

Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane

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Sphingolipid microdomains are thought to result from the organization of plasma membrane sphingolipids and cholesterol into a liquid ordered phase, wherein the glycosylphosphatidylinositol (GPI)-anchored proteins are enriched. These domains, resistant to extraction by cold Triton X-100, can be isolated as buoyant membrane complexes (detergent-resistant membranes) in isopycnic density gradients. Here the effects of methyl- β -cyclodextrin (MBCD), a specific cholesterol-binding agent that neither binds nor inserts into the plasma membrane, were investigated on the sphingolipid microdomains of lymphocytes. MBCD released substantial quantities of GPI-anchored Thy-1 and glycosphingolipid GM1, and also other surface proteins including CD45, and intracellular Lck and Fyn kinases. From endothelial cells, MBCD released GPI-anchored CD59, and CD44, but only a negligible amount of caveolin. Most MBCD-

released Thy-1 and CD59 were not sedimentable and thus differed from Thy-1 released by membrane-active cholesterol-binding agents such as saponin and streptolysin O, or Triton X-100. Unlike that released by Triton X-100, only part of the Thy-1 molecules released by MBCD was buoyant in density gradients and co-isolated with GM1. Finally, treatment of Triton X-100-isolated detergent-resistant membranes with MBCD extracted most of the cholesterol without affecting the buoyant properties of Thy-1 or GM1. We suggest that (1) MBCD preferentially extracts cholesterol from outside, rather than within the sphingolipid microdomains and (2) this partly solubilizes GPI-anchored and transmembrane proteins from the glycerophospholipid-rich membrane and releases sphingolipid microdomains in both vesicular and non-vesicular form.

INTRODUCTION

The current view of the three-dimensional organization of the mammalian cell plasma membrane envisages selective interactions among the membrane lipids and proteins resulting in physical and functional compartmentation into dynamic microdomains [1,2]. The presence of sphingolipid-rich microdomains in the plasma membrane of both polarized and non-polarized cells has been inferred by biochemical and biophysical methods. Sphingolipid microdomains isolated after non-ionic detergent extraction of membranes are enriched in glycosylphosphatidylinositol (GPI)-anchored proteins in the exoplasmic leaflet and harbour doubly acylated Src-family protein tyrosine kinases (PTKs) in the cytoplasmic leaflet (reviewed in [3,4]). The GPI-linked surface glycoproteins and acylated Src kinases of sphingolipid-rich microdomains remain associated with trilamellar vesicles after extraction with cold non-ionic detergent; these detergent-resistant membranes (DRMs) can be separated from solubilized membrane components by size [5,6] or density [7]. The caveolar membrane specialization is also detergent-resistant [8], but accumulated evidence now supports the notion that caveolae and sphingolipid microdomains represent distinct plasma membrane entities [9,10].

The physical properties of sphingolipid microdomains seem to arise from the interaction of sphingolipid hydrocarbon chains with cholesterol, which can form a liquid ordered phase in the liquid-crystalline phospholipid milieu of the plasma membrane outer leaflet [3,11,12]. Cholesterol associates with sphingolipids in the Golgi complex and stabilizes the microdomains to which GPI-anchored proteins become associated by way of lipid-lipid interactions [4,7]. A number of studies have examined the

influence of cholesterol on the physical and functional properties of GPI-anchored proteins in sphingolipid microdomains and caveolae by using different methods to lower cellular cholesterol levels such as (1) cholesterol deprivation by culturing cells in media devoid of exogenous cholesterol sources [13–15], (2) inhibition of cholesterol biosynthesis by compactin [16], lovastatin or squalenstatin [17,18] and (3) depletion of cholesterol by oxidation to cholestenone [19] or by cholesterol-binding agents such as filipin, nystatin, digitonin, saponin or streptolysin O (SLO) [16,20–24]. These studies have shown that lowering the cellular cholesterol levels markedly affected the properties of GPI-anchored proteins and led to their dispersion on the cell surface [16], a decrease in their cell-surface expression [20], their release as membrane vesicles [24], an increase in solubility in non-ionic detergents [15,20,22,23] and an impairment of their signalling properties [18]. On caveolae, cholesterol deprivation or depletion decreased the number of caveolae and decreased caveolae-mediated intracellular and transcellular transport of macromolecules [16,21,25].

The different ways in which cellular cholesterol levels were lowered had very different consequences on GPI-anchored proteins and caveolae. For example, treatment with cholesterol-binding agents such as filipin and saponins resulted in the dispersion of GPI-anchored proteins at the cell surface and facilitated their release from detergent-resistant sphingolipid microdomains [20], whereas the inhibition of cholesterol biosynthesis by lovastatin did not [18]. Interestingly, certain cholesterol-binding agents such as SLO released membrane vesicles enriched in GPI-anchored proteins and probably released whole sphingolipid microdomains as well [24]. Likewise, cholesterol depletion by filipin or nystatin decreased the number of caveolae

Abbreviations used: DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; HRP, horseradish peroxidase; MBCD, methyl- β -cyclodextrin; PTK, protein tyrosine kinase; SLO, streptolysin O.

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[16,21], whereas cholesterol oxidation to cholestenone did not affect the number or morphology of caveolae but dispersed caveolin from plasma membrane to intracellular vesicles [19]. In contrast, cellular cholesterol depletion by biosynthetic inhibition did not affect the membrane-associated caveolin [26]. Whereas cholesterol deprivation and inhibition of cholesterol biosynthesis both decreased the steady-state level of membrane cholesterol to approx. 70% [15,18], the polyene antibiotics filipin and nystatin [27,28], the sapogenin glycosides saponin and digitonin [29,30] and thiol-activated cytolysin SLO [31] complexed with cholesterol *in situ* to form multimeric globular deposits in the membrane.

In the present study we examined the effects of the cholesterol-binding agent methyl- β -cyclodextrin (MBCD) on the detergent insolubility and buoyancy of GPI-anchored proteins and the GM1 glycosphingolipid (GSL) in murine lymphocytes. Unlike other cholesterol-binding agents that incorporate into membranes, cyclodextrins are strictly surface-acting and selectively extract membrane cholesterol by including it in a central, non-polar cavity of cyclic oligomers of glucopyranoside in α -1,4 glycosidic linkage [32]. α , β and γ cyclodextrins are crystalline, water-soluble compounds that differ in having six, seven and eight glucose residues respectively. Additional substitutions on the external hydroxy groups of glucose and the degree of substitution improve the solubility of the cholesterol-loaded cyclodextrin molecules. Although cyclodextrins have been in use for many years as carriers of lipophilic drugs in pharmacological research, their potential use in membrane studies has been appreciated only recently. β -Cyclodextrins have been shown to extract membrane cholesterol selectively from a variety of cell types [33–35] and represent unique tools for membrane studies in that they neither bind nor insert into the plasma membrane [33]. Recently, with the use of MBCD as a cholesterol-depleting agent, the association of influenza virus haemagglutinin was shown to be critically dependent on cholesterol [36] and necessary for apical sorting [37]. In our experiments with non-polarized, murine T lymphocytes, we observed that cholesterol extraction by MBCD led to the release of endogenous GPI-anchored proteins, transmembrane glycoproteins and membrane-associated PTKs with discernible changes in cellular morphology, but without lysis. The physical properties of Thy-1 and GM1 released by MBCD were markedly different from those released by non-ionic detergents or other cholesterol-binding agents. In addition, a substantial amount of cholesterol was also extracted from Triton X-100-isolated DRMs without affecting the buoyancy of the DRM-associated Thy-1 and GM1, suggesting that the cholesterol extracted by cyclodextrin is not required for the organization of GPI-anchored proteins and sphingolipids in the buoyant membranes.

EXPERIMENTAL

Materials

MBCD was purchased from Aldrich Chemie (Steinheim, Germany). Triton X-100, bicinchoninic acid protein assay kit and horseradish peroxidase (HRP) enhanced chemiluminescence reagent were from Pierce (Rockford, IL, U.S.A.). Rat monoclonal antibody against murine Thy-1.2 (30-H12; ATCC TIB107) and CD45 (M1/9.3.4HL.2; ATCC TIB122) were from the American Type Culture Collection (Rockville, MD, U.S.A.). Rabbit polyclonal antibodies against Lck and Fyn tyrosine kinases and HRP-conjugated goat anti-(rat IgG) and goat anti-(rabbit IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). SLO was from Calbiochem (La Jolla, CA, U.S.A.).

The murine T-lymphoma cell line P1798 was obtained from Litton Bionetics (Bethesda, MD, U.S.A.) and maintained in

syngeneic Balb/c mice as ascites. Cells collected from the ascitic fluid were washed in PBS twice and once in TKM buffer [50 mM Tris/HCl (pH 7.4)/25 mM KCl/5 mM MgCl₂/1 mM EDTA]. Human umbilical vein-derived endothelial cell line ECV304 was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum.

Cells, cyclodextrin treatment, detergent lysis, plasma membrane preparation and sucrose gradient centrifugation

P1798 cells (2×10^7) were treated with the indicated concentrations of MBCD in 1 ml of TKM buffer at 37 °C for 30 min with intermittent shaking, and the cell-free supernatant was collected after centrifugation at 2000 *g* for 10 min. Triton X-100 lysates were prepared by lysing 10^8 cells for 15 min on ice in 1 ml of TKM containing 1% (v/v) Triton X-100 (TKM/Triton X-100) and the protease inhibitors Pefabloc[®] SC (2 mM), leupeptin (1 μ M) and aprotinin (2 μ g/ml), all from Boehringer Mannheim (Mannheim, Germany). The post-nuclear supernatants, obtained after centrifugation of the lysate in a Microfuge for 5 min at 4 °C, were assayed for protein content by microtitre bicinchoninic acid assay and stored at –20 °C. SDS lysates were prepared by boiling 10^8 cells in 1 ml of 10 mM Tris/HCl, pH 7.4, containing 1% (w/v) SDS and then sonicated.

To prepare total plasma membrane, 10^9 cells suspended in 5 ml of hypotonic lysis buffer [10 mM Tris/HCl (pH 7.4)/10 mM KCl/5 mM MgCl₂/1 mM EGTA] containing protease inhibitors were homogenized with a Dounce homogenizer and centrifuged at 2000 *g* to sediment the nuclei. The homogenate was layered over a 40% (w/v) sucrose cushion and centrifuged at 100000 *g* for 1 h. The light-scattering band above the sucrose cushion was collected, washed and resuspended in TKM buffer.

Equilibrium gradient centrifugation of detergent lysates or MBCD-released material was performed as described previously [38]. In brief, 1 ml of the Triton X-100 lysate was mixed with an equal volume of 80% (w/v) sucrose, loaded into SW41 tubes and overlaid with 6 ml of 36% (w/v) sucrose and finally topped with 3.5 ml of 5% (w/v) sucrose in TKM not containing Triton X-100. After centrifugation at 250000 *g* for 16–18 h, 1 ml fractions were collected from the top and stored at –20 °C. The top fractions (fractions 3 to 5) corresponding to the 5–36% sucrose interface, which contained membrane complexes enriched in GSLs and GPI-anchored proteins [38], were pooled and are referred to as DRMs. MBCD-released material was subjected to sedimentation gradient centrifugation in the same manner.

Detection of membrane-associated proteins and GM1 ganglioside

Detection of membrane proteins in the MBCD-released material and detergent lysates was performed by Western blotting. After SDS/PAGE under non-reducing (for cell surface antigens Thy-1, CD45 and CD59) or reducing (for intracellular proteins Lck, Fyn and caveolin) conditions, the separated proteins were transferred to nitrocellulose membrane. The blots were blocked with 5% (w/v) non-fat milk powder in TBST [10 mM Tris/HCl (pH 7.5)/100 mM NaCl/0.1% (v/v) Tween 20], probed with the primary antibody in TBST/0.5% milk powder followed by appropriate HRP-conjugated second antibody and the enhanced chemiluminescence reagent. GM1 in gradient fractions or MBCD-released material was detected by binding of cholera toxin: dot-blotted nitrocellulose filter strips were blocked with 5% (w/v) milk powder, incubated with biotinylated cholera toxin (Sigma Chimie, Buchs, Switzerland) in TBST/0.5% milk powder, washed and revealed by enhanced chemiluminescence.

Cholesterol detection

Total plasma membrane and DRMs were pelleted and extracted in chloroform/methanol (2:1, v/v). The organic extract was separated on TLC plates (Silicagel 60; Merck) with chloroform/acetone (96:4, v/v) and dried in air. Cholesterol was revealed by spraying with FeCl_3 and heating at 100 °C for 3–5 min [39].

RESULTS

Removal of membrane cholesterol by MBCD releases GPI-linked surface molecules, GSLs, transmembrane receptors and membrane-associated PTGs

To investigate the effects of cholesterol removal on membrane proteins, cells were first treated with MBCD, and then sequentially with Triton X-100 and SDS. Analysis of the cell-free supernatant after treatment with MBCD showed that a substantial amount of Thy-1 was released (Figure 1A, lane 3) compared with spontaneous release in buffer at 37 °C (lane 2) and approx. 5-fold more than that released by 1% (v/v) Triton X-100 at 4 °C (lane 4). Release of Thy-1 by MBCD was already

measurable at 1 min and reached a maximum after 20 min (Figure 1B). The smaller amount of Thy-1 measured in the cell-free supernatant after 40 min of incubation with MBCD could be due to reincorporation [40] in a form no longer releasable by MBCD. Extraction of the cell pellet with Triton X-100 after treatment with MBCD released additional Thy-1 (Figure 1A, lane 6) in amounts not significantly different from that released from untreated cells by Triton X-100 directly (lanes 4 and 5). Subsequent extraction of the remaining nuclear pellets with SDS showed that the cells treated with Triton X-100 alone still retained the bulk of Thy-1 (Figure 1A, lanes 7 and 8), whereas cells treated with MBCD and Triton X-100 (lane 9) contained no more Thy-1, indicating that cholesterol depletion by MBCD followed by extraction with Triton X-100 led to a quantitative release of cell-surface Thy-1. MBCD at 20 mM released 6-fold more protein than buffer alone (see the legend to Figure 1). Flow cytometric analysis showed a significant decrease in the volume of cells treated with 2.5 and 20 mM MBCD, as seen by a decrease in the right-angle light scattering (Figure 1C). Approximately half of the transmembrane receptor CD45 was also released by MBCD treatment (Figure 1A, lane 3) and the remainder was

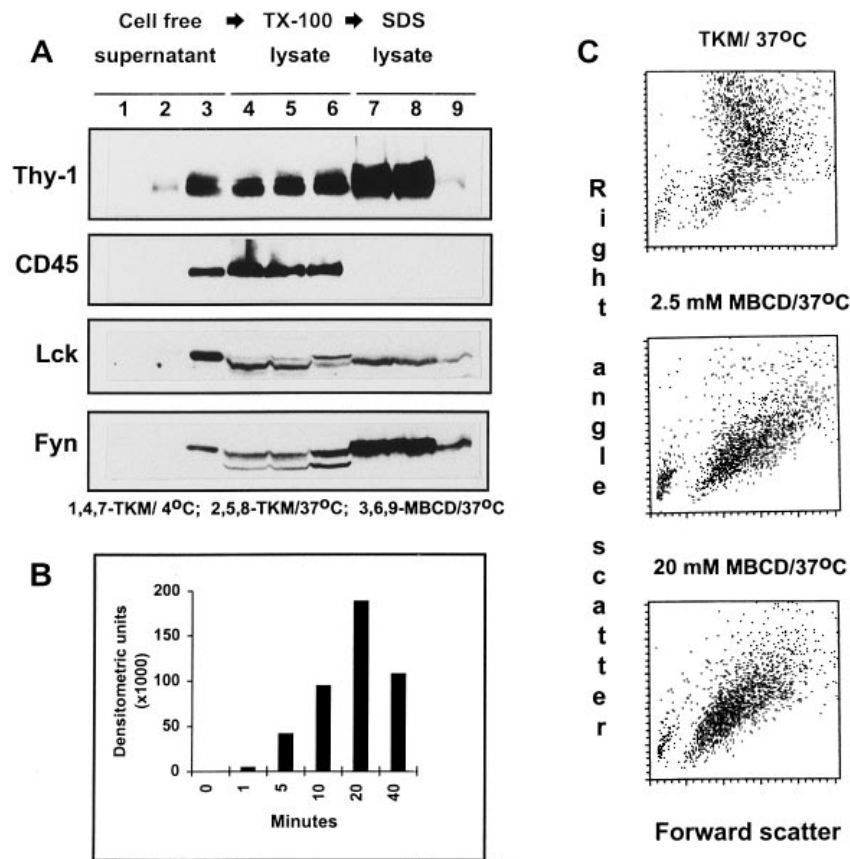


Figure 1 MBCD releases Thy-1 and other membrane-associated proteins with a concomitant decrease in cell volume

(A) P1798 murine T lymphoma cells (10^8) were incubated for 30 min in TKM buffer on ice (lanes 1, 4 and 7) or at 37 °C (lanes 2, 5 and 8), or in TKM buffer containing 20 mM MBCD at 37 °C (lanes 3, 6 and 9) in a 5 ml volume. The cell-free supernatants in a typical experiment contained 0.27, 0.4 and 2.5 mg/ml proteins, amounting to the release of 10.5 mg of protein by MBCD from 10^8 cells. The cell pellets were washed in cold TKM buffer and lysed in 1 ml of TKM/1% (v/v) Triton X-100 (TX-100) for 15 min on ice. After centrifugation the Triton X-100 lysates were saved and the remaining cell pellets were lysed in 1 ml of SDS lysis buffer [10 mM Tris/HCl (pH 7.4)/1% (w/v) SDS], boiled and then sonicated. Cell-free supernatants, Triton X-100 lysates and SDS lysates (20 ml of each) (note that the volume ratio was 5:1:1) were Western blotted for Thy-1, CD45, Lck and Fyn. (B) Cells (2×10^7) were incubated in 1 ml of TKM buffer containing 20 mM MBCD at 37 °C for the indicated durations, and Thy-1 detected by Western blotting in 20 ml of the cell-free supernatants was quantified densitometrically. (C) P1798 cells (2×10^7) were incubated in 1 ml of TKM either alone or in the presence of 2.5 or 20 mM MBCD for 30 min at 37 °C. The cells were washed and analysed by flow cytometry for forward and right-angle light scattering.

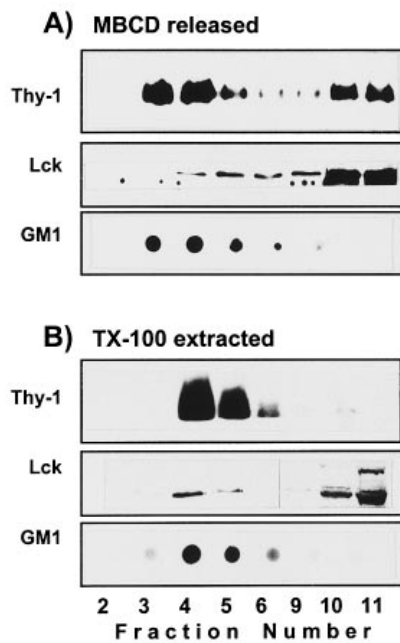


Figure 2 Buoyant properties of the MBCD-released and Triton X-100-extracted plasma membrane-associated molecules

P1798 cells were treated with 20 mM MBCD for 30 min at 37 °C in TKM buffer at 2×10^7 cells/ml (A) or extracted by TKM/1% (v/v) Triton X-100 (TX-100) for 15 min on ice at 10^6 cells/ml (B). Cell-free supernatant (1 ml) was adjusted to 40% (w/v) sucrose, overlaid with 36% and 5% (w/v) sucrose (not containing Triton X-100) in SW41 tubes and subjected to equilibrium gradient centrifugation as described in the Experimental section. Fractions of 1 ml were collected from the top; 20 ml of the indicated fractions were analysed for the distribution of Thy-1 and Lck by Western blotting. GM1 in 5 ml of the fractions was detected by dot-blotting with HRP-conjugated cholera toxin. Fractions 3–5 correspond to the low-density membrane complexes at the 5–36% (w/v) sucrose interface; fractions 9–11 correspond to the high-density membrane complexes and solubilized membrane and cytosolic proteins at the sample loading zone.

released quantitatively by further extraction of MBCD-treated cells with Triton X-100 (lane 6). Cell-surface biotinylation and immunoprecipitation with specific antibodies showed that MBCD released a number of cell-surface molecules including CD26 and MHC class I (results not shown). Substantial amounts of the intracellular, membrane-associated PTKs Lck and Fyn were also released by MBCD (Figure 1A, lane 3). Subsequent extractions with Triton X-100 and SDS showed that MBCD-treated cells yielded significantly less PTKs in the final SDS extract than in the control cells (Figure 1A, lanes 4–9). These results showed that MBCD released the membrane-associated proteins in a manner distinct from that of the non-ionic detergent Triton X-100.

To define the form in which the GPI-linked glycoprotein Thy-1 was released by MBCD, the cell-free 2000 g supernatant was subjected to equilibrium density gradient centrifugation and compared with that of Triton X-100-extracted material (Figure 2). The distribution of GM1, which co-isolates with GPI-anchored proteins in density gradients [38], was also examined. More than 60% of the Thy-1 and all of the GM1 in the MBCD-released material were recovered in the low-density fractions, which contained a small part of the released Lck as well (Figure 2A). In comparison, all of the Thy-1 and GM1, and a significant quantity of the Lck, extracted by Triton X-100 were present in the low-density fractions as buoyant membrane complexes.

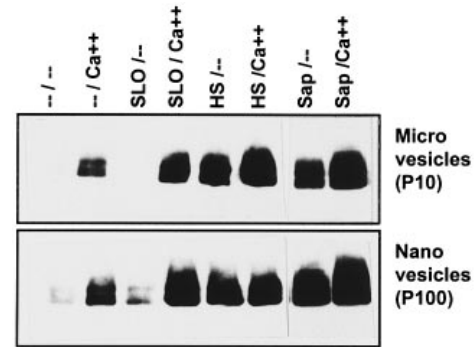


Figure 3 Saponin, SLO and heat shock release sedimentable plasma membrane vesicles rich in Thy-1

P1798 cells in 10 mM Hepes-buffered saline, pH 7.4 (5×10^6 cells/ml) were incubated either alone or in the presence of 1 mM CaCl_2 at 37 °C for 45 min. After this, aliquots of cells were treated with SLO (20 i.u./ml) or saponin (Sap., 0.15 mg/ml) for 15 min on ice, and SLO-treated cells were washed. Another aliquot was given a heat shock (HS) at 42 °C for 15 min. All were shifted to 37 °C and incubated for 45 min. The 2000 g cell-free supernatants were subjected sequentially to centrifugation at 10000 g (P10) and 100000 g (P100) for 30 min and 1 h to sediment microvesicles and nanovesicles respectively. The amount of released Thy-1 in the sedimented vesicles was determined by Western blotting. The 100000 g supernatant did not contain a significant amount of Thy-1 (results not shown).

Approx. 40% of the Thy-1 and 90% of the Lck released by MBCD were non-buoyant and remained in the sample loading zone of the gradient, suggesting their minimal association with lipids and/or association with protein-rich complexes.

Treatment with MBCD releases heterogeneous membrane complexes from caveolin-free lymphocytes and caveolin-containing endothelial cells

Treatment of erythrocyte membranes with calcium ionophores and nucleated cells with cholesterol-binding drugs have been shown to induce the release of membrane vesicles enriched in GPI-anchored glycoproteins sedimentable at 10000 g (microvesicles) and 100000 g (nanovesicles) [24,41]. We observed that heat-shock treatment of T lymphoma cells at 42 °C for 15 min can induce vesiculation similar to that induced by the cholesterol-binding agent saponin, a phenomenon that is augmented only marginally by Ca^{2+} ions (Figure 3). In contrast, the pore-forming and cholesterol-binding agent SLO released such vesicles only in the presence of Ca^{2+} ions. In all these cases most of the Thy-1 was pelleted at 100000 g and only a negligible quantity was recovered in the supernatant (results not shown), indicating that most of the released Thy-1 was in the form of sedimentable membrane vesicles.

To examine the vesicular or non-vesicular nature of the MBCD-released membrane components, sequential sedimentation at different centrifugal forces was performed (Figure 4A). Most of the GM1 was released as vesicular complexes sedimenting at 100000 g (P100), because only a very small amount was present in the 250000 g supernatant (S250). In contrast, a minor proportion of Thy-1 and substantial amounts of Lck and Fyn were sedimented at 100000 g. Increasing the centrifugation to 250000 g did not sediment a further subset of these molecules (Figure 4A), whereas under similar conditions most of the Triton X-100-released Thy-1 and Lck were sedimentable (results not shown). In sucrose density gradients, these different sedimentable

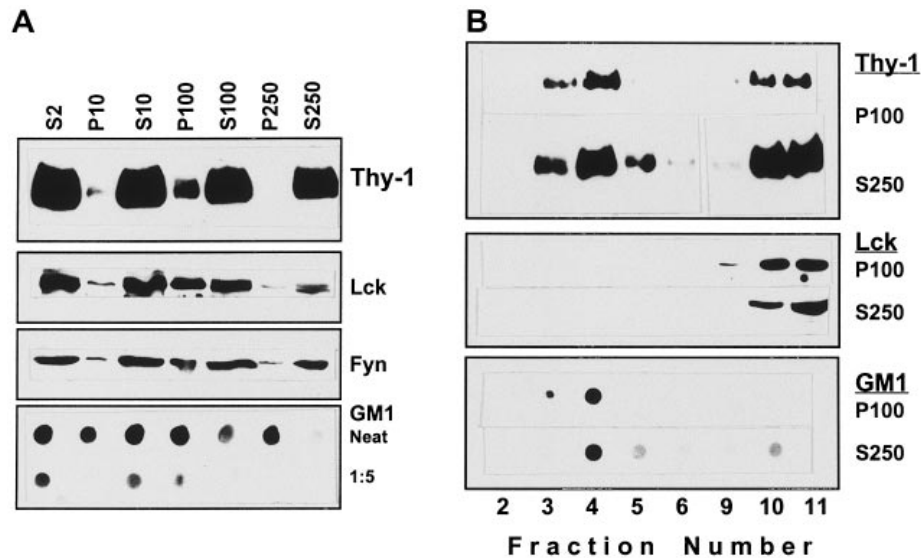


Figure 4 MBCD-released, plasma membrane-associated molecules are heterogeneous in their physical properties

(A) Sedimentability of Thy-1, GM1 and Src-family PTKs. After treatment of 10^8 P1798 cells with 20 mM MBCD at 37 °C for 30 min in a 5 ml volume, the 2000 *g* cell-free supernatant (S2) was subjected to sequential centrifugation at 10000 *g*, 100000 *g* and 250000 *g*. The pellet at each step was resuspended in the original volume of TKM buffer. An equal volume (20 ml) of the supernatant (S) and pellet (P) fractions from each sedimentation step (S10, P10; S100, P100; S250, P250) were tested for Thy-1 and for Lck and Fyn kinases by Western blotting. GM1 was detected by dot-blotting from 5 ml (undiluted) and 1 ml (diluted 1:5) of the samples with HRP-conjugated cholera toxin. (B) Buoyancy in sucrose density gradient. P100 or S250 fractions from (A) (1 ml of each) were subjected to equilibrium gradient centrifugation and the presence of the indicated molecules in the gradient fractions was tested as described for Figure 2.

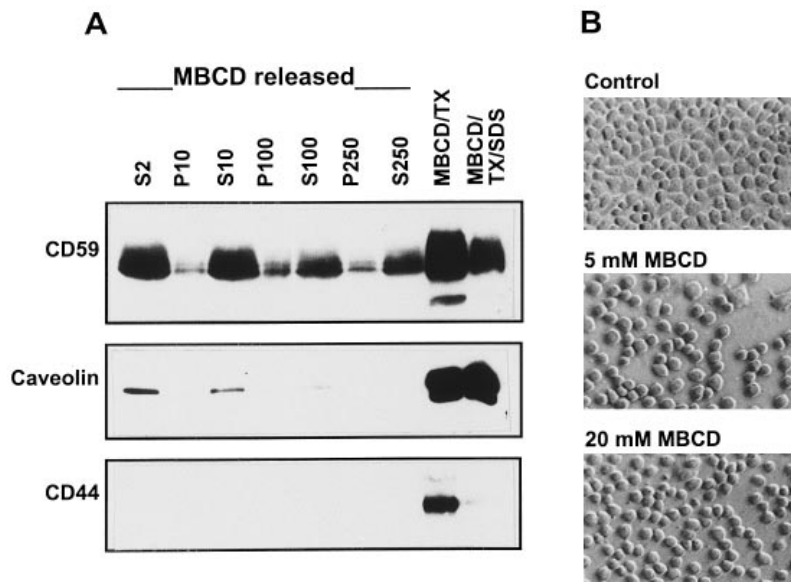


Figure 5 Effects of MBCD on endothelial cells

(A) MBCD releases GPI-anchored CD59. ECV304 human endothelial cells grown to confluency in Petri dishes 10 cm in diameter were washed in PBS and incubated in 5 ml of 20 mM MBCD in PBS for 30 min at 37 °C. The cell-free supernatant (2000 *g*; S2) was subjected to sequential centrifugation at 10000 *g*, 100000 *g* and 250000 *g*. The pellet at each step was resuspended in the original volume of PBS. The cell pellet was sequentially lysed in 1 ml of 1% (v/v) Triton X-100 (TX) and 1% (w/v) SDS as described for Figure 1. The supernatant (S) and pellet (P) fractions (20 ml of each) from each sedimentation step (S10, P10; S100, P100; S250, P250), and the Triton X-100 and SDS lysates, were tested for CD59, caveolin and CD44 by Western blotting. (B) Effect of MBCD on the morphology of ECV304 cells. Confluent cultures of ECV304 cells were washed in PBS and incubated with 5 or 20 mM MBCD in PBS at 37 °C for 30 min and examined by phase-contrast microscopy. Magnification $\times 100$.

and non-sedimentable fractions were again separated into heterogeneous subsets of molecules (Figure 4B). Thy-1 distributed into low-density and high-density fractions in the 100000 *g* pellet (P100) and 250000 *g* supernatant (S250) as in the first supernatant

(S2) (compare Figure 2A). In contrast, all of the Lck in both the P100 and S250 fractions was not buoyant and remained in the sample loading zone (Figure 4B). These results suggest that in the process of extracting cholesterol, MBCD releases GPI-

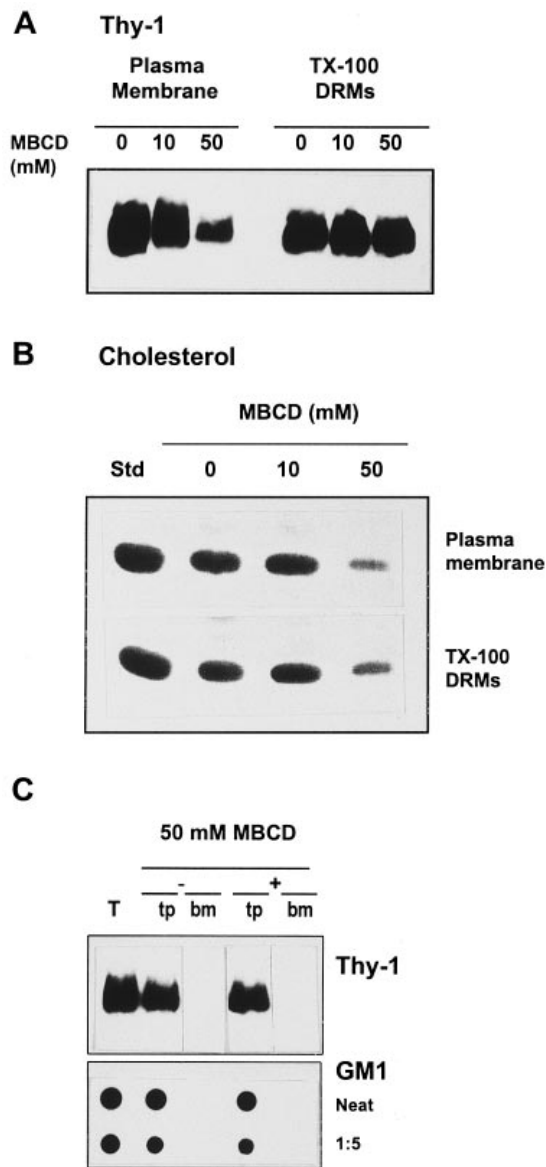


Figure 6 MBCD extracts cholesterol from DRMs without affecting the buoyant properties of Thy-1 and GM1

(A) Aliquots of total plasma membrane and DRMs (sedimented from pooled low-density fractions 3–5 from a gradient of Triton X-100 (TX-100)-lysed cells as shown in Figure 2B) containing equivalent amounts of total protein were treated with the indicated concentrations of MBCD in TKM buffer for 30 min at 37 °C. The amount of Thy-1 in the sedimentable membrane complexes was tested by Western blotting. (B) Lipids from the sedimented membrane complexes were extracted and the amount of cholesterol remaining was analysed by TLC as described in the Experimental section. (C) Gradient-isolated DRMs from 10^8 Triton X-100-lysed cells were sedimented, resuspended in 1 ml of TKM buffer without or with 50 mM MBCD, and incubated for 30 min at 37 °C. After a second gradient centrifugation the distribution of Thy-1 and GM1 in pooled top (tp, buoyant) and bottom (bm, non-buoyant) fractions were analysed as described for Figure 2.

anchored proteins that are heterogeneous in their physical properties: (1) the buoyant Thy-1 in P100 is similar to the Triton X-100-released vesicular Thy-1, whereas the non-buoyant Thy-1 in P100 seems to remain associated with the Triton X-100-resistant membrane matrix, and (2) the non-buoyant Thy-1 in S250 is likely to be completely solubilized, whereas the buoyant

Thy-1 in S250 might be complexed with sphingolipids, and possibly other lipids, in a non-vesicular form.

When the human ECV304 endothelial cells expressing caveolin were incubated with MBCD, a significant amount of GPI-anchored CD59 was released (Figure 5A), which showed a sedimentation behaviour identical to that of MBCD-released Thy-1 (Figure 3A). Subsequent lysis in Triton X-100 did not completely extract the remaining CD59, unlike Thy-1 in lymphocytes. A negligible quantity of caveolin, but no CD44, was released by MBCD (Figure 5A), showing that the release of membrane proteins by MBCD was selective. About half of the caveolin and all of the CD44 were released by subsequent Triton X-100 extraction, as reported previously [42]. Examination of the cellular morphology after MBCD treatment revealed discernible changes, including rounding up of cells and detachment from the substratum (Figure 5B).

MBCD releases cholesterol from DRMs but does not affect their buoyant properties

About 60% of the MBCD-released Thy-1 was buoyant in density gradients, indicating that the association of Thy-1 with membrane lipids was only partly disrupted by MBCD (Figures 2A and 4B). This suggested either that MBCD is ineffective in extracting cholesterol from the GPI-rich membrane domains, or that the buoyant properties of these domains are independent of their cholesterol content. To investigate these possibilities, we treated total plasma membrane and DRMs with MBCD and evaluated the Thy-1 and cholesterol contents of the sedimented membrane complexes. As shown in Figure 6(A), MBCD markedly decreased the amount of sedimentable Thy-1 from total plasma membrane but not from the DRMs, suggesting heterogeneity in the cholesterol/lipid environment of Thy-1 in lymphocyte plasma membranes. Nevertheless MBCD at 50 mM decreased the cholesterol contents of DRMs to almost the same extent as that of total plasma membranes (Figure 6B). Lastly, we investigated the buoyant properties of Triton X-100-isolated DRM domain constituents with or without prior treatment with MBCD. Results presented in Figure 6(C) show that the buoyancies of Thy-1 and GM1 were not affected by treatment with MBCD despite the loss of cholesterol. These results suggest that the cholesterol removed by MBCD is not required for determining the buoyant properties of Triton X-100-isolated DRMs.

DISCUSSION

The bulk of the cellular cholesterol is localized in the plasma membrane [43], where one of its functions is to maintain the liquid ordered state of sphingolipid microdomains [3,12,44]. Without cholesterol the hydrocarbon chains of sphingolipids would pack tightly to form a rigid gel-like phase. Hydrogen-bonding between the 3-OH group of cholesterol and the amide group of sphingolipids, but not the ester bonds of glycerophospholipids, presumably underlies the formation of sphingolipid-cholesterol liquid ordered domains [12] and contributes to their resistance to extraction with non-ionic detergent [45,46]. The second and main pool of plasma membrane cholesterol is found among the fluid, liquid-crystalline-phase glycerophospholipids [12] and a third pool of cholesterol is postulated to exist between the sphingolipid rafts and the glycerophospholipid milieu to provide a smooth transition in the energy states of the two phases [12,47].

Mammalian GPI-anchored proteins usually have saturated acyl chains in their lipid anchor [48] and should find the liquid ordered phase of sphingolipid-rich microdomains suitable for dynamic association [3]. The interaction of GPI-anchors with the

sphingolipid microdomains occurs in the *trans*-Golgi network and the interacting molecules acquire their detergent-resistant properties during biosynthetic transport to the cell surface [7]. However, it should be noted that Triton X-100 releases only a part of the GPI-anchored proteins as buoyant membrane complexes, whereas the remainder is bound to the membrane matrix still rich in GSLs (reviewed in [49]), probably corresponding to plasma membrane stably linked to cellular superstructures. Treatment with MBCD followed by extraction with Triton X-100 released most of the Thy-1 from the cell surface (Figure 1), whereas even after two sequential extractions Triton X-100 did not release Thy-1 completely (results not shown). It seems that the additional Thy-1 released by MBCD originated from the Triton X-100-resistant membrane matrix. This is in line with earlier observations that extraction with Triton X-100 in the presence of cholesterol-depleting saponin led to the complete release of Thy-1 from mast cells [23], alkaline phosphatase from transfected BeWo choriocarcinoma cells [20] and a number of GPI-anchored proteins from Madin–Darby canine kidney cells [22].

MBCD by itself released nearly half of the Thy-1 molecules from the cell surface. However, the physical nature of MBCD-released Thy-1 seems to be different from that released by Triton X-100 because it was mostly non-sedimentable and presumably not in a vesicular form, whereas almost all of the Triton X-100-released Thy-1 was vesicular and sedimentable. Similarly, in equilibrium density gradients most of the Triton X-100-extracted Thy-1 was buoyant, compared with only 60% of the MBCD-released Thy-1. However, inclusion of all of the MBCD-released GM1 in the buoyant material strongly suggested that only 60% of Thy-1 was present as GSL–GPI-rich complexes of high lipid-to-protein ratio. The actual proportion of glycerophospholipids in these complexes and their acyl chain composition have not been investigated.

Interestingly, most of the cholesterol (more than 80%) associated with Triton X-100-released vesicular complexes was still extractable by MBCD, but the remaining cholesterol resistant to MBCD extraction was sufficient to keep the DRM-associated Thy-1 and GM1 buoyant (Figure 6). This stands in contrast with the observations made on whole cells and reconstituted model membranes, in which cholesterol depletion by saponin led to the complete solubilization of GPI-anchored proteins by non-ionic detergents [20,23,46]. Unlike saponin, MBCD does not insert into the plasma membrane yet it has been reported to be very potent in extracting cholesterol: more than 90% depletion has been observed for erythrocytes [33] and influenza virus particles budding from Madin–Darby canine kidney cells [36]. Glycerophospholipids and glycoproteins are also released after treatment with MBCD, although no direct binding of MBCD to glycerophospholipids has been reported [50]. The release of non-cholesterol molecules from the plasma membrane by MBCD is thus likely to be indirect and to result from the disorganization brought about in the membrane by the removal of cholesterol. Non-ionic detergents (Triton X-100 and Nonidet P-40) include glycerophospholipids in mixed micelles, whereas MBCD releases them indirectly. In erythrocytes, where the plasma membrane interacts tightly with the cytoskeleton, MBCD nevertheless releases 30% of the total glycerophospholipids [33] and it is expected that in lymphocyte membranes, which are less stably anchored to the cytoskeleton, the MBCD-induced release of glycerophospholipids will be greater. We can envisage that after the extraction of lymphocyte membranes with MBCD, glycerophospholipids are released from bilayers and sphingolipids enriched in the remaining sedimentable vesicles. However, this does not exclude the possibility of the presence of glycerophospholipids in the MBCD-resistant membranes, as in DRMs [7].

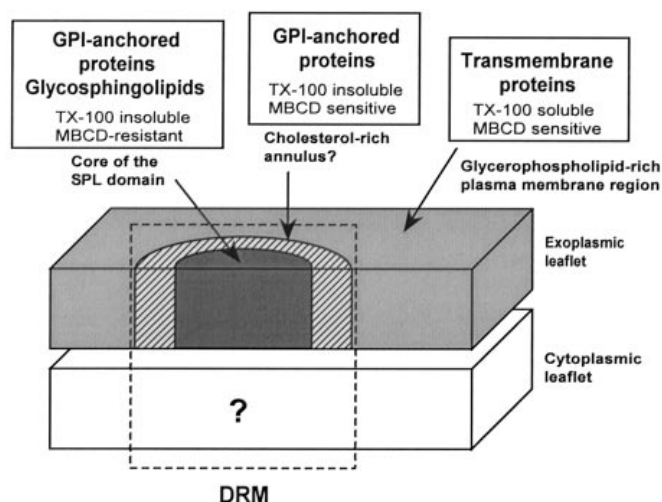


Figure 7 Hypothetical model for the organization of sphingolipids, cholesterol and GPI-anchored and transmembrane proteins in the plasma membrane on the basis of their behaviour towards Triton X-100 and MBCD

Sphingolipids, cholesterol and GPI-anchored proteins interact to form the core of the sphingolipid domains (SPL domain). GSLs and GPI-anchored proteins within this core are resistant to Triton X-100 (TX-100), and MBCD depletes all but a small fraction of cholesterol associated with the sphingolipids. Cholesterol outside this core seems to be completely released by MBCD. The presence of a cholesterol-rich annulus is inferred from the difference in the buoyant properties of MBCD- and Triton X-100-released Thy-1. Significant amounts of Thy-1 should reside within this annulus and be releasable by MBCD but not by Triton X-100 [compare the buoyant properties of Thy-1 released by MBCD from DRMs (Figure 6) and from whole cells (Figure 2)]. Most of the transmembrane proteins are released by both Triton X-100 and MBCD and reside in the glycerophospholipid-rich plasma membrane region, which contains as much cholesterol as the sphingolipid-rich regions. Solubility in Triton X-100 and sensitivity to release by MBCD of the respective component of these predicted membrane subdomains are indicated. The inner leaflet of the plasma membrane corresponding to the outer-leaflet sphingolipid-rich regions remains poorly defined (indicated with '?').

phospholipids in the MBCD-resistant membranes, as in DRMs [7]. Actually, when we removed cholesterol from DRMs with MBCD, the buoyant properties of Thy-1 were not altered, strongly suggesting that after treatments with Triton X-100 or MBCD the resistant membranes harbouring Thy-1 owed their buoyancy essentially to sphingolipids, and secondarily to glycerophospholipids.

We propose that the MBCD-resistant cholesterol in the total plasma membrane or Triton X-100-isolated DRMs represents the cholesterol tightly associated with the sphingolipids to form the core of the sphingolipid microdomains, whereas the rest of the plasma membrane cholesterol is readily extracted by MBCD; the MBCD-extracted cholesterol in the Triton X-100-isolated DRMs might represent the 'third pool' at the boundary of the sphingolipid microdomains discussed by Brown [12]. These possibilities are represented schematically in Figure 7. This model proposes that the Triton X-100-isolated DRMs contain both the core sphingolipid microdomain and the surrounding cholesterol-rich annulus. The fact that MBCD released Thy-1 (as buoyant and non-buoyant fractions) and GM1 from whole cells but not from Triton X-100-isolated DRMs suggests that (1) Thy-1 and GM1 are also present in the annulus surrounding the core of the sphingolipid domains and (2) the removal of glycerophospholipids from the annulus by Triton X-100 might render them refractory to subsequent MBCD-mediated release. An assessment of the role of glycerophospholipids in the buoyancy of GPI-anchored proteins and association with the sphingolipid,

cholesterol-rich environment would require the use of liposomes with defined lipid compositions and containing inserted GPI-anchored proteins.

In conclusion, the depletion of membrane cholesterol by the surface-acting MBCD alone appears to disrupt selectively the glycerophospholipid-rich regions of the membrane, sparing the sphingolipid-rich regions. This apparently more stringent approach to sphingolipid microdomain isolation by MBCD still shows that GSLs, GPI-anchored surface receptors and Src-family PTKs co-isolate in the form of buoyant vesicles, and thus contributes to validating the previous work with non-ionic detergents.

This work was supported by the Swiss League against Cancer, grant no. SKL 462-2-1997.

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