in the absence of cholesterol. In fact, DRMs can be isolated from membranes lacking cholesterol [43, 44]. However, some authors have described a differential redistribution of GPI-anchored proteins from DRMs into the TX-soluble fractions in response to acute depletion of cholesterol [45, 46]. In various cell types, the ceramide moiety of GM1 determines whether the molecular variants of the glycosphingolipid require cholesterol in order to fractionate in DRMs [47].

In conclusion, the use of TX for studying stable lipid domains at the cell surface should be carefully interpreted and necessarily complemented with high-resolution methodologies. We propose that the use of mixtures of detergents with different solubilization and/or fixation properties could provide an additional biochemical criterion for the study of stable and ordered domains at the PM.

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