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Cholesterol: Coupling between membrane microenvironment and ABC transporter activity

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Abstract

Lipid composition of biological membranes is closely related to the function of the ATP-binding cassette (ABC) transporter P-Glycoprotein (Pgp). Herein, we studied how membrane physico-chemical properties affect Pgp-activity. We effectively modulated the cellular cholesterol content using methyl-β-cyclodextrin (MβCD) and MβCD–cholesterol-inclusion complex. Pgp was not liberated from the plasma membrane during cholesterol modulation and functional inhibition of Pgp was related to varying cholesterol levels in the plasma membrane. Our data indicate that membrane fluidity does not solely account for cholesterol dependent modifications of Pgp-activity. Therefore, we isolated lipid rafts and examined distinct membrane microdomains. Both depletion and cholesterol enrichment induces a disassembly of lipid rafts. In cholesterol-depleted cell membranes a shift in the Pgp localisation to detergent soluble fractions was observed. Enrichment of membrane cholesterol changed lipid raft distribution but not the localisation of Pgp. From our data we conclude that Pgp-transport capacity depends on accurate lipid raft properties.

Keywords: Lipid raft; ABC transporter; ABCB1; DRM; Pgp; Cholesterol; Membrane; Fluidity

The ABC transporter Pgp (ABCB1), a 170-kDa transmembrane protein, couples ATP hydrolysis [1] with the transport of a wide spectrum of comparatively chemically unrelated drugs [2]. The transport substrates gain access to the multiple drug binding sites which are located inside the cytoplasmatic leaflet [3] after partitioning into the lipid phase of the membrane [4]. Strong evidence indicates, that the lipid composition of biological membranes is closely related to Pgp function [5], which is accompanied by overall changes in the structural organisation of the plasma membranes from chemoresistant lymphoblastic leukaemia cells [6]. Surrounding lipids modulate the ATPase activity of Pgp [7] and its interaction with its substrates [8]. Cholesterol seems to play an exceptional role in Pgp function; the active cholesterol redistribution across the membrane appears to be mediated by Pgp [9]. Furthermore, ATPase activity [10] and drug binding [8] are affected by cholesterol. Pgp is predominantly localised in low-density detergent resistant microdomains [10]. These lipid rafts are specialised membrane microdomains enriched in cholesterol and sphingolipids. The incorporation of cholesterol in the membrane is thought to induce a liquid-ordered phase, with properties between those of the liquid disordered and solid ordered phase [11]. Proteins with long saturated acyl moieties and GPI anchor partition into lipid rafts and those interactions are prevalently required for proper function. In the present study, the cholesterol content of Pgp expressing lymphoblastic leukaemia VLB cells was modulated and the effects on transport capacity and membrane fluidity were investigated.

Materials and methods

Cell culture. We used the drug-sensitive cell line CEM-CCRF, which is derived from a human T-cell lymphoblastic leukaemia, and the multidrug-

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resistant cell line variant VLB, which over-expresses Pgp. These cells present a well-known model for modulation experiments on Pgp [12] and were cultivated in RPMI-1640 medium supplemented with 10% FCS and penicillin/streptomycin (100 U/ml; 100 μg/ml), as previously described [13].

The sensitive parental cell line CEM was used as control and did not display any expression of Pgp, as assessed by Western blot analysis (data not shown).

Preparation of MβCD–cholesterol and in vitro cholesterol modulation. MβCD–cholesterol-inclusion-complexes were prepared and cholesterol levels were modulated as previously reported [14]. Briefly, 2×10^7 cells were suspended in MβCD–cholesterol-inclusion-complexes for 1 h at 37 °C. To deplete cholesterol, cells were incubated with 5 mM MβCD solution.

Calcein-AM assay. The calcein-AM assay was performed as previously reported [15,16]. In cells expressing Pgp, calcein-AM, but not calcein, is accepted by Pgp as a substrate, leading to an extrusion of calcein-AM and less intracellular calcein fluorescence will be observed.

Isolation of plasma membranes. 3×10^8 cells were suspended in 6 ml 5 mM Tris buffer (pH 8.5) and homogenised with a Teflon pestle. Thereafter, the cell suspension was vigorously stirred every 15 min for 30 s and finally centrifuged at 50,000*g* for 30 min. The resulting pellet was resuspended in 4 ml water and layered under 4 ml 10 mM Hepes buffer containing 1.5 M sucrose and 0.25 mM EDTA (pH 7.4). In the subsequent centrifugation at 68,000*g* for 40 min in a SW28 rotor using a Beckman L8-7 M ultracentrifuge the plasma membranes were enriched at the interface between the water and the sucrose layer.

Isolation of lipid rafts. Lipid rafts were isolated using a flotation method [17]. 5×10^8 cells were pooled and homogenised at 4 °C in 1 ml of a Triton X-100 (1%) solution in 25 mM MES buffer containing 0.15 M NaCl (pH 6.5) using a Dounce glass homogeniser with a Teflon pestle. The homogenates were sonicated and then adjusted to 45% sucrose by addition of 90% sucrose solution in 25 mM MES buffer containing 0.15 M NaCl (pH 6.5). The homogenate was layered under a two-step gradient consisting of 4 ml of a 35% sucrose solution and 2 ml of a 5% sucrose solution in 25 mM MES buffer containing 0.15 M NaCl (pH 6.5). Samples were centrifuged at 140,000*g* for 20 h in a SW28 rotor using a Beckman L8-70 M ultracentrifuge. One millilitre fractions were collected and characterised.

Marker enzyme activity assay. Alkaline phosphatase (APA) activity was determined as previously reported using *p*-nitro-phenyl phosphate [17]. Protein concentrations were determined with a BCA assay kit (Pierce, Rockford, IL).

Western blot analysis. Samples were mixed with Tris/glycine reducing buffer, denaturing loading buffer, loaded and electrophoresed on NuPAGE® 4–12% Bis–Tris Gels (Invitrogen, Germany). Gels were transferred to PVDF membranes, incubated with the respective primary antibodies anti-Pgp (C219, Alexis, Germany), anti-Na^+/K^+-ATPase (sc-21712, Santa Cruz, Germany), anti-GAPDH (MAB374, Chemicon, Germany), anti-LAT (06-807, Upstate, Germany), and anti-flotillin-1 (610820, BD Transduction, Germany), followed by secondary antibodies (Calbiochem, Germany) conjugated to horseradish peroxidase and processed for visualisation by ECL® reagent (Amersham Bioscience). The intensities of the samples were quantified by densitometry (ImageJ) in relation to the intensities of the loading controls.

Membrane fluidity measurements. DPH (1,6-diphenylhexa-1,3,5-triene) anisotropy measurements and pyrene bulk- and annular-fluorescence were measured as previously described [18]. Steady-state fluorescence polarisation P_S was expressed as the anisotropy r_S of the probe according to the equation: $r_S = 2P_S/3 - P_S$. The ratio of excimer (Em, 482 nm) to monomer (Em, 373 nm) fluorescence values F_e/F_m was calculated, which is proportional to pyrene-excimer formation.

Cholesterol determination. Unesterified cholesterol was determined with a special enzyme kit developed in our laboratory following the CHOD-PAP-method [18].

Statistical analysis. All data are expressed as mean values ± SEM. For direct comparison of differences between two groups, Student's *t*-test was calculated. All calculations were performed with Graph Pad Prism 4.03® software.

Results and discussion

MβCD treatment of VLB cells depleted the cholesterol content of plasma membranes significantly to about 50% compared to the untreated control (Supplementary Fig. 1A). Incubation of VLB cells with the MβCD–cholesterol-inclusion complex significantly increased the cholesterol levels of plasma membranes to about 160%. Cholesterol levels of membranes from untreated control cells were 124.20 ± 18.33 μg cholesterol/mg protein. Furthermore, the plasma membrane marker protein Na^+/K^+-ATPase was significantly enriched in plasma membrane preparations compared to the crude cell lysate (Supplementary Fig. 1B), demonstrating the successful membrane preparation. Neither depletion, nor enrichment of cellular cholesterol exerted a significant effect on the detected immunoreactivity of Na^+/K^+-ATPase protein levels in plasma membrane preparations, revealing that cholesterol modulation procedures did not disrupt the integrity of the plasma membrane surrounding the cells. Moreover, modulation of cellular cholesterol levels showed no significant effect on immuno-detected Pgp protein levels in isolated plasma membranes (Supplementary Fig. 1C). We concluded that Pgp was not liberated from the plasma membrane during cholesterol modulation as was suggested recently [19].

Different fluorescent probes were deployed to monitor fluidity in different areas of the plasma membrane. DPH (1,6-diphenylhexa-1,3,5-triene) anisotropy measurements were performed to assess the physico-chemical properties in the region of membrane acyl-chains. Hereby, fluorescence anisotropy is inversely correlated with membrane fluidity. We measured a linear inverse relationship between DPH anisotropy and the cholesterol concentration of the membrane (Fig. 1A). Enhanced anisotropy measurements indicated a reduced flexibility of the membrane acyl side chains. Cholesterol depletion increased, whereas an enhancement of cholesterol content reduced, the acyl chain flexibility. Hence, cholesterol inserted into the hydrocarbon core and rigidified the plasma membrane in a dose dependent linear manner.

Pyrene-excimer formation within the membrane hydrocarbon core was used as an indicator of membrane bulk-fluidity which is based on the hyperchromic effect that is observed after the generation of pyrene excimers [20]. An increase in the amount of membrane cholesterol led to a reduced formation of pyrene excimers and thus a reduced bulk fluorescence (Fig. 1B). This suggested a reduced fluidity close to the interleaflet space. Detection of exciplex formation between pyrene and idolyl moieties of tryptophan comprising membrane spanning domains of the embedded proteins was used to determine annular fluidity close to membrane proteins [20]. Again, levels of cholesterol in the membrane were inversely correlated with the pyrene annular fluorescence (Fig. 1B), which indicated that cholesterol reduces the fluidity in those membrane domains. Expression of Pgp has no impact on membrane fluidity parameters (data not shown).

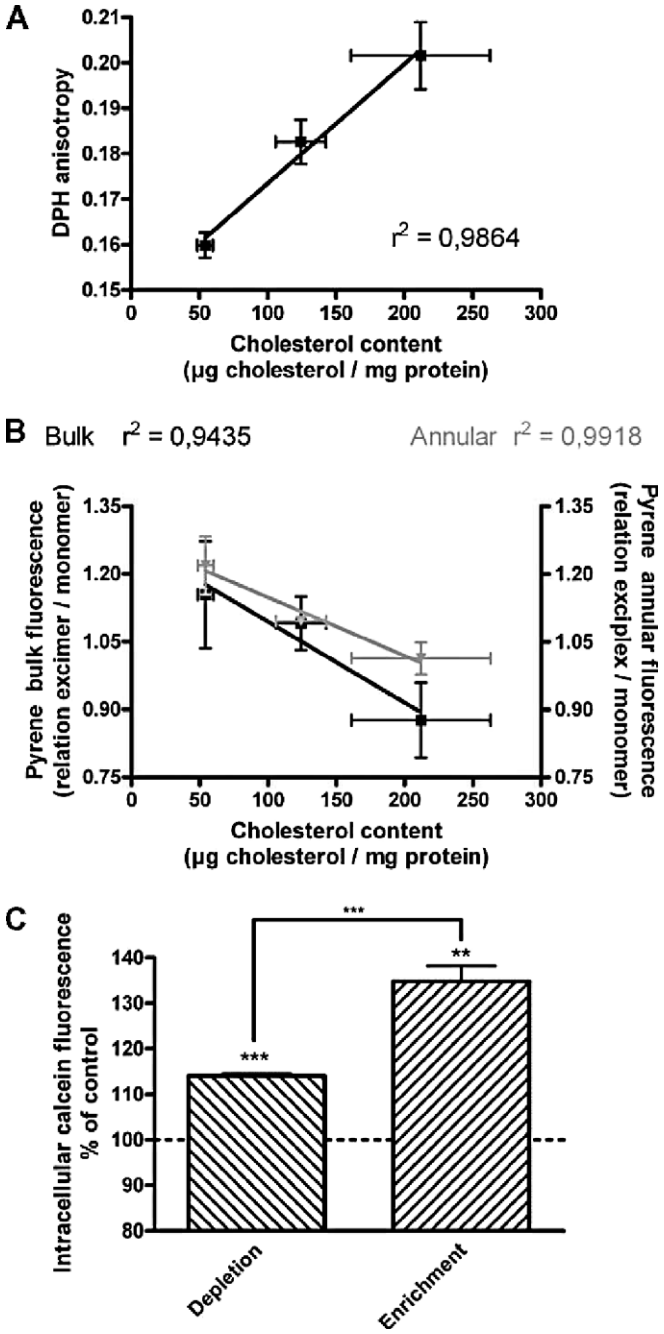

Fig. 1. Membrane properties and ABC transporter activity. Cholesterol content and fluidity parameters of isolated plasma membranes after modulation of cellular cholesterol levels (A,B). Plasma membrane fluidity properties were assessed by DPH anisotropy (A) and pyrene bulk and annular fluorescence measurements (B). Pgp transport activity was assessed by intracellular Calcein-AM accumulation (C). Data are given as means ± SEM for $n = 5$ experiments performed in triplicates ** $p < 0.01$, *** $p < 0.001$.

The calcein-AM assay was used to determine the effect of the varying cellular cholesterol contents on the transport capacity of Pgp [21]. Modulation of cellular cholesterol levels, both depletion and enrichment, increased the intracellular calcein fluorescence compared to the control cells, suggesting an inhibition of Pgp transport activity (Fig. 1C). Inhibition of Pgp transport activity was significantly more pronounced in cells that are enriched in cholesterol. From our data we can conclude that optimal Pgp function is only achieved at physiological cholesterol levels, since both increasing as well decreasing of cellular cholesterol levels impaired the calcein efflux out of the cells. The calcium channel blocker and well-known Pgp-inhibitor verapamil was used as a positive control (data not shown).

Consistent with our results, an inhibition of the extrusion of Pgp substrates after cholesterol depletion was described using different direct and indirect transport and uptake assays [22–25]. In agreement to the published data, we report herein, that the depletion of membrane cholesterol by MβCD fluidised all investigated areas of the plasma membranes and impaired the calcein-AM transport activity of Pgp.

Very few published results describe the effect of an additional supplementation of cholesterol to cells: cholesterol enrichment increased the transport of BODIPY-Verapamil [26] and rhodamine123, with no effect on transport activity upon depletion [27]. In another report the rhodamine123 uptake was not affected by cholesterol addition, however, the same treatment impaired the daunomycin transport [28]. The authors suggest a direct interaction of cholesterol with the daunomycin binding site.

In a simpler system consisting of Pgp reconstituted in proteoliposomes, the lipid composition and cholesterol content can be controlled more precisely compared to biological cell membranes. In this system, a biphasic-behaviour in the specific binding of various substances to Pgp was observed: photoaffinity labelling of Pgp with azidopine worked optimal at 20% weight ratio cholesterol and virtually no effect was seen at 40% [29]. Consonantly, adding cholesterol to purified reconstituted Pgp impeded the binding of the allosteric modulator nicardipine and the high-affinity inhibitor XR9576 [5], indicating that an optimal cholesterol concentration accomplishes full Pgp function.

Since membrane fluidity does not solely account for cholesterol dependent variations of Pgp activity, we examined the membrane microenvironment in close proximity to Pgp. Lipid rafts were isolated from cholesterol depleted or enriched cells (Fig. 2). Detergent resistant membrane fractions were defined by means of alkaline phosphatase (APA) activity [30]. For lipid rafts isolated from untreated VLB cells (Fig. 2B), a co-localisation of cholesterol and APA-activity was observed. As confirmed by Western blot analysis, the lipid raft marker flotillin was exclusively enriched in the lipid raft fractions (Fig. 2B), whereas Pgp was predominantly located in lipid raft fractions [10]. After cholesterol depletion, the majority of Pgp immunoreactivity was detected in the detergent soluble membrane fractions (Fig. 2A, lower panel). Flotillin immunoreactivity showed a very similar pattern of distribution in the isolated sub-fractions (Fig. 2A). Depletion of cholesterol further changed cholesterol distribution in subcellular fractions. A second cholesterol peak, distinct from the lipid raft location was detected (Fig. 2A). The cholesterol extraction

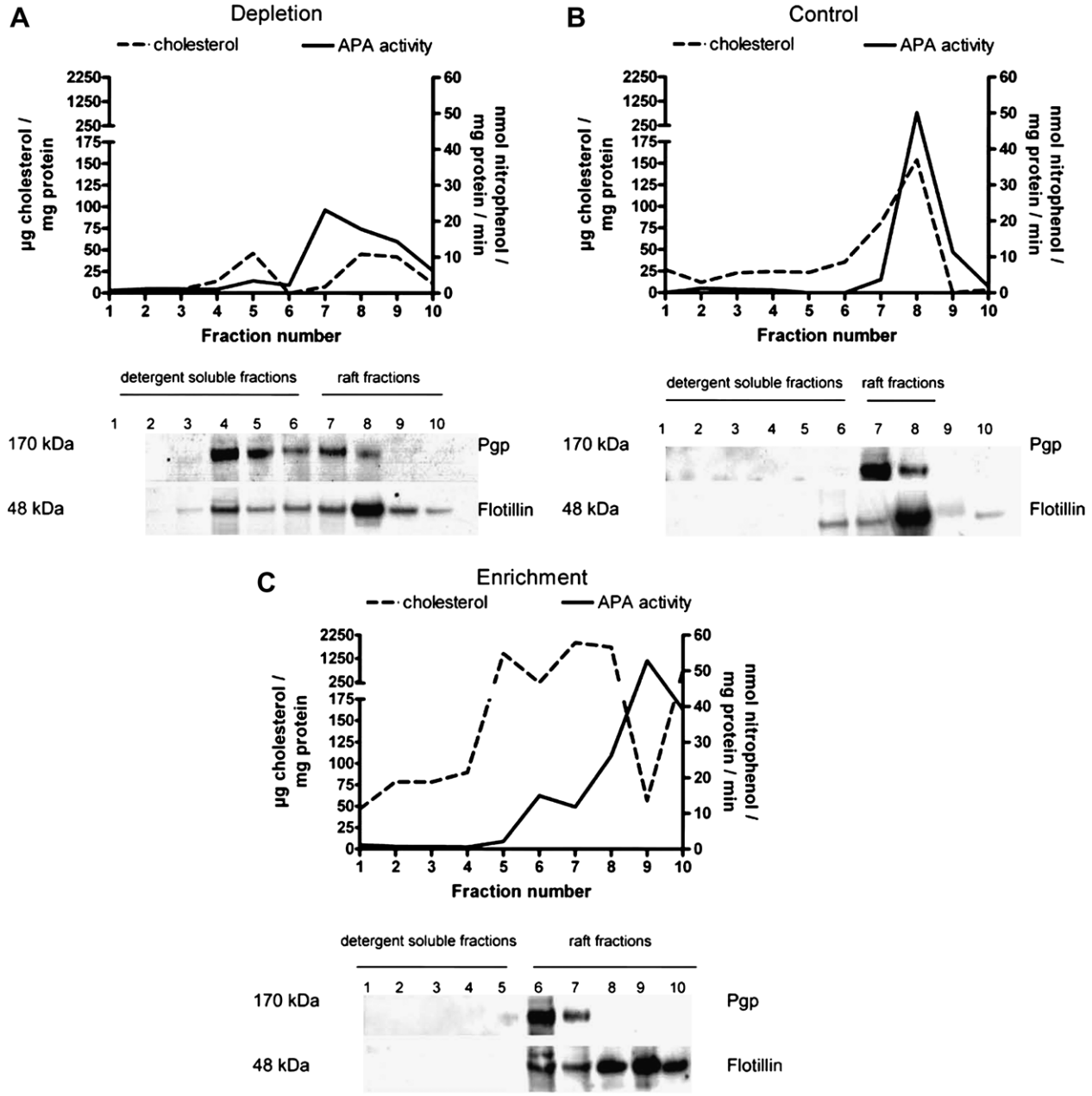

Fig. 2. Distribution of Pgp in lipid raft fractions. Cholesterol levels and alkaline phosphatase (APA) activity in detergent soluble and insoluble fractions (upper graphs). Representative western blot analysis for Pgp and the lipid raft marker flotillin after depletion (A) and enrichment (C) of cholesterol compared to untreated control (B).

appears to induce a disassembly of the lipid rafts. This is associated with a shift in the Pgp localisation out of the lipid rafts to detergent soluble fractions of the plasma membrane, which may account for impaired Pgp-transport capacity. MβCD extracts cholesterol preferentially from the exofacial membrane leaflet [31] and here out of the detergent insoluble areas of the plasma membrane, as suggested by the stronger reduction in cholesterol content of the lipid raft fractions after MβCD incubation (~88% reduction compared to untreated control) compared to the membrane preparations (~50% reduction) and crude cell lysate (~30%).

A shift in APA-activity and flotillin immunoreactivity was detected after cholesterol enrichment (Fig. 2C), suggesting the formation of two locally discrete pools of lipid rafts. After cholesterol enrichment, the majority of Pgp immunoreactivity was completely detected in one of the detergent insoluble membrane fractions, similar to the control (Fig. 2C, lower panel). Hence, exogenous supplemented cholesterol appears to change lipid rafts distribution, which is likely to account for impaired Pgp-transport capacity. Neither depletion, nor the enrichment treatment displayed any modified amount of Pgp immunoreactivity in the crude cell extract (data not shown) or the membrane

preparations (Supplementary Fig. 1). Rather, a significant shift in the localisation of Pgp in different subdomains of the plasma membrane was observed. The depletion treatment of the cells disrupts the structural organisation of the lipid rafts, as was supposed by Gayet et al. [24] and a shift of the Pgp immunoreactivity from the lipid rafts fraction to the membrane fraction of higher density was observed (Fig. 2 and Supplementary Fig. 2), which is similar to the lipid raft marker flotillin (Supplementary Fig. 2). The definition of lipid raft fractions along the sucrose gradient was based on the activity of the GPI-anchored raft marker APA. LAT (linker for activation of T cells), which is expressed in VLB cells was used as a loading control for Western blot analysis of lipid raft fractions [32]. LAT did not segregate out of the lipid raft fractions due to cholesterol depletion (Supplementary Fig. 2). Consequently, these proteins demonstrate different sensitivity towards the MβCD treatment.

Our Western blotting analysis suggests that exogenous supplemented cholesterol does not insert physiologically into membrane subdomain locations. This is supported by repletion experiments: atomic force microscopy experiments showed that the supply of exogenous cholesterol after depletion did not restore the initial state [33]. Overall, the fluidity of the membrane does not solely account for the functional inhibition of Pgp. Depletion did induce a significant shift in the localisation of Pgp in different subdomains of the plasma membrane by disrupting the structural organisation of the detergent insoluble lipid rafts. In contrast to Pgp and flotillin, LAT did not segregate out the APA defined lipid raft fractions due to cholesterol depletion, demonstrating different sensitivity toward MβCD treatment. The *in vivo* correlation of the biochemical defined lipid rafts is still being discussed controversially. It has been proposed that lipid rafts are organised in layers [34], composed of a highly ordered core region, rich in sphingolipids and cholesterol, which are surrounded a by less structured layer and then will pass into the bulk liquid disordered membrane phase [11,34]. This suggested *in vivo* organisation of the lipid rafts would account for the various compositions of the lipid raft preparations, dependent on the particular detergent used for extraction and furthermore, the unequal distribution of Pgp between raft and non-raft fractions, that was reported. For instance, the use of Brij98 instead of Triton X-100 shifts the Pgp localisation to intermediate density domains [35], which are structurally distinct from the classical lipid rafts isolated using Triton X-100. Our results support the hypothesis of lipid rafts in layers: in lymphoblasts, lipid rafts are composed of a core region, containing the GPI-anchored APA and LAT and a vicinal less structured layer, which harbours flotillin and Pgp. According to the model of lipid raft in layers, we suppose that accessory cholesterol predominantly inserts in the bulk liquid-disordered phase of the plasma membrane and induces a partial conversion of the liquid disordered to a liquid-ordered state. Thereby, the membrane area accessible to Pgp expands and transport capacity of Pgp is disturbed. The cholesterol enrichment of the membrane affects the lipid raft distribution and composition but not the localisation of Pgp. Therefore, we propose that cholesterol acts on Pgp transport activity by structuring the plasma membrane and structural organisation of lipid rafts. Our approach does not allow to discriminate between such structural effects and a possible direct interaction of cholesterol with the calcein-AM binding site of Pgp, as proposed in the case of daunomycin [28] or a reduced diffusion coefficient of the substrate close the binding site. Depletion of cholesterol disassembled the Pgp containing lipid rafts layer and resulted in the observed local shift of Pgp from the raft fractions to the non-raft fractions with subsequent inhibition of Pgp's transport capacity. Thus, strict regulation of membrane cholesterol levels appears to be essential for optimal Pgp activity. Our results are of substantial interest, since activities of Pgp and other ABC transporter generally determine the pharmacokinetic profile of drugs in therapy and especially multidrug resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.202.

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