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Effect of Cholesterol Depletion and Temperature on the Isolation of Detergent-Resistant Membranes from Human Erythrocytes

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Abstract Transient lateral microdomains or lipid rafts play important roles in many physiological membrane-mediated cell processes. Detergent-resistant membranes (DRMs) are good models for the study of lipid rafts. Here we report that DRMs can be obtained by treating human erythrocytes with the nonionic detergents Triton X-100 or octaethylene glycol monododecyl ether ($C_{12}E_8$) at 37°C, and by treatment at 4°C of cholesterol-depleted erythrocytes. Electron paramagnetic resonance with spin labels inserted at different membrane depths (5- and 16-doxyl stearic acids, 5-SASL and 16-SASL) were used to measure the order parameter (S) of the cell membranes and DRMs. We previously reported significantly higher S values in DRMs with respect to intact erythrocyte membranes. Here we show that higher S values were still measurable in DRMs prepared from intact erythrocytes at 37°C, or from cholesterol-depleted cells at 4°C, for both detergents. For 5-SASL only, increased S values were measured in 4°C DRMs obtained from cholesterol-depleted versus intact erythrocytes. Flotillin-2, a protein marker of lipid rafts, was found in DRMs from intact cells in trace amounts but it was sensitively increased in $C_{12}E_8$ DRMs prepared at 4°C from cholesterol-depleted erythrocytes, while the membrane-skeletal proteins spectrin and actin were excluded from both Triton X-100 and $C_{12}E_8$ DRMs. However, contrary to the 4°C treatment results, flotillin-2 and stomatin were not resistant to Triton X-100 and $C_{12}E_8$ treatment at physiological temperature. The role of cholesterol in DRMs formation is discussed and the results presented provide further support for the use of $C_{12}E_8$ to the study of DRMs.

Keywords Cholesterol · Lipid rafts · Erythrocytes · Electron paramagnetic resonance · Flotillin · Membrane skeleton

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Lipid rafts are transient liquid-ordered (*lo*) phase microdomains observed in biological membranes that are rich in cholesterol, sphingolipids, and membrane proteins, such as acylated and glycosylphosphatidylinositol–anchored proteins (Simons and Ikonen 1997; Brown and London 1998a; Simons and Vaz 2004; Lichtenberg et al. 2005) and lack glycerophospholipids, mainly the unsaturated ones. Rafts are thought to be small (10–200 nm) and heterogeneous structures, but they can be stabilized to form larger platforms through protein–protein and protein–lipid interactions (Pike 2006). The driving force for raft formation is phase separation caused by the favored association between cholesterol and sphingolipids, as shown by the phase induction observed when cholesterol was added to liposomes of phospholipids and sphingolipids (Brown and London 1998b, 2000) or by the spontaneous formation of cholesterol and sphingolipid-rich domains observed in monolayers composed of phospholipids, sphingolipids, and cholesterol (Dietrich et al. 2001, 2002).

Detergent-resistant membranes (DRMs) obtained from the low-density fractions of sucrose gradients (5–30%) are also composed of cholesterol and sphingolipids in the *lo* phase (Brown and Rose 1992; Schroeder et al. 1994) and have been extensively used in lipid-raft research. Although DRMs do not reflect lipid rafts in vivo (Lichtenberg et al. 2005), these membrane fractions are good models to understand the lipid–lipid and lipid–protein interactions present in rafts. Specific proteins associate to DRMs as a result of their inherent affinity for the ordered membrane state of those lipid domains. Furthermore, raft-targeting signals identified by DRMs analysis are often required for protein function, implicating rafts in a variety of cell processes (Brown 2006, 2007). Mild detergents such as the nonionic Triton X-100, Brij, Lubrol WX, and the zwitterionic 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) have been commonly used to isolate DRMs (Schuck et al. 2003; Delaunay et al. 2007) in various cell types, with detectable differences in their protein composition (Pike 2004). In a recent work, we have reported the isolation of Triton X-100 and octaethylene glycol monododecyl ether ($C_{12}E_8$) DRMs from human erythrocytes with a threefold increase in the cholesterol/protein mass ratio and high lipid ordering, compatible with the *lo* phase, as assessed by electron paramagnetic resonance (EPR) measurements of DRMs labeled with nitroxide spin labels. Despite the presence of lipid-raft marker proteins in those DRMs, flotillin-2 was selectivity enriched in Triton X-100 DRMs but poorly detected in $C_{12}E_8$ DRMs (Crepaldi Domingues et al. 2009).

Different treatments can affect the affinity and association of a particular protein to the DRMs (Ilangumaran and Hoessli 1998). Cholesterol seems to play a central role in stabilizing the sphingolipid/glycosphingolipid domains and its depletion or enrichment in biological membranes has resulted in destabilized membrane rafts (Ilangumaran et al. 1999; Xu and London 2000; Matkó et al. 2002). Methyl-β-cyclodextrin (MBCD) has been extensively used for disrupting raft integrity by selectively extracting membrane cholesterol. Unlike other cholesterol-binding agents, MBCD neither binds nor inserts into the plasma membrane (Ohtani et al. 1989). Methylated cyclodextrin is strictly surface acting and it is able to form inclusion complexes with cholesterol (Nishijo et al. 2003).

Here we report results on the role played by cholesterol depletion of human erythrocytes in determining integral and membrane-skeletal protein association to DRMs prepared with Triton X-100 and $C_{12}E_8$. Moreover, the acyl chain packing in the DRMs obtained from cholesterol-depleted erythrocytes was determined by means of EPR through the order parameter (*S*) sensed by a reporter nitroxide molecule inserted at different depths of the bilayer. The characterization of cholesterol and protein contents of DRMs prepared at physiological temperature (37°C), as well as their *S* values, are also described. At a given detergent concentration, temperature effects on membrane solubilization are complex, being a trade-off of opposing factors. Although phospholipids are "curvophobic" and tend to form planar bilayers, detergents are in general "curvophilic" and tend to form micelles with positive curvature. High temperatures tend to render the spontaneous curvature of lipid molecules more negative by disordering the acylic tails, and thus disfavor the formation of mixed detergent–phospholipid micelles with positive curvature. Therefore, low temperatures should improve solubilization by influencing this factor. Conversely, high temperatures can also favor solubilization by promoting the energy-consuming annealing of the void formed in the curved monolayer of the mixed micelle (Lichtenberg et al. 2005) and by decreasing the critical micelle concentration of the detergent. To our knowledge, this is the first time DRMs from erythrocytes were isolated at physiological temperature.

Materials and Methods

Preparation of Erythrocytes and Cholesterol Depletion

Blood was collected from healthy human donors after informed consent was obtained. Blood was mixed with either 0.1 volumes of 3.8% (w/v) sodium citrate or with 0.1 volumes of acid–citrate–dextrose (130 mM citric acid, 152 mM trisodium citrate, 112 mM glucose). After centrifugation at 1,000×*g* in a fixed-angle rotor for 5 min at 20°C, plasma was removed, and the packed cells were resuspended with an equal volume of phosphate-buffered saline (PBS) buffer (5 mM Na-phosphate, 154.5 mM NaCl, 4.5 mM KCl, 300 mOsmol/kg H_2O, pH 7.4). The suspension was then filtered through α-cellulose/microcrystalline cellulose to isolate erythrocytes from platelets and leukocytes (Beutler et al. 1976). The purified erythrocyte suspension was washed three times in PBS, and the packed cells were used to prepare DRMs.

Cholesterol depletion was performed by using MBCD (Sigma–Aldrich, Milan, Italy): purified packed erythrocytes were suspended up to 20% hematocrit in 5 mM MBCD in PBS buffer and then incubated for 30 min at 37°C. The cells were then washed three times with PBS, by centrifugation at 1,000×*g*, to remove the MBCD–cholesterol complexes (supernatant). After that, MBCD-treated and nontreated (control) erythrocytes were lysed and subjected to lipid extraction (Rose and Oklander 1965). Cholesterol was quantified using a colorimetric assay kit (N. 10139050035, R-Biopharm Italia Srl, Milan, Italy). White ghost membranes were prepared as previously described (Crepaldi Domingues et al. 2009).

Preparation of DRMs

DRMs were prepared from either intact or cholesterol-depleted erythrocytes following a previously detailed protocol (Crepaldi Domingues et al. 2009). Briefly, the packed cells (approximately 1.25×10^9) were incubated with TNE buffer (25 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA [ethylenediaminetetraacetic acid] pH 7.4) containing either Triton X-100 or $C_{12}E_8$, so that the final volume was 0.625 ml and the final detergent concentration 16 mM, giving a detergent/lipid molar ratio of approximately 12. After 30 min at 4, 25, or 37°C, the samples were mixed with an equal volume of 80% sucrose solution containing 0.3 M Na_2CO_3. The sample was then transferred to ultracentrifuge tubes and 2.5 ml of a 30% sucrose solution in TNE was gently layered on top of the sample, followed by 1.25 ml of a 5% sucrose solution in TNE. The samples were subjected to ultracentrifugation in a bench-top ultracentrifuge (Optima Max, equipped with a swinging-arm MLS50 rotor, Beckman Coulter, Milan, Italy) at $225{,}000 \times g_{max}$, for 16 h at 4°C. The top 1 ml was aspirated as the first fraction. Proceeding down the gradient, fractions 2–6 were aspirated as 0.8-ml aliquots and saved for subsequent characterization. DRMs were usually collected in fractions 2 and 3.

Detection of DRM-associated Proteins

Samples were separated in 10, 12.5, or 5–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels according to Laemmli (1970). The gels were either stained with Coomassie brilliant blue or electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h in TNE containing 5% nonfat milk and 0.05% Tween 20, then incubated with the relevant primary antibody: mouse monoclonal anti-flotillin-2 (BD Italia, Milan, Italy), goat polyclonal anti-human stomatin and mouse monoclonal (BRIC 10) anti-human glycophorin C (Santa Cruz Biotechnology, Tebu-bio, Italy) or mouse monoclonal anti-human band 3 (Sigma–Aldrich, Milan, Italy). The membranes were then washed and incubated with the appropriate peroxidase-conjugated secondary antibody, and the proteins were revealed with the Amersham ECL Western blotting kit (GE Healthcare, Milan, Italy).

EPR Experiments

The n-doxyl-stearic acid spin labels, SASL probes (Sigma–Aldrich, Milan, Italy) with $n =$ carbon 5 or 16, were incorporated up to approximately 2 mol% of lipids into the samples (intact cells, cholesterol-depleted cells, and their respective DRM fractions). To reach that condition, 100 µl of DRM samples or erythrocyte suspensions at 20% hematocrit in PBS buffer were placed in tubes containing a film of SASL (prepared by evaporating a stock chloroform solution of the spin label under a stream of N_2). After 30 min incubation under gentle agitation at room temperature, the samples were used to obtain EPR spectra, which were recorded at 20–25°C in 0.1 ml flat quartz cells at 9.4 GHz (X Band) and 3.4 kg in a Bruker EMX spectrometer (Bruker GMbH, Germany). Field modulation frequency was 100 kHz, and modulation amplitude was 1.0 gauss. The order parameter (S) was calculated from the hyperfine splittings of the spin label spectra (Hubbel and McConnel 1971). The order parameter is directly related to the tilt angle of the acyl chains and (indirectly) to the *trans-gauche* distribution of chain dihedrals so that larger S values (i.e., values near unity) correspond to small amplitudes of motion and more ordered lipid chains (Schreier et al. 1978).

Results

Because cholesterol has been shown to play an important role in the formation of lipid rafts (London and Brown 2000; Matkó et al. 2002; Pike 2004) and DRMs are cholesterol enriched (approximately 30–40% of the total erythrocyte membrane cholesterol remains in the detergent-insoluble fraction according to Ciana et al. 2005; Koumanov et al. 2005; Crepaldi Domingues et al. 2009), we have investigated the composition and organization of DRMs from cholesterol-depleted erythrocytes. When erythrocytes were incubated with nonlytic amounts of MBCD (5 mM), approximately 40% of the cholesterol was removed from the cell membrane (Fig. 1), a finding in good agreement with the results of Vazquez et al. (2002). Although visually slight differences could be observed in the low buoyant density fractions (interface between 5 and 30% sucrose gradient) obtained from cholesterol-depleted or intact erythrocytes, DRMs were obtained from both samples with either Triton X-100 or $C_{12}E_8$. Figure 1 shows the cholesterol content of DRMs obtained from intact erythrocytes: no significant differences were observed in their cholesterol content depending on the temperature of detergent treatment. Moreover, Fig. 1 interestingly demonstrates that the decrease of approximately 40% of cholesterol amount in DRMs from cholesterol-depleted cells in comparison to the cholesterol present in DRMs from intact erythrocytes, at 4°C, occurred in the same proportion as that observed for MBCD-treated cells in comparison to intact cells. This means that the cholesterol amount present in DRMs was approximately 30% of that in the starting material (MBCD-treated or intact cells).

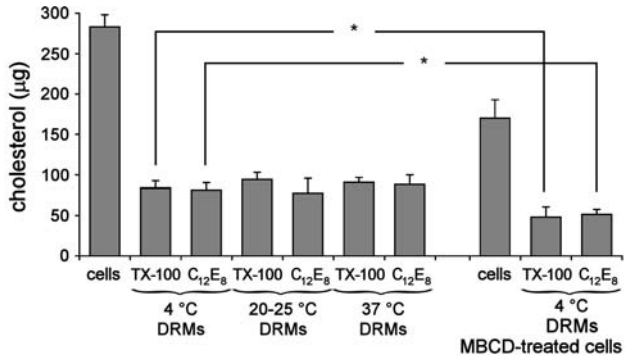

Fig. 1 Quantification of cholesterol content in DRMs obtained from intact erythrocytes by treatment with Triton X-100 or $C_{12}E_8$ at various temperatures, and from cholesterol-depleted erythrocytes (MBCD-treated cells) by treatment with Triton X-100 or $C_{12}E_8$ at 4°C. Histogram columns labeled "cells" correspond to the cholesterol contained in the amount of cells from which the DRM fraction was obtained. Error bars represent the standard deviation ($n = 3$). Statistical differences between DRMs from MBCD-treated cells and their respective control DRMs, unpaired Student's t test, * $P < 0.01$

Effect of Cholesterol Depletion on Different Proteins in Triton X-100 and $C_{12}E_8$ DRMs

Figure 2 ("4°C" and "MBCD, 4°C" panels) shows the distribution of specific proteins in Triton X-100 and $C_{12}E_8$ DRMs from intact (untreated) and cholesterol-depleted erythrocytes, isolated as fractions 2 and 3 from the sucrose gradient; the proteins revealed in fractions 5 and 6 correspond to the soluble membrane fractions. Stomatin association with DRMs was essentially unaffected by MBCD treatment, irrespective of the detergent used. Flotillin-2, which is almost completely solubilized by $C_{12}E_8$ treatment of intact cells, was also significantly present in the DRM fraction when cholesterol-depleted cells were treated with $C_{12}E_8$. Conversely, although flotillin-2 partitions almost exclusively in the DRM fraction when Triton X-100 is used on intact cells, it was partially redistributed to the detergent-soluble fraction(s) in MBCD-treated cells (Fig. 2). This behavior agrees well with previous reports (Samuel et al. 2001; Rivas and Gennaro 2003), indicating that cholesterol is critical for the assembly of flotillin-2 into DRMs.

As evidenced in Fig. 2, almost all band 3 and glycophorin C are found in the soluble fractions (5 and 6), irrespective of cholesterol depletion, for any detergent used. These results suggest that the environment of these proteins is not affected by cholesterol removal.

The effects of cholesterol removal on membrane-skeletal proteins are illustrated in Fig. 3a. Interestingly, spectrin and actin were virtually absent in the DRMs from cholesterol-depleted erythrocytes, irrespective of the detergent used, suggesting a possible role for cholesterol in modulating the association of the membrane skeleton with DRMs. However, it is known that MBCD has the side effect of releasing some small cholesterol-binding proteins from the membrane (Ilangumaran and Hoessli 1998). This must be considered in the preparation of DRMs because the organization of such domains in vivo is likely controlled by cholesterol-binding proteins (Anderson and Jacobson 2002; Pike 2003).

Effect of Temperature on the Isolation of Triton X-100 and $C_{12}E_8$ DRMs

Although low temperature (4°C) is required in the conventional protocols used for DRM isolation, we obtained DRMs from human erythrocytes treated at physiological temperature (37°C) with either Triton X-100 or $C_{12}E_8$ detergents. Visually, slight differences could be observed in such DRMs in comparison to the low-density material obtained at 4°C (data not shown).

The results in Fig. 1 revealed that the cholesterol content of Triton X-100 and $C_{12}E_8$ DRMs prepared at 37°C was approximately 30% of the total cholesterol in the original cells, in good agreement with DRMs prepared at 4°C (Crepaldi Domingues et al. 2009), showing that cholesterol is detergent resistant not only at low temperature. On the other hand, the content of specific lipid raft-marker proteins, flotillin-2 and stomatin, was reduced in both

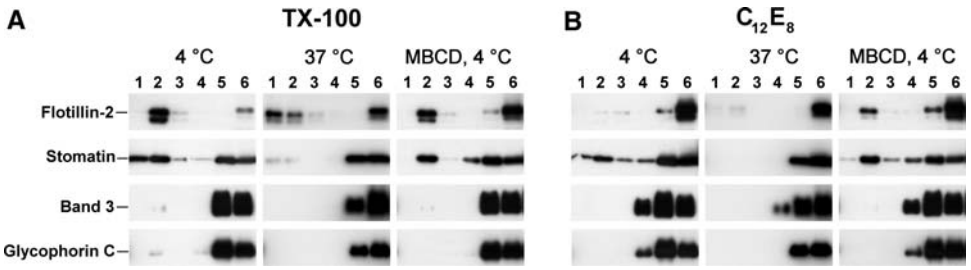

Fig. 2 Distribution of flotillin-2, stomatin, band 3, and glycophorin C in density gradient fractions 1 to 6. Fractions 1–3 correspond to DRMs obtained from whole erythrocytes treated with Triton X-100 (**a**) or $C_{12}E_8$ (**b**) at 4°C (*left*) or 37°C (*middle*). Results for DRMs obtained at 4°C from cells previously treated with MBCD are also shown (*right*). Results are representative of five independent experiments

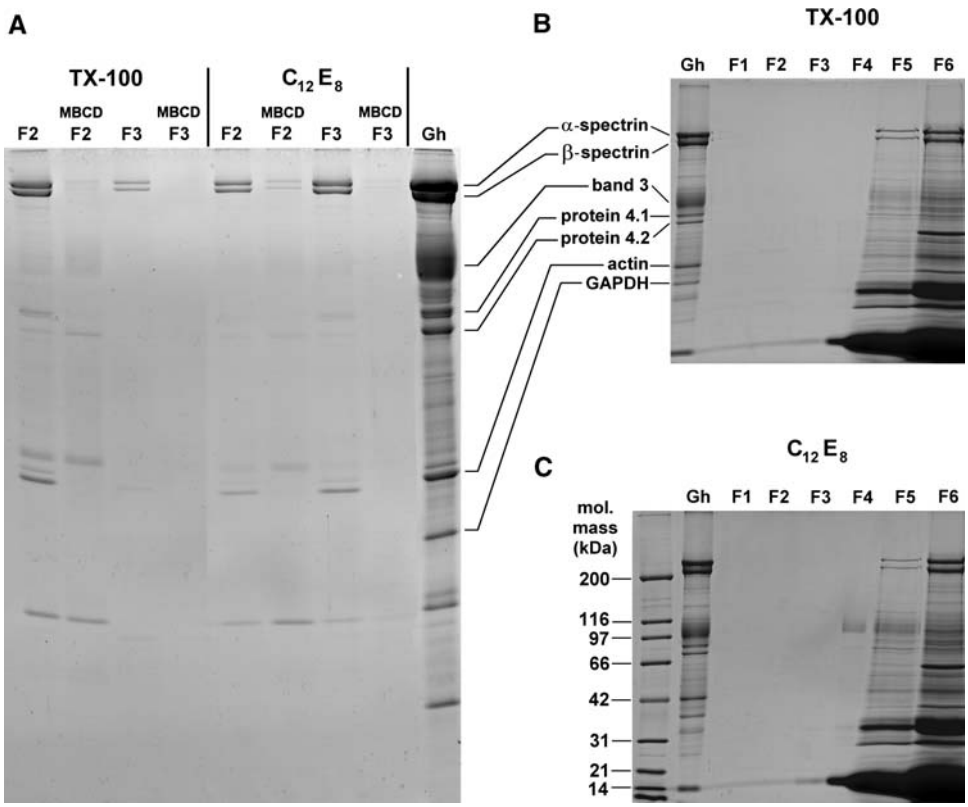

Fig. 3 SDS–PAGE of proteins present in sucrose gradient fractions obtained after erythrocyte lysis in different conditions. **a** Proteins associated with DRMs fractions 2 and 3 (F2 and F3) from intact and MBCD-treated erythrocytes obtained with Triton X-100 (*left*) and $C_{12}E_8$ (*right*) at 4°C. **b**, **c** SDS-PAGE of proteins present in sucrose gradient fractions 1 to 6 (F1–F6) from intact erythrocytes treated with Triton X-100 and $C_{12}E_8$, respectively, at 37°C. An amount of ghosts (Gh) proportional to the quantity of cells processed for DRM isolation was also loaded in all gels. Results are representative of three independent experiments

Triton X-100 and $C_{12}E_8$ DRMs prepared at 37°C (Fig. 2). In addition, SDS–PAGE showed that membrane-skeletal proteins were absent in those DRMs (Fig. 3b, c). These results indicate that at physiological temperature, the solubilization of membrane proteins is facilitated while the resistance of cholesterol is maintained.

EPR Results

To further understand the effect of cholesterol depletion and temperature in DRM formation, we have monitored the lipid packing of these structures using EPR spectroscopy with the nitroxide spin probes 5- and 16-doxyl stearic acids, which sense the degree of lipid packing at, respectively, less deep and deeper levels within the lipid bilayer. Figure 4a shows typical EPR spectra for 5-SASL in intact erythrocytes and in DRM fractions prepared with Triton X-100. We have shown before that the increase in DRM cholesterol content (threefold increase in the cholesterol/protein mass ratio in comparison to intact cell membrane) as well as the increased lipid packing of these DRMs, sensed by nitroxide spin labels inserted in between the lipids and measured by EPR, were similar no matter what detergent—Triton X-100 or $C_{12}E_8$—was used (Crepaldi Domingues et al. 2009).

Although we observed a slight variation in order parameter (*S*) among intact and MBCD-treated erythrocytes sensed by the 5-SASL (Fig. 4b), no significant changes were registered ($P > 0.05$). On the other hand, for 16-SASL (Fig. 4c), a significant decrease ($P < 0.001$) in the *S* value was found in MBCD-treated with respect to control cells, indicating a less ordered acyl chain packing in the bilayer core after cholesterol depletion (see "Discussion" section). Figure 4 also shows that the order parameter values were significantly higher in DRMs prepared from cholesterol-depleted erythrocytes than in the MBCD-treated whole membrane. The high *S* values measured in DRMs from cholesterol-depleted cells obtained with Triton X-100 and $C_{12}E_8$ detergents in Fig. 4b reveal that lipids are in the *lo* phase, a situation similar to that observed in DRMs prepared from control erythrocytes (Crepaldi Domingues et al. 2009).

Interestingly, the order parameter sensed by 5-SASL was significantly increased in the DRMs from cholesterol-depleted versus non-cholesterol-depleted cells, prepared with both detergents (Fig. 4b; $P < 0.001$). Because the content of cholesterol in the DRMs is approximately 30% of that in the respective original membrane (MBCD-treated or untreated cells), the higher *S* values of DRMs from cholesterol-depleted cells must reflect the increased sphingomyelin/cholesterol ratio in such DRMs and possibly the changes in protein composition, as shown in Figs. 2 and 3.

Finally, the order parameter values of DRMs obtained at 37°C (Fig. 4b, c) revealed that these structures are as ordered as DRMs prepared at 4°C with either Triton X-100

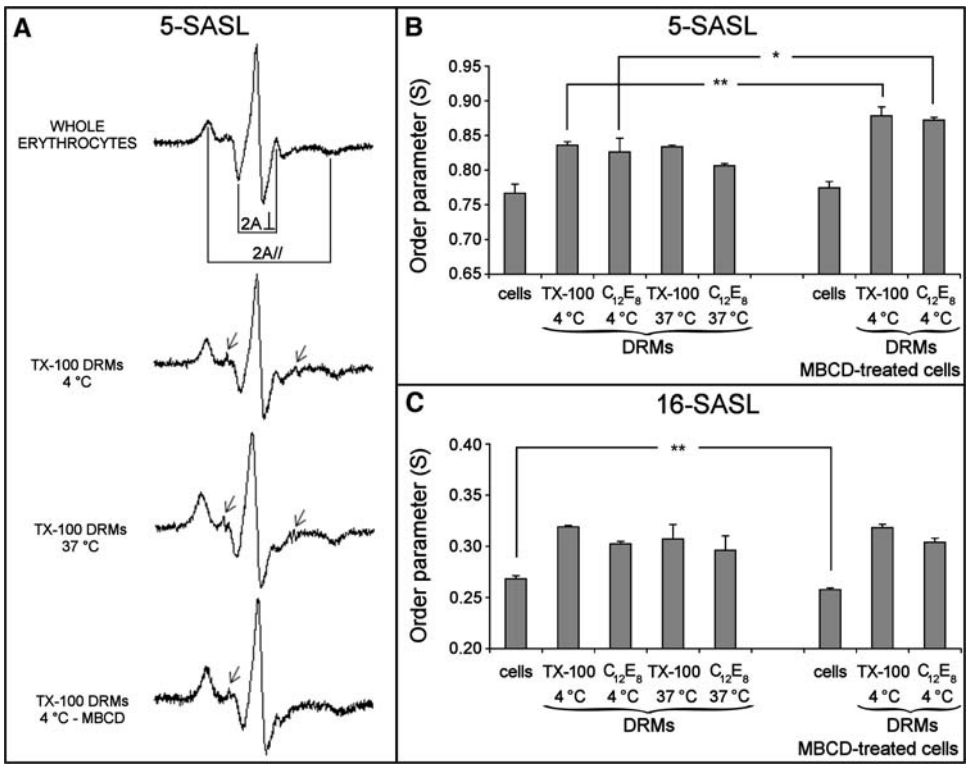

Fig. 4 **a** EPR spectra of the spin label 5-SASL in intact erythrocytes (hematocrit 20%, top), their respective DRMs prepared with Triton X-100 at 4 and 37°C (*middle*) and DRMs prepared with Triton X-100 at 4°C from cholesterol-depleted cells (*bottom*). *Arrows* indicate the spin probe's remaining water signal (after applying spectral subtraction). **b** Order parameter, *S*, calculated from the hyperfine splittings (A// and A⊥) EPR spectra of the spin labels 5-SASL and **c** 16-SASL in intact erythrocytes (cells), MBCD-treated erythrocytes and their respective DRMs (prepared with Triton X-100 or $C_{12}E_8$) at 4°C and DRMs prepared from intact erythrocytes by detergent treatment at 37°C. Error bars represent the standard deviation. In (**b**) and (**c**), the statistical differences between *S* values of DRMs and their corresponding cells were significant for all samples ($P < 0.001$, unpaired Student's *t* test, $n = 8$–10 for intact cells and 3–5 for DRMs). Additionally, in (**b**), statistical differences between DRMs from MBCD-treated cells and the corresponding 4°C DRMs from control cells were observed: ** $P < 0.001$; * $P < 0.01$ (unpaired Student's *t* test, $n = 3$–5). In (**c**), statistical differences were found between S values of intact erythrocytes and S values of MBCD-treated erythrocytes ($P < 0.001$, unpaired Student's *t* test, $n = 8$ for intact cells, $n = 4$ for MBCD-treated cells)

or $C_{12}E_8$. Therefore, the high acyl chain packing of DRMs is maintained even for samples prepared at physiological temperature.

Discussion

Triton X-100 is a widely used detergent to isolate DRMs from model and biological membranes, especially from erythrocytes. Recently, we have reported the characterization of DRMs from human erythrocytes prepared with either Triton X-100 or $C_{12}E_8$. These nonionic detergents of equivalent hemolytic activity (Preté et al. 2002) lead to DRMs of different protein composition and lipid packing compatible with the *lo* phase (Crepaldi Domingues et al. 2009). Here, we provide results on the erythrocyte membrane proteins associated with DRMs from cholesterol-depleted cells as well as with DRMs prepared at 37°C after treatment with Triton X-100 or $C_{12}E_8$. Moreover, the lipid organization of these DRMs prepared in different experimental conditions was also analyzed.

Modified cyclodextrin such as MBCD has been extensively used for cholesterol trapping (Ilangumaran and Hoessli 1998; Cassera et al. 2002; Schuck et al. 2003). Our results show that MBCD partially extracts cholesterol from the membrane in a nonselective manner. DRMs obtained from cholesterol-depleted erythrocytes have approximately 30% of the total cholesterol present in those from MBCD-treated cells. Samuel et al. (2001) have also reported a decrease in the cholesterol content in DRMs from erythrocyte ghost membranes treated with MBCD. This proportion in the cholesterol content in DRMs versus the original membrane (30%) for cholesterol-depleted erythrocytes treated with Triton X-100 and $C_{12}E_8$ was the same to that observed in DRMs obtained from untreated erythrocytes.

After MBCD treatment, if a reduction in the association of a protein to DRMs is observed, one can easily infer a preferential binding of the protein to membrane domains in

the *lo* phase, which is weakened by the decrease in cholesterol content. On the other hand, if no changes in protein association are found, no conclusions can be drawn because the remaining cholesterol in the DRMs can be enough to keep such domains intact (Schuck et al. 2003). For flotillin and stomatin, which are two of the cholesterol-binding and raft-organizing proteins, we found different results regarding their association with DRMs. MBCD treatment affected only flotillin-2, increasing its resistance to $C_{12}E_8$ and partial solubilization by Triton X-100 (Fig. 2). On the other hand, Schuck et al. (2003) found no changes in DRMs association of several proteins tested after treating intact MDCK cells with MBCD to extract more than 70% of the membrane cholesterol. Therefore, these results show that the question whether the association of a particular protein with DRMs (and, extrapolating, with lipid rafts) reflects its ability to bind to cholesterol and/or ordered domains is still open. Because cholesterol has been shown to modulate attractive forces between reversibly palmitoylated signaling proteins and lipids in rafts (Fragoso et al. 2003), herein it might have modulated flotillin-2, which has no transmembrane domains but is a multiply palmitoylated protein acyl anchored to the internal leaflet of the membrane, and that tends to form oligomers (Neumann-Giesen et al. 2004; Delaunay et al. 2007).

In the literature, there is no consensus on the presence of membrane-skeletal proteins in DRMs. We and other authors have shown the presence, in variable amounts, of such membrane-skeletal proteins as actin, spectrin, tropomodulin, and protein 4.1 and 4.2 in DRMs from human erythrocytes (Salzer and Prohaska 2001; Murphy et al. 2004; Ciana et al. 2005; Wilkinson et al. 2007; Crepaldi Domingues et al. 2009). However, the physiological relevance of this observation remains unclear. What was confirmed in the present work is that DRMs are linked to the membrane skeleton, from which they can be dislodged only after increasing the ionic strength and pH of the medium during the detergent treatment (Ciana et al. 2005; Crepaldi Domingues et al. 2009). Cholesterol depletion has global effects on membrane and cell properties. Perturbation of the membrane skeleton would explain the results in Figs. 2 and 3, and this hypothesis is based on the modulation of the membrane-skeleton/membrane interaction by phosphatidylinositol 4,5-bisphosphate (PIP_2). According to Kwik et al. (2003), depletion of cholesterol reduces the lateral mobility of membrane proteins by disruption of their highly regulated interactions with PIP_2, controlling the state and organization of the actin cytoskeleton. Actually, An et al. (2005, 2006) reported that PIP_2 alters the formation of the spectrin–actin–protein 4.1R junctional complex in erythrocytes. Thus, because a regulated PIP_2 activity depends on cholesterol domains, cholesterol depletion results in failed regulation of the cell membrane skeleton, facilitating a release of spectrin and/or actin from DRMs.

Our previous indication that DRMs are connected to the membrane skeleton and the present evidence of a possible role for cholesterol in modulating such interaction are also supported by Diakowski et al. (2006), whose data have evidenced that erythrocyte spectrin interacts with model membranes prepared from a raftlike mixture of lipids. Those authors have shown that beside cholesterol content, the chemical structure of the membrane lipids would contribute to that interaction so that cholesterol depletion mainly from the outer leaflet of the bilayer should have an effect on the lipid distribution in the inner leaflet, leading to the disruption of spectrin interaction with DRMs. Beside cholesterol removal, other strategies to study the association of DRMs with the membrane skeleton are under investigation in our laboratory.

Another interesting critical point about DRMs is the temperature at which they are prepared. Almost all protocols described in the literature use a low temperature (4°C) and claim that at 37°C these insoluble membrane fractions cannot be obtained; this is a serious pitfall for DRMs to be considered a good lipid-raft model. Here, we have demonstrated the preparation of DRMs from erythrocytes at physiological temperature. The cholesterol content of such DRMs is very similar to that of DRMs prepared at 4°C (Fig. 1). However, in this case, markers of lipid rafts such as flotillin-2 and stomatin were not found to be resistant to the detergent, as observed at 4°C (Fig. 2). Glycosylphosphatidylinositol-linked proteins are also known to resist solubilization by Triton X-100 at 4°C and selectively partition into the lipid rafts, but they were shown to be completely solubilized by 1% Triton X-100 at 37°C (Brown and Rose 1992).

Our results show for the first time that the lipid-raft-specific, non-glycosylphosphatidylinositol-linked proteins stomatin and flotillin-2 are also associated to the lipid-raft phase in a temperature-sensitive manner. Although these membrane proteins were solubilized by Triton X-100 and $C_{12}E_8$ at high temperature (37°C), we found DRM structures to be as ordered as those prepared at low temperature (Fig. 4), being the order parameter values of DRMs obtained at 37°C compatible with the high lipid packing of the *lo* phase.

Cholesterol is known to increase the acyl chain order of pure phospholipids bilayers at the fluid phase as well as of biological membranes (Warren 1987). The cholesterol molecule is shorter than the other major lipids of the membrane (diacylglycerol or sphingolipids) and has to keep its 3-hydroxyl group in the membrane surface, where it is believed to fill interlipid spaces in the more rigid superficial bilayer region (polar head, glycerol and first acyl chain carbon regions), favoring the packing of the

trans-gauche acyl chains of surrounding lipids. Otherwise, cholesterol depletion of the erythrocyte membrane causes an increase in the order parameter in the upper regions of the bilayer, while a decrease is observed in deeper regions (Cassera et al. 2002). In this study, we show that DRMs from cholesterol-depleted erythrocytes are more ordered structures than control DRMs from intact cells, as sensed by the 5-SASL probe (Fig. 4b) in the glycerol moiety of the bilayer. This increase in the *S* values measured with 5-SASL for Triton X-100 or $C_{12}E_8$ DRMs obtained from cholesterol-depleted erythrocytes is different from what observed by Rivas and Gennaro (2003) in Triton X-100 DRMs from cholesterol-depleted membranes. However, those authors analyzed an insoluble pellet obtained by centrifugation after detergent extraction, not a fraction of purified DRMs isolated in a sucrose gradient, as in our case.

We have not measured the cholesterol:phospholipid ratio in the DRMs. However, it can be inferred that cholesterol is present in the cell membrane at approximately 45 mol% with respect to total lipids.[1] The order parameter we measured in intact erythrocytes was lower with respect to DRMs, which is indicative of a liquid disordered state of the membrane. In DRMs from normal cells, cholesterol is present at approximately 54–57 mol% with respect to total DRM lipids. Thus, although our EPR measurements were conducted at a temperature (25°C) at which sphingomyelin should be in a solid ordered state, being 32°C its phase transition temperature (Radhakrishnan et al. 2001), the phase of the system should be liquid-ordered even at 25°C when more than 50 mol% cholesterol is present. For DRMs from cholesterol depleted cells, which should also be, at least in part, cholesterol depleted (see below), the order parameter we have measured by EPR at 25°C with 5-SASL, was significantly higher than the corresponding values for DRMs from control cells. This may indeed reflect the existence of a solid ordered phase due to the higher proportion of sphingomyelin in these samples, but further studies would be needed to decide whether this is the case.

The fact that DRMs from cholesterol-depleted cells were obtained in lower amounts (in terms of cholesterol) and yet they had approximately 30% of the cholesterol of cells from which they were extracted (the same proportion of cholesterol found in DRMs from intact cells) suggests that MBCD does not selectively deplete the nonraft cholesterol. If MBCD could discriminate between raft and nonraft cholesterol and selectively extracted the latter, then the total amount of cholesterol extracted as DRMs from MBCD-treated cells would be the same as in DRMs from normal cells (in other words, all the DRM histogram columns in Fig. 1 would have the same height), which is not the case. Therefore, the DRMs we have isolated from cholesterol-depleted cells are likely also cholesterol depleted. A relative enrichment in sphingolipids would result, which should be reflected by an increase in the order parameter measured with the 5-SASL. This is indeed what we have observed.

In summary, the cholesterol content decreased in DRMs from cholesterol-depleted cells, relative to DRMs from normal cells, but it was never lower than 10% (to curb liquid-ordered phase formation). Koumanov et al. (2005) have reported that the sphingomyelin:cholesterol molar ratio in DRMs from human erythrocyte ghosts is approximately 1:1, and it does not change in DRMs from erythrocytes of various other animal species, regardless of differences in the cholesterol content of the respective erythrocyte membranes. But as explained above, sphingomyelin/cholesterol ratios higher than 1:1 can be obtained after MBCD treatment. The highly ordered milieu of DRMs from cholesterol-depleted erythrocytes (as sensed by 5-SASL) is then justified by the proportional increase in sphingolipids, which are composed of long and saturated acyl chains (Rietveld and Simons 1998), resulting in a tighter packing.

As for 16-SASL, which monitors a deeper bilayer region (Godici and Landsberger 1974), no changes were detected, except for a slight decrease in the *S* value of MBCD-treated cells with respect to control cells. Such phenomenon has been described to occur (Cassera et al. 2002; Rodi et al. 2008) as a result of the lack of cholesterol spacer effect at upper bilayer regions, close to the lipid head groups (Kessel et al. 2001), resulting in a lower order and increased dynamics of the membrane core (Fraceto et al. 2002). However, the probe was unable to detect a similar decrease in the order parameter of DRMs obtained from cholesterol-depleted cells with respect to DRMs from intact cells. In our opinion, this probably occurs because DRMs from cholesterol-depleted cells still contain enough cholesterol to fill the interlipid spaces in the apical region

[1] Free cholesterol in human red blood cells is 3.15 μmol/ml cells, while total lipid phosphorus is 3.90 μmol/ml cells (Dodge and Phillips 1967). Cholesterol is therefore present in the cell membrane at approximately 45 mol% with respect to total lipids. In DRMs from normal cells, we have 30% of the cell cholesterol, corresponding to approximately 0.945 μmol (per milliliter of cells). If we assume that the sphingolipid-enriched, detergent-resistant phase early described in the literature (before the notion of lipid rafts was introduced) contained what we now call DRMs (and can be released from the membrane skeleton only by treatment with carbonate), then the sphingomyelin in the detergent-resistant portion of the erythrocyte, obtained with Triton X-100 at concentrations comparable to those we used, corresponds approximately to 70–80% of the cell sphingomyelin (Yu et al. 1973; Sheetz 1979). Because the latter corresponds to 25 mol% of total cell lipid phosphorus (Dodge and Phillips 1967), it amounts to 1.0 μmol (per milliliter of cells), and its content in the DRMs should be approximately 0.7–0.8 μmol. Therefore, the cholesterol percentage with respect to the total of DRM lipids (i.e., sphingomyelin plus cholesterol, with the reasonable approximation that the other phospholipids are present in DRMs in much lower amount) is 54–57%.

of the bilayer, and/or because the inner core of the bilayer remains in a more ordered state, even at decreased cholesterol levels, thanks to the stronger interactions of the long saturated acyl chains of sphingolipids (which are characteristically enriched in the DRMs).

Recently, Rodi et al. (2008) described that the detergent resistance of erythrocyte membranes is dependent of both cholesterol and sphingomyelin membrane molar content. Nevertheless, our EPR results confirm that cholesterol depletion is not sufficient to weaken the interactions responsible for the characteristic liquid-ordered state of DRMs, indicating that the stability of DRMs is influenced by, but not strictly dependent on, the cholesterol content.

Although our EPR results for cholesterol-depleted DRMs revealed changes in the order parameter only in the upper acyl chain region of the bilayer (sensed by 5-SASL), they can provide insights to explain aspects related to association of proteins with DRMs. Changes in the lipid environment induced by cholesterol depletion could affect the extractability of membrane proteins by detergents. Highly ordered DRMs seem to favor the association of flotillin-2, making $C_{12}E_8$ less powerful in solubilizing it. Furthermore, the effect of each detergent per se cannot be excluded to explain our results. Hägerstrand and Isomaa (1989, 1992) have previously reported that surfactants induce membrane invaginations and endovesicles in human erythrocytes in different ways. $C_{12}E_8$ was shown to convert the human erythrocyte into a single large torocyte, while Triton X-100 was shown to induce the formation of many small spherical endovesicles (Hägerstrand and Isomaa 1989, 1992; Hägerstrand et al. 2004). Thus, Triton X-100 and $C_{12}E_8$, which are stomatocytogenic nonionic detergents, may solubilize membrane domains differently, revealing different associations of proteins with DRMs. As proposed by Pike (2004), the heterogeneity found in DRMs isolated by different detergents could reflect the existence of heterogeneous domains before extraction with detergent. Furthermore, the different membrane lipid composition and/or distribution after MBCD treatment could give rise to differential sensitivity to detergent extraction.

In conclusion, our data indicate that cholesterol depletion can affect the distribution of proteins in DRMs because it can induce the formation of more ordered domains. The organization of flotillin-2 domains seems to be strongly dependent on cholesterol, while stomatin domains are practically unaffected. DRMs from erythrocytes obtained at physiological temperature and at 4°C present similar cholesterol contents and similar acyl chain packing of the lipids, but on the other hand, those DRMs present differences in their associated proteins. Our results also suggest that both Triton X-100 and $C_{12}E_8$ may be used as good tools to study the association of specific proteins with DRMs.

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