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Interaction between glycophorin and a spin-labeled cholesterol analogue in reconstituted dimyristoylphosphatidylcholine bilayer vesicles

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The interaction between glycophorin and a spin-labeled cholesterol analogue has been investigated by EPR spectroscopy. In vesicles which were reconstituted by the freeze and thaw technique, direct evidence was obtained for a reorganisation of the membrane at low protein content (protein/lipid ratio less than 1:300). From the spin exchange interaction we were able to show a protein-induced clustering of the steroid in fluid and in gel state membranes. Tryptic cleavage of the complete N-terminus of glycophorin vanishes the effect. Whereas the removal of the sialic acid residues by neuraminidase digest had no influence on the EPR spectra. The interaction seems to be cholestane spin label specific since it was not observed with an androstane spin-label.

Introduction

The arrangement of membrane proteins within lipid bilayer membranes and the interaction of these proteins with different classes of lipids has been investigated intensively in the last decade [1,2]. The lipids that make up these bilayers vary from membrane to membrane and also from one organism to the other. Sterols are widespread membrane constituents in higher organism whereby cholesterol, the most common sterol, is mainly concentrated in plasma membranes. However, its exact role remains elusive. In erythrocyte membranes cholesterol amounts to nearly 30% of the lipid content. It is known to alter the mechanical viscoelastic properties of these lipid bilayer membranes [3]. Moreover, cholesterol is believed to be associated with proteins like bands 1, 2 and 5 [4,5]. Borochov et al. [6] clearly demonstrated that changes in erythrocyte membrane cholesterol alters the availability of protein sulfhydryl groups at the membrane surface which is interpreted by a cholesterol induced decrease in the lipid–protein interaction and correspondingly by an increased protein–water interaction. Band 3 which is the anion transport protein of erythrocyte membranes possesses a high-affinity steroid binding site which is proposed to be an inhibitory site of the anion transport [7]. Klugermann et al. [8] suggest that the level of cholesterol in the erythrocyte membrane influences the conformation of band 3.

A specific effect of cholesterol on protein function is not restricted to erythrocyte membranes. Reconstitution of the acetylcholine receptor from *Torpedo californica* requires the presence of cholesterol [9]. Without cholesterol liposomes did not form. The acetylcholine receptor rich membrane contains two pools of cholesterol [10]. One is an easily depleted fraction that influences only the bulk viscosity, whereas the second is a tightly bound fraction which perhaps surrounds the acetylcholine receptor oligomer.

Capping of surface Ig on murine lymphocytes is another example for a cholesterol controlled process [11]. The protein involved in capping is located in gel-like lipid domains. This gel-like nature of the bilayer membrane is restored by cholesterol. Removal of cholesterol inhibits capping by making the lipid domain less gel-like. Similar effects of cholesterol in determining the lateral organization of proteins in a membrane have been reported earlier [12] and strong evidence exists that cholesterol plays a keyrole in promoting viral infectivity [13]. The organization of the G protein of vesicular stomatitis virus, which is an externally oriented glycoprotein, is strongly affected by cholesterol.

Abbreviations: DMPC, 1,2-dimyristoylphosphatidylcholine; cholestane-SL, 3-d-xylcholestane; EPR, electron paramagnetic resonance; X_G, vesicular molar protein to lipid ratio; X_{Ch}, molar fraction of cholesterol or spin-labeled cholestane.

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In the present paper we report a strong interaction between a spin-labeled cholestane and glycophorin isolated from erythrocyte membranes Spin-labeled cholestane seems to be separated from the bulk lipid phase thus forming a heat stable protein-steroid domain in the surrounding fluid bilayer membrane

Materials and Methods

Materials Dimyristoylphosphatidylcholine and cholesterol were obtained from Fluka (Neu-Ulm, F R G) Spin-labeled cholestane (3-doxylcholestane) and androstane (3-doxylandrostanol) were from Aldrich (Steinheim, F R G) Glycophorin was isolated and purified from human erythrocytes as described earlier [14] following the procedure of Verporte [15] and Kapitza et al [16] The purity of the isolated protein was checked by sialic acid determination using the resorcinol method of Svennerholm [17] Remaining lipids were quantified by phosphate determination [18] Trypsin from bovine pancreas and neuraminidase from *Clostridium perfringens* were from Boehringer (Mannheim, F R G)

Reconstitution Glycophorin-containing vesicles were prepared by successive freezing and thawing of sonicated samples [19] Dried lipid films containing the spin-label probe were sonified in a 10 mM aqueous Tris-HCl buffer (pH 7 2) in the presence of glycophorin with a Branson sonifier (2 minutes, power 30 mW) until a clear suspension was formed The lipid concentration was 1 mg/ml The obtained suspensions of small unilamellar vesicles were slowly frozen from 4°C to −20°C in a refrigerator and thawn again at a temperature above the lipid phase transition temperature This cycle was repeated three times yielding fused vesicles with an average diameter of 100 nm [19] The obtained vesicles were freed from surface-adsorbed glycophorin by repetitive centrifugation and resuspension The pelleted vesicles were assayed for phosphate and sialic acid The analytical values were corrected for the remaining intermediate volume between the vesicles as was described earlier [14] Enzymatic digestion of lipid-protein sediments was performed with 50 μg trypsin within 5 min and with 0 1 mg neuraminidase within 30 min, both at 37°C and at pH 7 2

EPR measurements EPR measurements were performed with a computer controlled Varian E4 spectrometer Samples were transferred into 0 8 mm quartz tubes and centrifuged at 13000 × *g* The supernatant was discarded Temperature control (±0 1°C) and recording of spectra was performed automatically by the AT-PC Probe concentrations were varied and are given in Results

Results

Reconstitution

Glycophorin-containing vesicles were prepared by freezing and thawing small unilamellar vesicles obtained by ultrasonication in the presence of glycophorin In accordance with our earlier observations [14] the amount of glycophorin incorporated into the bilayer vesicles (X_G) was almost independent from the initial amount of glycophorin (X_G^0) added to the preformed vesicles in a molar fraction range between 5 10^{-3} and 2 10^{-2} (Fig 1) The molar amount of vesicular protein with respect to the lipid ranged from 1 10^{-3} to 2 10^{-3}

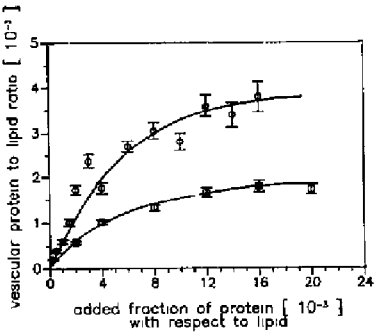

Fig 1 Incorporation of glycophorin into vesicles prepared by the freeze and thaw technique The molar vesicular protein to lipid ratio X_G is given as function of the initial molar protein fraction X_G^0 added to the vesicle suspension before the freeze and thaw cycles An increased X_G for a given X_G^0 was obtained in the presence of 5 mol% cholesterol or cholestane spin label (○——○) The lower curve (□——□) stands for proteoliposomes without steroids

Glycophorin incorporation was increased if cholesterol or cholesterol derivatives were present A linear dependence from the initial glycophorin content was observed up to 4 10^{-3} of the added protein fraction although the certainty of different preparations is low Compared to other techniques the freeze and thaw technique has the advantage to be a solvent-free and a detergent-free method

EPR measurements

Spin-labeled cholestane was used to investigate a glycophorin–cholesterol interaction Complete temperature scans for DMPC samples doped with a molar fraction of cholestane-SL of $X_{Ch} = 0\,01$ are given in Fig 2 in the presence of glycophorin ($X_G = 2.71 \cdot 10^{-3}$ with respect to the lipid) Thus very low protein content causes a strong line broadening at all temperatures below and above the lipid phase transition temperature The local concentration of the probe is obviously drastically increased in the presence of glycophorin which could be interpreted by a phase separation.

A summary of these results is given in Fig 3 where the EPR spectra of samples containing $X_{Ch} = 0\,01$ cholestane-SL are collated in the presence of different amounts of glycophorin within the membrane The temperature of $T = 36°C$ is well above the lipid phase

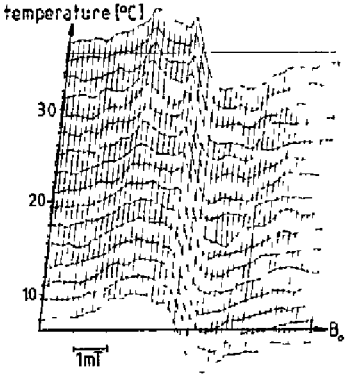

Fig 2 Temperature dependence of the EPR spectra of spin-labeled cholestane in DMPC-vesicles containing different amounts of glycophorin The probe concentration is $X_{Ch} = 0.01$ with respect to the lipid The mole fraction of membrane bound glycophorin is C_0 $X_G = 2.71\ 10^{-3}$

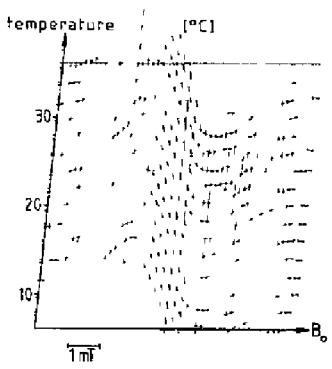

Fig. 4 Temperature dependence of the EPR-spectra of spin labeled cholestane in DMPC-vesicles containing different amounts of glycophorin The probe concentration is $X_{Ch} = 0.05$ with respect to the lipid The glycophorin content is $X_G = 2.96\ 10^{-3}$

transition temperature The three-line spectrum first broadens and is converted into a broad one-line spectrum at a glycophorin content of $X_G = 3.6\ 10^{-3}$ This effect is even more pronounced at a cholestane-SL content of $X_{Ch} = 0.05$ (Figs 4 and 5)

The observed formation of cholestane-SL-rich domains could be reversed by tryptic protein digestion (Fig. 6). The broadened EPR spectrum of DMPC vesicles containing $X_{Ch} = 0.05$ cholestane-SL and $X_G = 1.2\ 10^{-4}$ glycophorin (Fig 6a) becomes partially restructured after treatment with trypsin (Fig. 6b) Note that trypsin is added to the preformed vesicles and thus could only act on that protein which protrudes to the outside of the vesicles Application of a short sonication which allows the trypsin to enter the vesicles results in a three line spectrum (Fig. 6c) which is identical to the one in the absence of glycophorin Sonication in the absence of trypsin did not effect the spectral shape

A simple approach to the fraction of clustered cholestane-SL was made by spectral subtraction at a given glycophorin concentration The spectrum taken in the absence of glycoporin was subtracted from the one obtained in its presence Membrane preparations with $X_{Ch} = 0.5$ cholestane-SL were analyzed (Fig. 7) The fraction of clustered cholestane-SL increases from 0.5 to 1 in a glycophorin concentration range between $X_G = 5\ 10^{-4}$ and $4\ 10^{-3}$ Enzymatic digestion with trypsin from the outside of the vesicles reduces the fraction of clustered cholestane-SL by about 0.35 If we analyze the

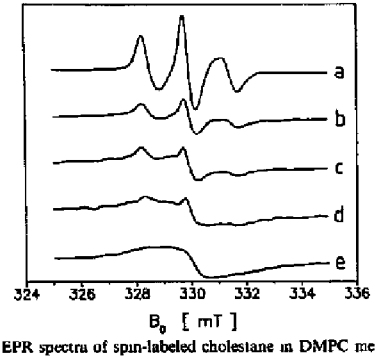

Fig. 3 EPR spectra of spin-labeled cholestane in DMPC membranes containing different amounts of glycophorin The probe concentration is $X_{Ch} = 0.01$ The spectra were taken at 36 °C The mole fractions of membrane bound glycophorin are (a) $X_G = 0$, (b) $X_G = 0.5\ 10^{-3}$, (c) $X_G = 1.7\ 10^{-3}$, (d) $X_G = 2.7\ 10^{-3}$, (e) $X_G = 3.6\ 10^{-3}$ The intensities of spectra (a) and (b) are reduced by a factor of two

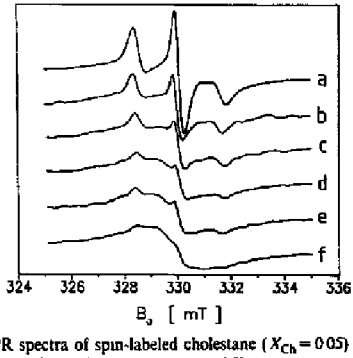

Fig 5 EPR spectra of spin-labeled cholestane ($X_{Ch} = 0.05$) taken at 36 °C in DMPC-membranes containing different amounts of glycophorin (a) Absence of glycophorin (b) $X_G = 0.5\ 10^{-3}$, (c) $0.9\ 10^{-3}$, (d) $1.3\ 10^{-3}$ (e) $1.7\ 10^{-3}$, (f) $3\ 10^{-3}$

content of intact glycophorin after trypsination and relate the fraction of clustered cholestane-SL to this value, an excellent agreement was observed with the original curve before trypsin treatment This clearly demonstrates that trypsin-digested glycophorin affects the distribution of the cholestane-SL in DMPC membranes Enzymatic digestion with neuraminidase which

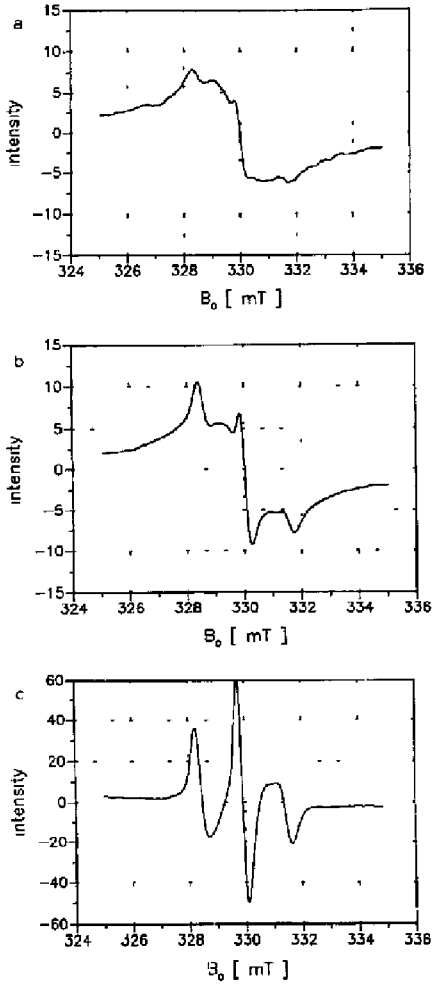

Fig 6 The effect of tryptic digestion on the EPR spectra of DMPC-vesicles containing cholestane spin label ($X_{Ch} = 0.05$) and glycophorin ($X_G = 1.2\ 10^{-3}$) Spectra were taken at $T = 36°C$ (a) Initial spectrum (b) Spectrum after incubation of the vesicles with a trypsin solution Only external protein fragments were cleaved (c) Spectrum after tryptic digest and treatment with ultrasonic pulses External as well as internal protein fragments are cleaved

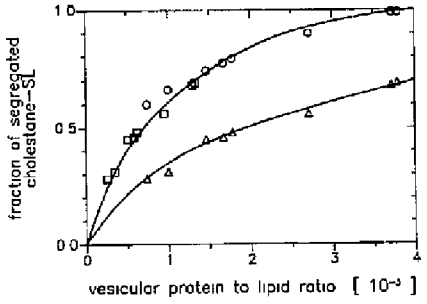

Fig 7 The fraction of segregated cholestane spin label is given as function of the membrane glycophorin content EPR spectra were analyzed by spectral subtraction of the narrow 3-line spectrum obtained in the absence of glycophorin from the broadened spectrum obtained in the presence of glycophorin Vesicles were doped with $X_{Ch} = 0.05$ (○———○) cholestane spin label. Tryptic digestion of the vesicle outside reduces the amount of segregated cholesterol (△———△) The corresponding X_G values for the triangles represent the initial undigested glycophorin content Sialic acid determination performed after external enzymatic digest gives the mole fraction of glycophorin where the N-terminus is oriented towards the inside of the vesicles and which is therefore not cleaved Curve (□———□) was obtained by taking X_G as the mole fraction of glycophorin with intact N-terminus This curve is almost identical to the one obtained before enzymatic digest With additional ultrasonication the fraction of segregated cholestane spin label approaches zero at all glycophorin concentrations

cleaves the sialic acid residues did not change the spectrum of cholestane-SL in glycophorin-containing membranes

Steroid clustering was only observed with cholestane-SL but could not be ascertained with the androstane-SL which lacks the hydrocarbon chain and which is oriented inversely within the membrane

Discussion

Numerous intrinsic proteins show a preferential interaction for some class of lipids, which whenever it exists has a relative weak specificity Marsh et al [20,21] reported that cytochrome oxidase or Na^+/K^+-ATPase, for example, have only a 5-fold higher relative affinity constant for cardiolipin over phosphatidylcholine Different spin-labeled lipids have widely been used to characterize such a lipid–protein interaction [22] by analyzing their EPR spectra Jost et al [23] were the first who demonstrated the coexistence of two components in the EPR spectra of spin-labeled lipids in protein-containing membrane systems A motionally restricted component was attributed to the lipids interacting directly with the intermembranous protein surface, whereas the second mobile component represents the fluid bilayer lipids The number of protein-interacting lipids bears a fixed stoichiometry with respect

to the amount of proteins and correlates roughly with their intramembranous protein perimeter [24]

Long-range interactions at very low protein contents have been described recently in glycophorin-containing membranes [14] The functional implications of the lipid–protein interactions, however, are still unclear as well as the role of the first shell lipids which may simply ensure good sealing of the protein into the fluid lipid environment

Matching of lipid chain length with the length of the hydrophobic protein core is sufficient to result in a preferential interaction [25] Lipids of a given chain length may thus adapt the protein to the lipid bilayer Another candidate for such an adaptation process is cholesterol Seigneuret et al [26] incorporated the spin-labeled cholesterol analogue 25-doxylcholestanol into intact erythrocyte membranes and obtained clear evidence for the existence of an immobilized spectral component The authors postulate domains of cholesterol and band 3 Interestingly this domain formation was reduced after extraction of the cytoskeletal proteins and it disappeared completely upon proteolysis of intrinsic proteins by chymotrypsin A possible role of cholesterol in regulating associations between integral membrane proteins was suggested by Muhlebach and Cherry [27] Decreasing the cholesterol content of erythrocyte ghosts resulted in an increased rotational mobility of band 3 However, this effect could only be observed after complete removal of the cytoskeletal proteins and after partial proteolysis with trypsin which clearly demonstrates the modulating role of these proteins Another example for the involvement of cholesterol in a lipid–protein interaction is the Ca^{2+}-ATPase Cholesterol is trapped in protein/protein interfaces in ATPase oligomers which is then not accessible to external phospholipids [28] To summarize cholesterol seems to be an important factor that controls lipid–protein as well as protein–protein interaction

Our present study provides strong evidence that one of the main glycoproteins of erythrocyte membranes, glycophorin, specifically binds the cholestane spin label The proteoliposome reconstitution is favoured by this steroid EPR spectroscopy directly proves a cholestane spin label segregation induced by glycophorin interaction The spin-labeled sterol analogue is a well suited substitute for cholesterol due to its unchanged cross-sectional area and the preserved polarity The change in its spectral shape in the presence of glycophorin can be interpreted unequivocally without computer simulation The strong increase of the spin exchange interaction after incorporation of glycophorin converts the three-line EPR spectrum into a broad one-line spectrum which is only possible if the probe molecules approach each other by the size of the lattice spacing of the lipid bilayer [29,30] A glycophorin-induced increase in the diffusional rate in the fluid phase is excluded from earlier photobleaching experiments [16] Thus a conclusive interpretation of our EPR data is the formation of glycophorin-cholesterol domains However, cholesterol exclusion from lipid-protein domains, as opposed to glycophorin-cholesterol domain formation, cannot be dismissed but has not been conclusively shown by the present experiments

Most interestingly the observed effect could be reversed by tryptic cleavage of the hydrophilic protein residues Cleavage of the protein segments on the outside of the vesicle reduces the spin–spin interaction drastically and the fraction of segregated cholestane-SL decreases by about 35% (Fig 7) Sialic acid determination after enzymatic digest revealed, that about 65% of the glycophorin was oriented with its N-terminus to the vesicle outside If we take our experimental data for the fraction of segregated cholestane-SL and if we relate them to the remaining amount of glycophorin with intact N-terminus (squares in Fig 7) we obtain an excellent fit with the original curve obtained before enzymatic digestion Values are identical to those which were obtained with a 65% reduced glycophorin content. We thus conclude that the N-terminus is important for this protein–cholestane-SL interaction Cleavage of the N-terminus may lead to an aggregation of the remaining more hydrophobic protein segments We assume that cholestane-SL is then excluded from the protein boundary and is partially dispersed in the bilayer membrane The clustering was completely reversed if tryptic digestion was performed from both sides of the vesicles leaving the pure hydrophobic segments within the bilayer membrane which are known to aggregate spontaneously [31] Cleavage of the sialic acid residues did not effect the EPR spectra

The observed clustering seems to be cholestane-SL specific Another steroid spin label, 3-doxylandrostane, which lacks the hydrocarbon chain and which is inversely oriented within the bilayer membrane did not show any of the effects observed with cholestane-SL This may again be taken as an indication for a hydrophobic interaction between cholesterol and glycophorin Such a protein–steroid interaction may well control the protein assembly between glycophorin and Band 3, the anion transporter of the red blood cell, which is also known to bind cholesterol [7]

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