THE INFLUENCE OF LIPID COMPOSITION ON GLYCOPHORIN-INDUCED BILAYER PERMEABILITY

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(1) Glycophorin was incorporated into large unilamellar vesicles and the bilayer permeability was measured as a function of the lipid composition. (2) In agreement with previous data (Van der Steen, A.T.M., De Kruijff, B. and De Gier, J. (1982) Biochim. Biophys. Acta 691, 13–23) it was found that glycophorin greatly increased the bilayer permeability of DOPC vesicles. This effect was observed for a large variety of phosphatidylcholines, differing in their fatty acid composition and homogeneity. (3) In sharp contrast, it was observed that variations in the polar headgroups by incorporation of DOPE, DOPS and, to a lesser extent, cholesterol, into the DOPC/glycophorin vesicles restored the barrier function. (4) These results are compared to the size of the particles, revealed by freeze-fracture electron microscopy on the glycophorin-containing bilayer and are discussed in the light of various types of lipid-protein interactions and protein aggregation state.

Introduction

The primary function of biological membranes is to form permeability barriers. Concerning their molecular organization it is generally accepted that lipid molecules are arranged in a bilayer, and that membrane proteins are embedded in this lipid matrix. From studies on model systems it is known that lipid bilayers (which are formed spontaneously when e.g. phosphatidylcholines are dispersed in water) act as efficient permeability barriers. However, when a membrane protein is incorporated in such a bilayer, often a large increase in nonspecific permeability of the barrier can be noticed [1–7].

To obtain information on the effect of lipid-protein interactions on the general barrier functions of membranes, model systems containing glycophorin have been studied. Glycophorin is a membrane-spanning protein and the major sialoglycoprotein of the human erythrocyte membrane [8]. This protein is suitable for studies on the barrier properties of model membranes since it has no known transport function. Moreover, it can be purified in sufficient quantities with very little contamination of native erythrocyte lipids and it can be incorporated in bilayer organizations without the use of detergents.

From earlier studies it is known that incorporation of glycophorin into DOPC bilayers by the method of MacDonald and MacDonald [9], which results in large unilamellar vesicles, increases the permeability for electrolytes like K^+ [1] and Ca^{2+}, and non-electrolytes with a molecular weight of up to 900. This suggests that the incorporation of the protein induces the formation of pore-like struc-

tures of 15–18 Å diameter, formed by glycophorin aggregates or packing defects at the protein/lipid interface [7]. It has been reported that such a glycophorin-induced permeability is dependent on the phospholipid composition of the bilayer. Incorporation of 10 mol% dioleoylphosphatidylethanolamine into dioleoylphosphatidylcholine vesicles decreases the permeability for Dy^{3+} [3], while glycophorin reconstituted with a mixture of the native erythrocyte lipids, with or without cholesterol, resulted in vesicles which were much less leaky than glycophorin/DOPC vesicles [1].

To get a better understanding of this lipid dependency of the glycophorin-induced bilayer permeability, we systematically investigated the effect of fatty acid chain composition of the phospholipids, variation in the polar headgroup of the phospholipids and cholesterol content on the permeability of large unilamellar glycophorin-containing lipid vesicles. It is shown that the increased permeability is virtually independent on the acyl chain composition of the phospholipids, but is highly dependent on the nature of the polar headgroup of the phospholipids and the presence of cholesterol.

Materials and Methods

Mateirals

1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (12:0,12:0 phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0,14:0 phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0,16:0 phosphatidylcholine), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (16:1,16:1 phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c,18 : 1_c phosphatidylcholine) and egg phosphatidic acid, were synthesized as described before [10].

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0,18:1_c phosphatidylcholine), 1-stearoyl-2-elaideoyl-*sn*-glycero-3-phosphocholine (18 : 0, 18 : 1_t phosphatidylcholine), 1,2-dielaideoyl-*sn*-glycero-3-phosphocholine (18 : 1_t,18 : 1_t phosphatidylcholine), 1,2-dieruceoyl-*sn*-glycero-3-phosphocholine (22 : 1_c,22 : 1_c phosphatidylcholine), were synthesized according to Warner et al. [11], with a slight modification [12].

1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (18 : 1_c, 8 : 1_c phosphatidylserine) was purified accord-

ing to Comfurius et al. [13].

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (18 : 1_c,18 : 1_c phosphatidylethanolamine) was prepared according to established methods [14].

All phospholipids were pure as they showed one spot on high performance thin-layer chromatography, using the appropriate solvent system. Cholesterol was purchased from Merck and bovine brain sphingomyelin was from Koch-Light Laboratories Ltd. Glycophorin was purified from human erythrocytes according to the method of Taraschi et al. [15].

D-[1-³H]Glucose (7.0 Ci/mmol) was purchased from Amersham, [³H(G)]Raffinose (7.8 Ci/mmol) and [*carboxyl*-¹⁴C]dextran (0.99 mCi/g) from New England Nuclear.

All other chemicals used were of analytical grade.

Methods

Reconstitution of glycophorin. Glycophorin was incorporated into unilamellar lipid vesicles by the method of MacDonald and MacDonald [9]. A mixture of 0.65 mg of glycophorin and 5 μmol lipid (lipid/glycophorin, molar ratio, 400:1), was dissolved in chloroform/ methanol/ water (150:75:1, v/v) and dried by evaporation. After hydration of the mixed glycophorin-lipid film at a temperature above the transition temperature of gel state to liquid-crystalline state in 0.5 ml 100 mM NaCl, 0.2 mM EDTA, 0.2 mM NaN₃, 0.1% (g/v) dextran, 1 mM glucose or 1 mM raffinose, 10 mM Na/Hepes, pH 7.4 buffer containing 0.25 μCi/ml [¹⁴C]dextran, 2 μCi/ml [³H]glucose or [³H]raffinose, the spontaneously formed glycophorin-containing vesicles were separated from multilamellar, glycophorin-poor vesicles by means of differential centrifugation.

Preparation of protein-free liposomes. As blank vesicles for the studied glycophorin/lipid vesicles, multilayered liposomes of the same lipid composition, but with 4 mol% egg phosphatidic acid, were used. The liposomes were prepared by vortexing 5 μmol lipid film (dried under vacuum from chloroform) with 0.5 ml buffer, described above, at a temperature above the gel-to-liquid phase transition temperature of the lipids, followed by centrifugation during 10 min at 10000 × g.

Efflux assay. The efflux of ³H-labeled glucose

and raffinose was determined, using the method of Van der Steen et al. [1] which is described in detail in Van Hoogevest et al. [7]. After centrifugation, the vesicles were washed four times by resuspending the vesicle pellet (0.5–5 μmol lipid) obtained after centrifugation (30 min, 35000 $\times g$) in 1 ml buffer (at indicated temperatures) without radioactive solutes. This washing procedure took 4 h and is essential to reduce the amount of non-trapped solutes to acceptable levels. Finally, the vesicle pellet was resuspended in 1 ml buffer from which samples for radioactivity (see Ref. 2) and phosphorus assay [16] were drawn. In this way, an estimate of the amount of solute (glucose, raffinose), relative to the trap of the large nonpermeable solute, still present in the vesicles after 4 h at the indicated temperatures is obtained.

Analytical methods. Phospholipids were determined as phosphorus according to Rouser et al. [16] or Fiske and SubbaRow [17].

In general, the glycophorin content of the vesicles was determined via a sialic acid assay [18], using a value of 2.1 μmol of sialic acid/mg of protein.

Freeze-fracture electron microscopy was performed as outlined previously [19]. 25%(v/v) Glycerol was added to the sample to prevent freeze damage.

Results

Reconstitution of glycophorin according to the method of MacDonald and MacDonald [9] as described in Methods, was performed with a variety of molecular species of phosphatidylcholine. For all the species tested, this resulted in the spontaneous formation of protein-containing vesicles with comparable trap of 5 $\pm$ 2 1/mol phospholipid, as measured with [^{14}C]dextran and comparable lipid-glycophorin molar ratios of 400 $\pm$ 50 : 1. The permeability properties of the protein containing membranes, have been studied by measuring the trapping ability of the vesicles for glucose and raffinose, relative to the trap of large impermeable dextran, after a washing procedure during 4 h at the indicated temperature (see Methods). For reason of comparison, trap measurements have also been done with multilayered protein-free liposomes of the same phosphatidylcholines. The re-

sults of these experiments are summarized in Table I.

Comparison of the permeability data of the glycophorin-containing vesicles with the pure lipid vesicles, clearly shows that incorporation of glycophorin induces a largely increased permeability for glucose as well as for raffinose. This confirms earlier results with reconstituted systems using 18 : 1$_c$,18 : 1$_c$ phosphatidylcholine [1,7], and shows that changes in the chain length and saturation of the paraffin chains of phosphatidylcholine do not influence the glycophorin-induced permeability defects in the bilayer. The protein-induced permeability found in the reconstituted system with 16 : 0,16 : 0 phosphatidylcholine, suggests that even the physical state of the bilayer seems to have no influence, since at 25°C (assay temperature) this lipid in the absence of protein is in the gel state. This increased permeability is possibly caused by similar packing defects present in the glycophorin/DOPC vesicles since it is known from differential scanning calorimetric analysis on glycophorin/lipid vesicles, that incorporation of the protein may remove a considerable amount of lipid molecules from the cooperative phase transition which results in a local, liquid, disordered environment around the protein [20].

Because of the similarity in polar headgroup between phosphatidylcholine and sphingomyelin we also tested the effect of glycophorin incorporation on the permeability properties of bovine brain sphingomyelin vesicles (see Table I). The limited increase in permeability, caused by glycophorin incorporation, possibly suggests that the ceramide skeleton of the phospholipid is more capable to confine the protein-induced permeability than the glycerol skeleton present in phosphatidylcholine.

The high relative trap values for raffinose (> 100%) in the case of the protein-free liposomes (which are multilayered) most likely are caused by a relatively low trapping ability for the large nonpermeable dextran molecule (molecular radius 30 Å, Ref. 7), caused by the small interlamellar repeat distance in the liposomes. The lower trap values for glucose are explained by earlier studies which showed that fluid phospholipid bilayers have a low, but significant, permeability for glucose [21].

The low trap values for liposomes prepared of 12 : 0,12 : 0 phosphatidylcholine, both for glucose

TABLE I

PERMEABILITY PROPERTIES OF GLYCOPHORIN/PHOSPHATIDYLCHOLINE VESICLES AS A FUNCTION OF THE FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE

Glycophorin/lipid vesicles and liposomes were prepared at 25°C as outlined in Methods. Liposomes of $22:1_c, 22:1_c$ phosphatidylcholine and $18:1_t, 18:1_t$ phosphatidylcholine were made in 25 mM NaCl, 250 mM sucrose, 0.2 mM NaN$_3$, 0.2 mM EDTA, 0.1% (g/v) dextran, 1 mM glucose or 1 mM raffinose, 10 mM Tris-HCl (pH 7.4) containing 0.25 μCi/ml [^{14}C]dextran, 2 μCi/ml [^{3}H]glucose or [^{3}H]raffinose and washed (see efflux assay, outlined in Methods) in 140 mM LiCl, 0.2 mM NaN$_3$, 0.2 mM EDTA, 0.1% (w/v) dextran, 1 mM glucose or 1 mM raffinose, 10 mM Tris-HCl, (pH 7.4), because the specific density of the liposomes was too low for a proper pelleting in the normal buffer (Ref. 35). The efflux assay was performed at 25°C as outlined in Methods.

Phosphatidylcholine species	Relative glucose trap		Relative raffinose trap	
	Glycophorin/lipid vesicles	Liposomes	Glycophorin/lipid vesicles	Liposomes
Di-saturated				
12:0,12:0	0	11	0	48
14:0,14:0	0	3	0.3	4
16:0,16:0	0	91	0	134
Di-unsaturated				
16:1,16:1	0.8	88	10	145
$18:1_t, 18:1_t$	0.5	77	6	164
$18:1_c, 18:1_c$	1.0	81	7	193
$22:1_c, 22:1_c$	3.5	79	6	157
Mixed species				
$16:0,18:1_c$	0.5	89	8	126
$18:0,18:1_c$	4.5	108	8	155
Bovine brain sphingomyelin	15	20	26	34

and raffinose, are consistent with the inability of this phospholipid to form a stable permeability barrier [22], whereas the low values for liposomes of 14:0,14:0 phosphatidylcholine can be explained by the assay temperature of 25°C which is close to the gel-to-liquid-crystalline transition temperature of 14:0,14:0 phosphatidylcholine (23°C, Ref. 20) at which bilayers of this phospholipid have been shown to exhibit a permeability maximum [24]. Also for bovine brain sphingomyelin vesicles an increased permeability at 25°C has been reported [36], possibly due to the fact that the assay temperature is close to the broad phase transition temperature ranging from 37 to 52°C, as measured with differential scanning calorimetry.

A possible dependency of the glycophorin-induced permeability on the nature of the polar headgroup has been studied by the incorporation of increasing amounts of, respectively, DOPS and DOPE into glycophorin/DOPC vesicles.

Fig. 1 shows the effect of replacement of DOPC

by increasing concentrations of DOPS with identical paraffin chains. It can be noted that the glucose leak decreases from 99% in the DOPC/glycophorin vesicles to 35% in vesicles with high concentrations of DOPS, whereas the vesicles become already completely impermeable to raffinose when an equimolar mixture of the two phospholipid species is reached. This decrease in permeability is not caused by a decreasing amount of incorporated glycophorin, since the lipid/glycophorin molar ratio is found to be constant up to very high concentrations of DOPS (compare inset, Fig. 1). The trapped volumes, calculated from the amount of [^{14}C]dextran which is enclosed, decreases with increasing DOPS content (Fig. 1). This indicates that smaller vesicles are formed at high concentrations of this phospholipid.

Freeze-fracture electron microscopy shows ill-defined particles at fracture faces of glycophorin/DOPC vesicles (diameter 50 Å, Ref. 25). Glycophorin/DOPS vesicles show fracture faces with
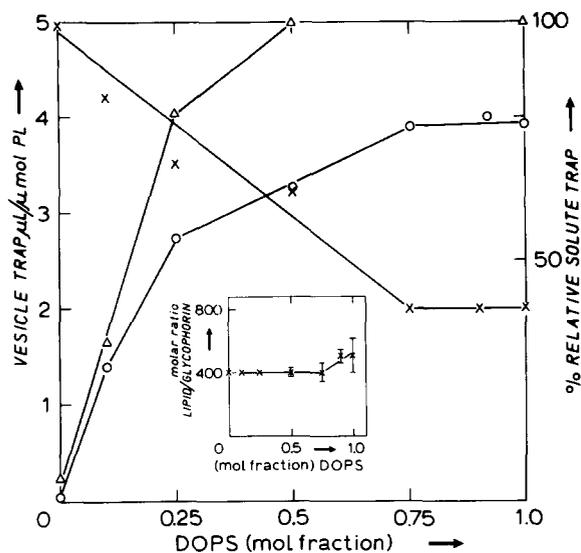
Fig. 1. Effect of increasing concentrations of DOPS on the permeability properties and incorporation of glycophorin in DOPC vesicles ×———×, vesicle trap in μl/μmol phospholipid as measured with ¹⁴C-labeled dextran; O———O, glucose trap relative to dextran trap; Δ———Δ, raffinose trap relative to dextran trap. Vesicles were prepared and the efflux assay was performed at 0°C as outlined in Methods.
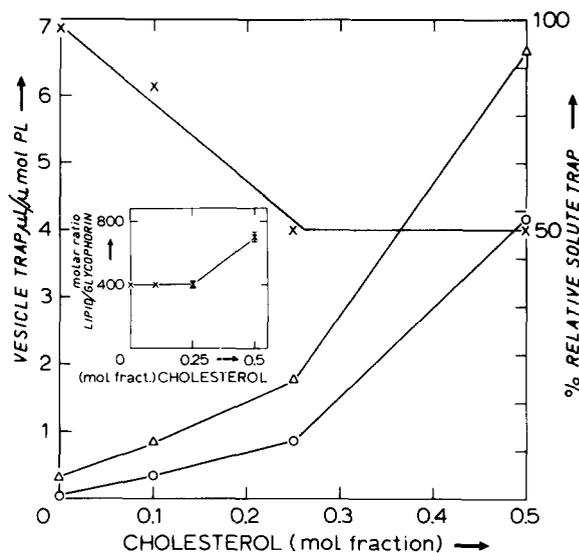

Fig. 3. Effects of increasing concentrations of cholesterol on the permeability properties and incorporation of glycophorin into glycophorin/DOPC vesicles. ×———×, vesicle trap in μl/μmol lipid as measured with ¹⁴C-labeled dextran; O———O, glucose trap relative to dextran trap; Δ———Δ, raffinose trap relative to dextran trap. Vesicles were prepared and the efflux assay was performed at 0°C as outlined in Methods.
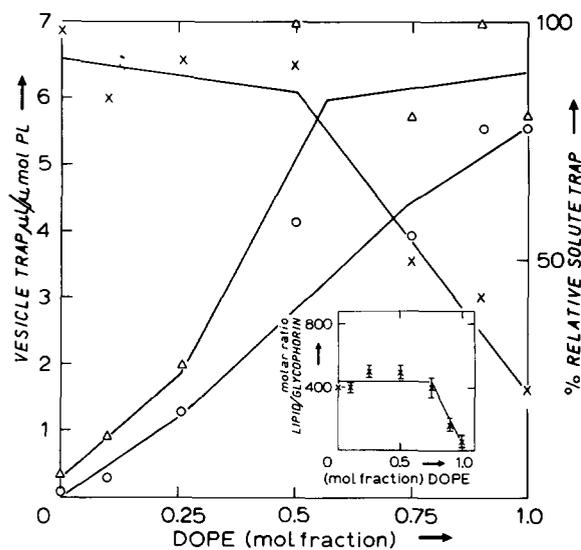

Fig. 2. Effect of increasing concentrations of DOPE on the permeability properties and incorporation of glycophorin into glycophorin/DOPC vesicles. ×———×, vesicle trap in μl/μmol phospholipid, as measured with ¹⁴C-labeled dextran; O———O, glucose trap relative to dextran trap; Δ———Δ, raffinose trap relative to dextran trap. Vesicles were prepared and the efflux assay was performed at 0°C as outlined in Methods.

protein particles with comparable size (not shown).

Replacement of DOPC by increasing concentrations of DOPE, up to 50 mol% results in a gradual permeability decrease for glucose as well as raffinose (Fig. 2), while the incorporated amount of glycophorin in the vesicles is not influenced by the increasing DOPE concentration (see inset Fig. 2).

Further increase of the DOPE concentration results finally (at 100 mol% DOPE) in vesicles, which are not leaky for raffinose and only slightly for glucose (Fig. 1). This is in agreement with previous results which showed that the glycophorin/DOPE vesicles are impermeable for K⁺ [15].

The further increase of DOPE from 50 to 100 mol% is accompanied with a dramatic decrease in the phospholipid/glycophorin molar ratio (inset Fig. 2).

The vesicle trap hardly changes up to 50 mol% DOPE, but decreases to 1.8 μl/μmol phospholipid upon further increasing the DOPE content (Fig. 2). Such a relatively low trap for unilamellar vesicles was also noticed in K⁺ trap measurements

and agrees with the smaller size of these vesicles, as observed by freeze-fracture electron microscopy [15].

In agreement with earlier observations [15], it was found that in glycophorin/DOPE vesicles much larger particles (about 100 Å) were present at the fracture face than in the case of glycophorin/DOPC and glycophorin/DOPS vesicles.

Earlier studies [1] showing differences in barrier function upon incorporation of glycophorin into a mixture of erythrocyte lipids with or without cholesterol, prompted us to study also the effect of cholesterol on the glycophorin-induced permeability in DOPC vesicles. Fig. 3 shows that incorporation of up to 25 mol% of cholesterol into glycophorin/DOPC vesicles has a moderate decreasing effect in the protein-induced permeability. Further increasing the cholesterol concentration from 25 mol% to 50 mol% had a much more pronounced effect on the permeability both with respect to glucose and raffinose. However, increasing the amount of cholesterol from 25 mol% to 50 mol% also decreases the amount of incorporated glycophorin and, therefore, the permeability reducing effect of cholesterol may partially be caused by a reduction in protein content of the bilayer. These results are in agreement with a previous study of Van der Steen et al. [1] in which a permeability decrease was observed, after addition of cholesterol to glycophorin/erythrocyte phospholipid vesicles.

Discussion

Reconstitution of glycophorin into DOPC bilayer results in an increased permeability allowing polar components with diameters of up to 15–18 Å to pass the barrier [7]. In an attempt to explain this increased permeability two possibilities have been considered. Firstly, the increased permeability could be the result of dynamic disorientations of the lipid molecules on the lipid-protein interface. Secondly, the 'pores' could be formed by aggregated protein molecules in the bilayer. Considering the large size of the pores it has been suggested [7] that they most likely consist of protein aggregates. The packing defects at protein/lipid interfaces were thought to be more unlikely [7], since packing defects are generally believed to be smaller than the participating lipid

molecules. However, recent experiments (Van Hoogevest, P., unpublished observations) indicate the presence of pores of similar size in a lipid bilayer at the liquid-crystalline-to-gel phase transition temperature.

The present results indicate that the formation of 'pores', present in the glycophorin/lipid vesicles, is not influenced by the nature of the paraffin chains of the membrane lipids, but that variation in the polar regions of the bilayers can promote a sealing of the barrier. Moreover, it is quite remarkable to note that only phosphatidylcholine is not capable of sealing the glycophorin/lipid vesicles properly. In contrast, the glycophorin/DOPS and glycophorin/DOPE vesicles are properly sealed. In attempt to explain this phenomenon, we subsequently discuss the glycophorin/lipid interaction in terms of (1) the so-called shape model, which relates the overall dynamic shape of the lipid molecule, including intra- and intermolecular interactions, to the phase preferred by that lipid in aqueous dispersion and which has been used to explain the presence of specially shaped lipid molecules at the protein-lipid interface, in order to match the irregular shape of the protein [27]; (2) hydrophobic interactions between the protein and phospholipid; (3) aggregation of the protein in the plane of the membrane; (4) hydrogen bond formation and electrostatic interactions between the polar headgroup of the phospholipid and the protein.

The shape model predicts a cylindrical form for DOPC and DOPS, since these lipids adopt, in aqueous medium, the lamellar phase and a cone form for DOPE as this lipid prefers the H_{II} phase. It could be suggested that defects occur between the cylindrical DOPC molecule and an irregularly shaped protein surface, which could be sealed by the cone shaped DOPE molecule. These packing defects could give rise to the observed increased permeability. Besides the fact that the shape of the lipid molecule could be distorted by the protein, there are two other arguments to oppose this view: firstly the DOPS molecule is cylindrical and it is still capable to seal the glycophorin/lipid vesicles properly (see Fig. 1). Secondly, the permeability for glucose as well as raffinose decreases gradually upon increasing the concentration of DOPE in the glycophorin/DOPC bilayer, whereas in terms of

the shape concept one would expect to observe a dramatic sealing effect at low concentrations of DOPE.

It is known that, upon reconstitution into a lipid bilayer, glycophorin causes extensive perturbations of membrane phospholipid hydrocarbon chain conformation and packing [29,30]. These perturbations may contribute to a certian extent to the increased permeability caused by the incorporation of glycophorin. This study indicates that the permeability properties of the vesicles are not influenced by changes in the length and unsaturation of the hydrocarbon chains of the phospholipids and suggests the presence of such perturbations in all phosphatidylcholines tested.

Freeze-fracture electron microscopy of glycophorin/lipid vesicles shows a lipid dependency of the protein particle size. In glycophorin/DOPE and glycophorin/DOPS vesicles small particles are observed, whereas in glycophorin/DOPE vesicles the particles are much larger. If the particle size is a measure for protein aggregation in the plane of the membrane, this means that in the glycophorin/DOPC and glycophorin/DOPS vesicles the protein is much less aggregated as compared to the protein in the glycophorin/DOPE vesicles. This is also suggested by rotational diffusion measurements by means of phosphorescence depolarization (Van Hoogevest, P. and co-workers, unpublished observations). If the degree of permeability is governed by the aggregation state of the protein, the glycophorin/DOPE vesicles would be more permeable than the glycophorin/DOPC and glycophorin/DOPS vesicles. However, the reverse is the case. At present it cannot be excluded that the particles, observed by freeze-fracturing, represent not only protein but protein-lipid complexes. In the latter case it could be that the presence of lipids enclosed in the aggregate could change the permeability properties of the system. Furthermore it could be that due to the strong intermolecular interaction in PE the glycophorin molecules are squeezed together so tightly, that a possible pore within the aggregate would close. In addition, the effective lipid-protein binding zone would be decreased by the increased size of the glycophorin aggregate in the PE vesicles. Possibly the intermolecular PS and PC interactions are much weaker, which results in a lower tendency for protein aggregation.

Another possible explanation for the low permeability of the glycophorin/DOPS and glycophorin/DOPE vesicles (see Fig. 2) is the formation of hydrogen bonds between the polypeptide and the serine or ethanolamine headgroup. The high permeability of the glycophorin/PC vesicles could then be understood as the lack of capacity of the choline headgroup to undergo hydrogen binding. Besides hydrogen bond formation, electrostatic interactions between the negatively charged headgroup of PS and the positively charged amino acids, present in the hydrophilic C-terminal part of glycophorin, possibly located at the membrane/water interphase [25] could further stabilize the protein-lipid interaction, leading to a decreased bilayer permeability. Specific interactions between glycophorin and PS is furthermore suggested by the fact that this lipid copurifies with glycophorin [31–33] and has a strong and specific interaction with glycophorin in monomolecular films at the air/water interface [34].

With respect to a possible participation of hydrogen bonds in the lipid-protein boundary phase it is interesting to compare permeability properties of pure lipid bilayers at the gel-to-liquid-crystalline phase transition temperature with the glycophorin/lipid vesicles. Whereas phosphatidylcholines show an enhanced bilayer permeability [24], phosphatidylethanolamines are not leaky at the phase transition temperature [28]. This has been explained by the hydrogen bonds present between the PE molecules [28] which would stabilize packing defects at the phase boundaries. This may resemble the situation encountered in the glycophorin-containing PE vesicles. Whether phosphatidylserine show an enhanced permeability in the phase transition is not yet known.

The present study indicates the complexity of glycophorin-lipid interactions with respect to the permeability properties of the recombinant vesicles. In addition, it shows that the three phospholipids studied in most detail, e.g. DOPC, DOPS and DOPE, all show to a certain extent specific types of interaction with glycophorin. At present no unifying concept can be offered to explain all aspects of the observed permeability changes.

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