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Short communication

Detergent resistant domains in erythrocyte membranes survive after cell cholesterol depletion: an EPR spin label study

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Abstract

We use electron paramagnetic resonance (EPR) with liposoluble spin labels in order to study the lipid structures obtained after Triton X-100 extraction of erythrocyte membranes. The apparent order profile in these detergent resistant membranes (DRM) is very similar to that of the parent membrane, although with higher absolute values, consistent with a liquid-ordered state. DRM could also be obtained from erythrocytes previously depleted in a 40% of their membrane cholesterol, in apparent opposition to the phenomenon of raft disruption reported by other authors. However, the protein profile of these samples showed important differences with that of DRM from untreated cells. The analysis of our results suggests that the effect of Triton X-100 on cholesterol depleted erythrocytes is limited to the solubilization of raft proteins, without disrupting the lipid matrix of DRM.

Keywords: DRM; Membrane rafts; Erythrocyte; Cholesterol; EPR or ESR; Triton X-100

1. Introduction

The concept of lipid rafts, laterally segregated plasma membrane microdomains enriched in cholesterol, sphingolipids and GPI anchored proteins, has gained great importance recently (Simons and Ikonen, 1997; Brown and London, 1998; London and Brown, 2000). These domains are also called detergent resistant membranes (DRM) because they are resistant to cold non-ionic detergent extraction. They have been implicated in a variety of sorting and signaling processes in cells (Brown and London, 1998; Brown and London 2000, and references therein). Their resistance to detergent solubilization has been attributed to a liquid-ordered state of the lipid components (Ge et al., 1999; London and Brown, 2000). Recently, the existence of DRM in erythrocytes has been demonstrated (Civenni et al., 1998; Salzer and Prohaska, 2001).

The usual method to isolate DRM structures is to perform Triton X-100 extraction, subsequently submitting the detergent extract to ultracentrifugation in a sucrose step gradient (Samuel et al., 2001; Salzer and Prohaska, 2001). After these procedures, raft proteins are found in a low-

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density gradient zone, due to their association to membrane material.

In this work, we propose a simpler way to detect membrane-like structures by labeling the whole detergent insoluble pellet with the stearic acid spin labels 5, 12 or 16-SASL, and subsequently analyzing it by electron paramagnetic resonance (EPR) spectroscopy. We apply this technique to analyze DRM obtained from human erythrocyte membranes. We find that these lipid structures are able to survive to cell cholesterol depletion previous to detergent extraction.

2. Materials and methods

Freshly obtained erythrocytes were washed three times and afterwards extracted by incubation at 10% concentration (v/v) in ice-cooled 1% Triton X-100 solution in Tris buffer saline (TBS), pH 7.5. The insoluble pellet was obtained by centrifugation (15 000 $\times g$, 4 °C, 30 min), followed by three washings in TBS (Salzer and Prohaska, 2001).

To achieve membrane cholesterol depletion, intact erythrocytes were incubated at 5% (v/v) concentration in 3 mM methyl-β-cyclodextrin (MβCD, Sigma) solution in PBS buffer, at 37 °C for 30 min. In these conditions, 40% membrane cholesterol is removed (Vazquez et al., 2002). These cholesterol depleted cells were subsequently extracted with Triton X-100.

The presence of membrane structures in the insoluble pellet was detected by EPR, using the liposoluble spin labels *n*-doxyl stearic acid (*n*-SASL, Sigma), with $n = 5$, 12 or 16. The labels were incorporated to the pellet by 30 min incubation. The EPR experiments were carried at room temperature, and the microwave frequency was 9.7 GHz (X band). From the spectrum of each of the three labels, which sense the bilayer at different depths, an apparent order parameter S_{app} (Griffith and Jost, 1976) can be evaluated as:

$$S_{app} = \frac{(1/k_0)(A_{||} - A_{\perp})}{[A^c_{zz} - 1/2(A^c_{xx} + A^c_{yy})]}$$

where $A_{||} = A_{max}$; $A_{\perp} = A_{min} + 1.4(1 - S_0)$, with $A_{max} = 1/2(B_4 - B_1)$; $A_{min} = 1/2(B_3 - B_2)$; $S_0 = (A_{max} - A_{min})/[A^c_{zz} - 1/2(A^c_{xx} + A^c_{yy})]$, $A^c_0 = 1/3(A^c_{zz} + A^c_{xx} + A^c_{yy})$, $A_0 = 1/3(A_{||} + 2A_{\perp})$, and $k_0 = A_0/A^c_0$.

B_1–B_4 (pointed out with arrows in Fig. 1) are magnetic field values for spectrum structures related to the hyperfine interaction between the radical unpaired electron and the ^{14}N nucleus. The parameters $A^c_{zz} = 32.9$ G, $A^c_{xx} = 5.9$ G and $A^c_{yy} = 5.4$ G are the single crystal values of the ^{14}N hyperfine coupling tensor (Griffith and Jost, 1976). The apparent order parameter is related to the angular amplitude of motion of the labeled segment, determined by the degree of straightening of the acyl chains, and its values lie in the range $0 < S_{app} < 1$. Values of S_{app} near 1 would be

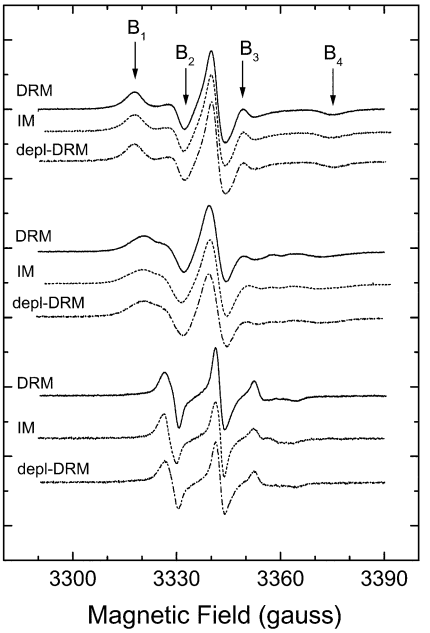

Fig. 1. EPR spectra of liposoluble spin labels in DRM and IM. Temperature, 25 °C; microwave frequency, 9.75 GHz. Upper group, 5-SASL (labeled moiety at carbon 5, closest to polar headgroups); medium group, 12-SASL (labeled moiety at carbon 12); lower group, 16-SASL (labeled moiety at carbon 16, closest to the bilayer center). For each group of spectra, the upper one corresponds to detergent resistant membranes from untreated erythrocytes (DRM), middle one corresponds to intact erythrocyte membranes (IM), and lower spectrum corresponds to DRM obtained from erythrocytes previously depleted in 40% of their original membrane cholesterol content (depl-DRM). The arrows point to the spectrum features whose magnetic field positions are used in the calculation of the apparent order parameter S_{app} (see text).

obtained for a label into a structure with fully aligned all-*trans* acyl chains, while S_{app} close to zero indicates unrestricted isotropic movements of the spin label at the corresponding position. These features are governed by *trans–gauche* ($t-g$) isomerizations, which confer flexibility to the acyl chains. The plot of S_{app} as a function of the position of the label, sometimes called 'flexibility gradient', represents a depth profiling showing how the order parameter decreases towards the membrane hydrophobic core, and it is the signature of a membrane-like structure (Griffith and Jost, 1976; Schorn and Marsh, 1996; Cassera et al., 2002).

The protein profile of DRM was obtained by electrophoresis. Aliquots of the Triton insoluble pellets containing approx. the same amounts of protein were run on 12% SDS-PAGE gels in a Mini-Protean II Bio-Rad system, and stained with Coomassie blue.

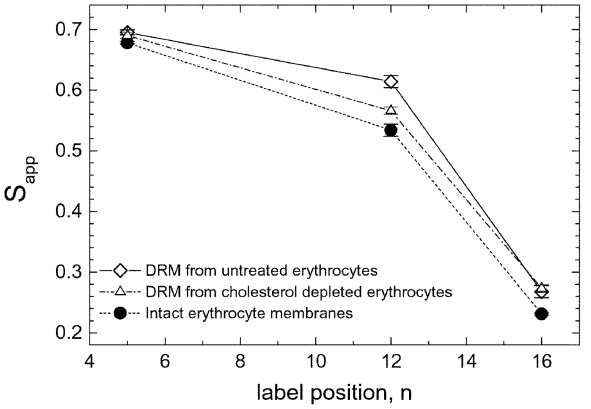

Fig. 2. Apparent order parameters calculated from the EPR spectra shown in Fig. 1, as a function of the position, n, of the labeled moiety along the acyl chain. Level of significance (Student's t-test, $n=3$) between DRM and IM is $P<0.01$ for 5- and 16-SASL, and $P<0.001$ for 12-SASL; difference between depl-DRM and DRM is only significant for 12-SASL ($P<0.01$).

3. Results and discussion

Fig. 1 shows the EPR spectra of the insoluble pellet for each of the three spin labels (upper spectra of each group). The corresponding spectra of intact erythrocyte membranes (IM) are also included for comparison, as the middle spectra of each group. The similitude between these two sets of spectra gives clear evidence of the presence of membrane-like structures in the Triton-insoluble pelleted material. These structures resisted extraction with Triton X-100, and thus they should be identified with DRM or rafts. Also, the spectra of 5- and 16-SASL are similar to those of 5- and 16-PC spin labels in DRM from RBL-2H3 cells reported by Ge et al. (1999).

The apparent order parameter evaluated by EPR spectroscopy showed systematic higher values in DRM in comparison with those of IM (Fig. 2, see levels of significance in the figure caption). This fact implies a straightening of the acyl chains (i.e. increased order) at all levels. In the light of these results, the higher S_{app} values in DRM are consistent with the postulated liquid-ordered state of these membrane structures (London and Brown, 2000).

We found that cholesterol depleted erythrocytes also yield an insoluble pellet when extracted in Triton X-100. This material (depl-DRM) was also labeled and analyzed by EPR (lower spectra of each group in Fig. 1). It can be observed that these spectra are rather similar to the upper ones, indicating the presence of DRM. The comparison of the order parameters of these depl-DRM with those of DRM from untreated erythrocytes (Fig. 2) showed a significant difference only for 12-SASL, S_{app} being lower in depl-DRM ($P<0.01$, $n=3$, Student's t-test).

Samuel et al. (2001) failed to isolate raft proteins in the floating zone after ultracentrifugation when analyzing detergent extracts from cholesterol depleted erythrocytes (reduced in one-third of their original cholesterol membrane content). They attributed this fact to a disruption of raft structure due to cholesterol depletion. However, our EPR spectra clearly indicate that the lipid matrix of erythrocyte rafts still remains after a 40% cholesterol depletion.

In order to clarify this apparent discrepancy, we searched for differences in the protein pattern of DRM from untreated and cholesterol depleted

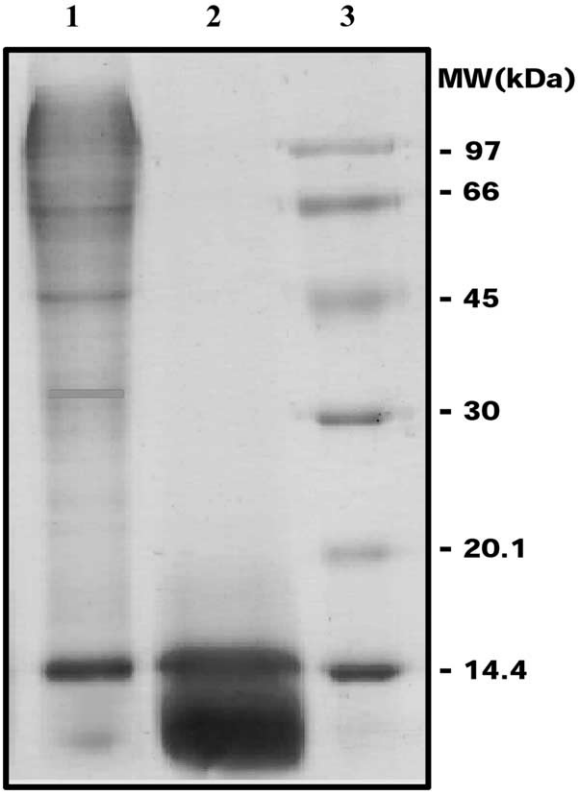

Fig. 3. Coomassie blue stained SDS-PAGE profile of erythrocyte DRM proteins. Lane 1, DRM from untreated cells; lane 2, DRM from cholesterol depleted cells; lane 3, molecular weight markers.

cells, performing SDS-PAGE electrophoresis. We found that the DRM fraction from untreated cells contains multiple protein bands (Fig. 3, lane 1). Resolved protein bands near 45 and 30 kDa likely correspond to flotillin-1,2 and stomatin, respectively. According to Salzer and Prohaska (2001) these are the major integral proteins of erythrocyte lipid rafts. These bands are not detected in the DRM fraction from depleted cells (Fig. 3, lane 2).

These observations suggest that Triton X-100 is able to solubilize raft proteins in cholesterol depleted erythrocytes. In this case, these proteins would no longer float in a low-density gradient zone after ultracentrifugation, and this fact would be interpreted as raft disruption (Samuel et al., 2001).

4. Conclusions

We have applied EPR as an alternative method to detect and study detergent resistant lipid domains (rafts) in the erythrocyte membrane. The behavior of acyl chain ordering at all levels is consistent with the proposed liquid-ordered state of raft lipids.

We found that DRM or rafts are also present in cholesterol depleted erythrocytes. Thus, previous evidences of raft disruption caused by cholesterol depletion should be taken with caution, as our results suggest that the effect of Triton X-100 on cholesterol depleted erythrocytes is only to solubilize raft proteins, without destroying the lipid matrix.

It is interesting to remark that this method is complementary to those relying on proteins as raft markers, because it is capable to detect DRM even in the absence of raft proteins. To our knowledge, this is the first time that such a case is reported. Work is in progress in order to understand the small differences between spectra of DRM from untreated and cholesterol depleted erythrocytes.

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