Biochimica et Biophysica Acta, 464 (1977) 482—492

BBA 77597

EVIDENCE FOR THE PREFERENTIAL INTERACTION OF GLYCOPHORIN WITH NEGATIVELY CHARGED PHOSPHOLIPIDS

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(Received July 13th, 1976)

Summary

Glycophorin, extracted from the erythrocyte membrane after treatment with lithium-diiodo-salicylate, still contains a significant amount of phospholipid, consisting predominantly of phosphatidylserine. Methods are described wich lead to a full delipidation of the protein.

After treatment with neuraminidase, delipidated glycophorin shows a preferential interaction with monolayers of negatively-charged phospholipids. This lipid-protein interaction is decreased by the presence of cholesterol in the lipid film.

Introduction

Of all biological membranes, the human erythrocyte membrane is best characterized, both with regard to the lipids and to the membrane-bound proteins [1—4]. The phospholipids are asymmetrically distributed between the two halves of the bilayer, with phosphatidylcholine and sphingomyelin preferentially on the outer and phosphatidylethanolamine and phosphatidylserine preferentially on the inner side of the membrane [3,5—7]. Furthermore, the membrane contains an almost equimolar amount of cholesterol, but the role and distribution of this lipid have not yet been elucidated.

As well as the lipids, the major membrane-bound proteins have also been shown to have an asymmetric distribution over the membrane [1,2,8]. Spectrin, a high molecular weight protein, is bound exclusively to the cytoplasmic side of the membrane. On the other hand the so-called 100 000 mol. wt protein, which is probably involved in water and anion transport [8,9] and glycophorin, the major glycoprotein of the human red cell, span the membrane with their sugar residues directed to the outside of the cell [10].

Of these membrane-bound proteins glycophorin is best characterized. Due to its high sugar content (about 60% by weight) and the relatively high proportion of hydrophilic amino acids, this protein is very water-soluble, in contrast to most of the other membrane-bound proteins.

Several purification methods have been described for glycophorin, all based on its water solubility [11,12]. Recently the complete amino acid sequence of glycophorin has been solved [13], showing a division of the protein into three distinct regions.

The C-terminal end of the protein, containing many charged amino acids, is located at the cytoplasmic side of the membrane. A second part of the protein spans the membrane and consists of 23 uncharged and mostly hydrophobic amino acids, probably in an α-helical structure. The N-terminal part of the protein is located at the exterior of the membrane. This last part contains all the places of attachment of the oligosaccharide residues.

Since this protein can be purified in a water-soluble and lipid-free form, it was chosen in this study to investigate whether special lipids are involved in the binding of an integral protein to the lipid matrix. This was done by studying the lipids that remain bound to the protein during the various purification steps, and by recombining the delipidated protein with lipids using the technique of monolayer penetration. Some preliminary conclusions of this study have been published elsewhere [14].

Materials and Methods

Materials

Fresh blood in acid-citrate dextrose was obtained from the bloodbank without special blood group preference. Ghosts were prepared according to Dodge et al. [15], dialysed against distilled water and lyophilized.

The major phospholipids of the human erythrocyte membrane were purified as described before [16].

Beef brain phosphatidylserine was purified according to Sanders [17] and freed of divalent cations according to Papahadjopoulos [18]. Egg phosphatidic acid was prepared from egg phosphatidylcholine according to Davidson [19]. Dioleoylphosphatidylglycerol was made synthetically as described [20].

Neuraminidase from *Clostridium perfringens* was obtained from Sigma (Type V) and freed of proteolytic activity according to Hatton [21]. All other reagents were of Analytical Reagent Grade and were used without further purification.

Purification of glycophorin

Glycophorin was purified according to Marchesi and Andrews [11]. Ghosts were treated with lithium-diiodo-salicylate (0.3 M), and extracted with 50% phenol in water at 4°C. The phenol-poor phase was dialysed against water and lyophilized. The protein was suspended in ethanol at a concentration of 3 mg protein/ml, stirred for 20 min at room temperature, and centrifuged for 15 min at 3000 $\times g$ at room temperature. The pellet was suspended in chloroform/methanol (2 : 1, v/v) and treated in a similar way. The pellet was now sus-

pended in 10 mM Tris · HCl pH 7.4, dialysed against water and lyophilized (see also ref. 11).

Total delipidation could be achieved in two alternative ways. (a) The protein was suspended in chloroform/methanol, 2 : 1 with 0.04 N HCl at a concentration of 3 mg protein/ml and stirred for 20 min at room temperature. The solution was neutralized and centrifuged at 3000 × *g* for 15 min (acid delipidation). (b) The protein was dissolved in 10 mM Tris · HCl pH 7.4 at a protein concentration of 0.5 mg/ml. An equal volume of 50% phenol in water was added and the solution was stirred for 45 min at room temperature. Subsequently the solution was centrifuged at 4° C at 4000 × *g* and the delipidated protein obtained from the phenol-poor phase (second phenol extraction).

Methods

Protein was determined according to Lowry et al. [22], using bovine serum albumin as a standard. Sialic acid was determined according to Warren [23], phosphorus according to a modification [24] of the procedure of Fiske-Subba-Row and cholesterol according to Liebermann-Burchard as modified according to Huang [25].

Phospholipids were separated by two-dimensional thin-layer chromatography according to Broekhuyse [26] in a nitrogen atmosphere using 20 × 20 cm plates of silica H/2% Mg SiO_3 (Merck). Fatty acid analysis was done using a Perkin-Elmer "F-11" gas chromatograph, as described in ref. 16. Isoelectric focussing in 8 M urea was done as described by Bhakdi [27].

Monolayer technique

Force-area curves were measured on a teflon trough 32.2 cm long and 17.2 wide. The trough was filled with a 1 mM Tris · HCl solution, pH 7.4 in double distilled water. 50 μmol lipid was spread from a chloroform solution; cholesterol was spread from an ether solution. The compression rate was 77.4 cm^2/min, and the surface tension was recorded by the Wilhelmy plate technique, using a Beckman L.M.C. 500 electrobalance.

Interactions of the protein with the lipid film were measured as described by Demel et al. [28]. A small circular glass trough was used containing 50 ml 1 mM Tris · HCl solution pH 7.4.

200 μl of a solution containing 0.5 mg protein/ml was injected under the lipid film. All experiments were carried out at 30° C.

Results

Purification and delipidation of glycophorin

The crucial step in the purification of glycophorin according to Marchesi [11] is the treatment of the erythrocyte ghost with the chaotropic reagent lithium-diiodo-salicylate, which leads to a disruption of the bilayer structure.

By means of the subsequent phenol extraction a water-soluble protein fraction, consisting predominantly of glycophorin, is obtained in the phenol-poor phase. This protein fraction still contains a rather significant amount of bound phospholipid (see Table I). In order to delipidate the protein and analyse this phospholipid fraction, subsequent extractions of the protein were carried out

TABLE I

PHOSPHATE, SIALIC ACID (AcNeu) AND CHOLESTEROL CONTENT OF GLYCOPHORIN AFTER THE VARIOUS DELIPIDATION STEPS

(For details see text.)

	Phosphate/protein (μmol/mg)	Ac Neu/protein (μmol/mg)	Cholesterol/ phospholipid *
1. Phenol-extraction	1.23	1.6	0.27
2. Ethanol and chloroform/ methanol, 2 : 1	0.92	2.1	trace
3A. Chloroform/methanol, 2 : 1 plus 0.04 N HCl	0.30—0.20	2.1	—
3B. 2nd phenol-extraction	0.25	2.1	—

* Molar ratio (in human erythrocyte membrane 0.74).

using organic solvents with an increasing power to delipidate. The extraction with ethanol and chloroform/methanol led to a decrease in the phosphate to protein ratio and to an almost complete release of cholesterol. Furthermore, these steps resulted in an increase in sialic acid to protein ratio, indicating an increased purity of the protein. The composition of the phospholipid released in this way is listed in Table II.

Other methods for delipidation, such as a lipid extraction according to Bligh and Dyer [29] or a treatment with chloroform/methanol near the isoelctric point of glycophorin failed to reduce the phosphate to protein ratio any further. In Materials and Methods two methods are described which result in a complete delipidation of the protein.

To analyse the strongly bound phospholipids, glycophorin was extracted with chloroform/methanol 2 : 1 containing 0.04 N HCl (acid delipidation). In this medium the phosphate to protein ratio was reduced to about 0.30 μmol/mg after 20 min and could be reduced to 0.20 μmol/mg by a prolonged exposure. Analysis of the released phospholipids after treatment for 20 min showed that it consists of over 90% phosphatidylserine or lyso-phosphatidylserine (see Table II). This lyso-formation is due to the exposure of the phospholipids to the acid medium, as could be shown by exposing a lipid mixture to the same

TABLE II

PERCENTAGE COMPOSITION OF THE PHOSPHOLIPIDS RELEASED FROM GLYCOPHORIN DURING TREATMENT WITH ETHANOL AND CHLOROFORM/METHANOL, 2 : 1 (I), AND DURING SUBSEQUENT TREATMENT WITH CHLOROFORM/METHANOL, 2 : 1 PLUS 0.04 N HCl (II)

	I	II
Phosphatidylcholine	20.6	0.5
Sphingomyelin	17.3	0.2
Phosphatidylethanolamine	17.2	3.9
Lyso-phosphatidylethanolamine	5.5	1.9
Phosphatidylserine	32.6	63.0
Lyso-phosphatidylserine	3.8	25.9
Phosphatidic acid	3.0	4.6

conditions. A prolonged exposure led to an increase in lyso-formation. The fatty acids of the phosphatidylserine, released during the acid delipidation, showed some striking differences from the phosphatidylserine of the bulk of the human erythrocyte membrane (see Table III). The enrichment of molecular species containing palmitic acid compared with species containing stearic acid was also found in the lyso-phosphatidylserine fraction, indicating that this shift in molecular species is not caused by a specificity in the acid hydrolysis. Analysis of the partially delipidated glycophorin (0.30 μmol phosphate/mg protein) showed that the remaining phospholipid had the same fatty acid composition as the released phosphatidylserine.

The protein could also be fully delipidated by carrying out a second phenol extraction at a low protein concentration for a prolonged period of time at room temperature.

This treatment resulted in a decrease of the phosphate to protein ratio to 0.25 μmol/mg. To determine whether this protein still contained bound phospholipids, fatty acid analysis was done on the protein using heptadecanoic acid as a standard. It could be shown that 0.05 μmol/mg originated from phospholipids, so the remaining 0.20 μmol/mg must originate from sources other than phospholipids.

Glycophorin, delipidated by this relatively mild method, was used for recombination studies with purified lipids. Because of the high water solubility of the protein, specificity in the binding of the delipidated protein with phospholipids was investigated using the technique of monolayer penetration.

Binding of glycophorin to lipid monolayers

Monolayers of lipids were spread at the air-water interphase, protein was injected in the subphase and the increase in surface pressure was measured.

Independent of the nature of the phospholipid, an increase in surface pressure was found when glycophorin was injected under the monolayer. It appeared, however, that this increase was partially caused by the interaction of the sialic acid groups of the protein with the phospholipids. This was shown by injecting sialic acid under the monolayer; particularly with the uncharged phospholipids phosphatidylcholine and sphingomyelin good interactions were measured. Because we were only interested in the interaction of the protein part of glycophorin with lipids, the effect of sialic acid was overcome by

TABLE III

PERCENTAGE FATTY ACID COMPOSITION OF PHOSPHATIDYLSERINE RELEASED FROM GLYCOPHORIN DURING ACID DELIPIDATION (I), COMPARED WITH THE PHOSPHATIDYLSERINE FRACTION OF THE HUMAN ERYTHROCYTE MEMBRANE (II)

	Glycophorin (I)	Ghosts (II)
16 : 0	13.2	2.7
18 : 0	56.4	37.5
18 : 1	8.4	8.1
18 : 2	1.2	3.1
20 : 4	5.3	24.2
22 : 4, 5, 6	9.4	17.5
Ratio 16 : 0/18 : 0	0.23	0.07

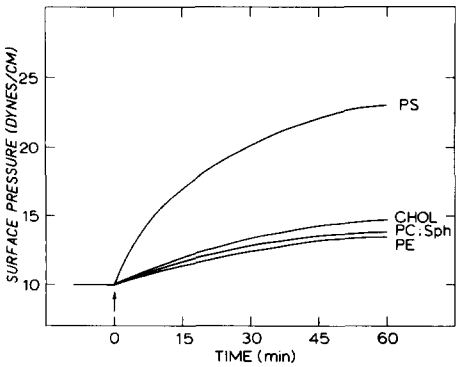

Fig. 1. Increase in surface pressure for the interaction of asialoglycophorin with purified lipids of the human erythrocyte membrane. Subphase: 1 mM Tris · HCl pH 7.4. Temperature, 30°C. At zero time 0.1 mg protein was injected. Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; Sph, sphingomyelin; PE, phosphatidylethanolamine; CHOL, cholesterol. The initial pressure of the film was 10 dynes/cm.

splitting off enzymatically the sialic acid groups of the protein (over 95%) using neuraminidase from *C. perfringens*. The so-formed asialoglycophorin was used throughout the following monolayer experiments. Asialoglycophorin (0.1 mg) was injected under a monolayer with an initial surface pressure of 10 dynes/cm (π_0), using a subphase of 1 mM Tris · HCl pH 7.4 at 30°C and the increase in surface pressure with time was measured. Addition of more protein did not change the final surface pressure any further.

After one hour, the largest increase in surface pressure was found with phosphatidylserine, although the surface pressures were still increasing (Fig. 1). The force-area curves for these phospholipids were also recorded (Fig. 2), because the measured increase in surface pressure due to the interaction of protein with the lipid film is very much dependent on the steepness of the slope of the force-area curve [29]. The purified phospholipids form a liquid-expanded curve, except sphingomyelin, which shows a gradual transition from the liquid-crystalline to gel phase at pressures above 20 dynes/cm. These results are in agreement with calorimetric data concerning the fluidity of these phospholipids [16]. A good value for the penetration rate can now be obtained by multiplying the

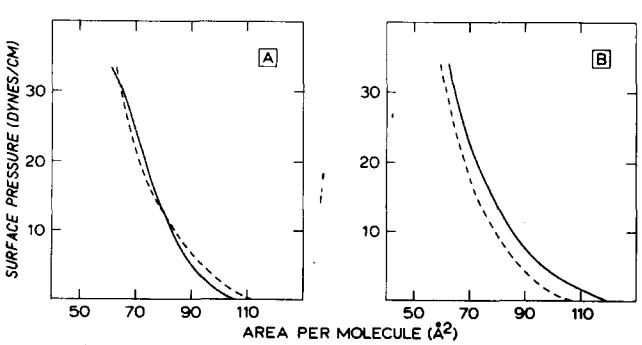

Fig. 2. Force-area curves for the main phospholipids of the human erythrocyte membrane: (A) phosphatidylcholine, (- - - - -); sphingomyelin, (———); (B) phosphatidylethanolamine, (- - - - -); phosphatidylserine, (———). Subphase: 1 mM Tris · HCl pH 7.4. Temperature, 30°C.

TABLE IV

RATE OF PENETRATION OF ASIALOGLYCOPHORIN INTO MONOLAYERS OF HUMAN ERYTHROCYTE LIPIDS

This quantity was calculated by multiplying the initial change in surface pressure $(d\pi/dt)_{t=0}$ with the reciprocal of the slope of the force-area curve at the initial pressure $(dA/d\pi)_{\pi_0}$

	Penetration rate (in $Å^2$/min)
Phosphatidylserine	0.79
Phosphatidylcholine	0.19
Sphingomyelin	0.11
Phosphatidylethanolamine	0.12
Cholesterol	0.02

initial change in surface pressure with the reciprocal of the slope of the force-area curve at the initial surface pressure. These values are plotted in Table IV, taking for cholesterol a slope of 6 dynes/cm $Å^2$ (ref. 31). Clearly, the strongest interaction is seen with erythrocyte phosphatidylserine.

Other negatively charged phospholipids like phosphatidic acid or phosphatidylglycerol also showed good interactions with the protein (Fig. 3). The difference in interaction between egg phosphatic acid and egg phosphatidylcholine which differ only in their polar head groups, is shown. No change in interaction was found when EDTA (1 mM) was present in the subphase, indicating that no divalent cations are involved in the binding of the net negatively charged asialoglycophorin (iso-electric point 4.2) with the negatively charged phospholipids.

Fig. 4 shows the effect on the penetration when mixed films of phosphatidylserine and cholesterol were used. In spite of the fact that an increase in the amount of cholesterol leads to a steeper slope of the force-area curve, a decrease in the change in the surface pressure is seen. A 1 : 1 mixture of phosphatidylserine and cholesterol hardly shows any interaction, while a 1 : 1 mixture of phosphatidylserine and an uncharged phospholipid (e.g. phosphatidylcholine or

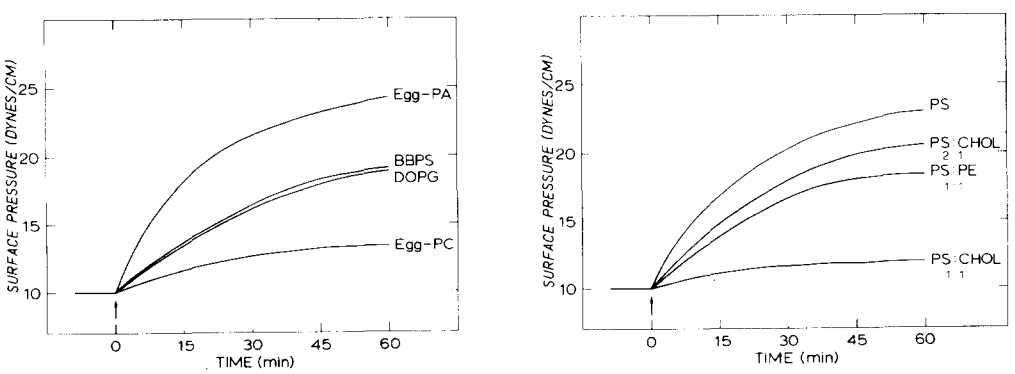

Fig. 3. Increase in surface pressure for the interaction of asialoglycophorin with lipids. Abbreviations: Egg PA, egg phosphatidic acid; Egg PC, egg phosphatidylcholine; BBPS, beef brain phosphatidylserine; DOPG, di-oleoyl-phosphatidylglycerol. Conditions, see legend Fig. 1.

Fig. 4. Increase in surface pressure for the interaction of asialoglycophorin with mixed films of erythrocyte phosphatidylserine (PS) and cholesterol (CHOL), as a function of the molar ratio. PE, phosphatidylethanolamine. Conditions, see legend Fig. 1.

phosphatidylethanolamine) still shows a good penetration. A stimulation of the interaction was never found when mixtures of phospholipids were used.

Also the ionic strength of the subphase proved to be very important. An increase in Tris concentration to 10 mM led to a 5-fold decrease in penetration rate.

The protein hardly showed any surface activity by itself. Under the conditions used, a collapse pressure of 2 dynes/cm was found for asialoglycophorin, and a value of 6 dynes/cm for the sialic acid-containing glycophorin.

When the phosphatidylserine-asialoglycophorin film at its final pressure was treated with trypsin, no change in surface pressure was observed. However, when asialoglycophorin was added after the injection of trypsin under the monolayer or when asialoglycophorin was first treated with trypsin before injection under the lipid film, no increase in surface pressure was found. Obviously, the tryptic peptides of asialoglycophorin can not give the same interaction with the lipid film as the intact protein. These two observations indicate that the interaction of the protein with the lipid film is not merely an electrostatic interaction between parts of the protein and the polar head group of the phospholipid, but that at least a part of the protein must be embedded in the lipid film.

Discussion

Glycophorin gives a strong interaction with negatively charged phospholipids, especially with phosphatidylserine. This can be seen from Table V, where the data from Table I and II are combined, and the composition of the phospholipids bound to glycophorin after the various delipidation procedures is listed. In this calculation it was assumed that all lyso-phosphatidylserine originated from phosphatidylserine. It is shown in this table that the gradual enrichment of phosphatidylserine compared to other phospholipids already begins to occur during the first phenol extraction. This indicates that the shift is not caused by a specific extraction of the uncharged phospholipids during the extraction of the protein with the strongly hydrophobic solvents.

The enrichment of molecular species containing palmitic acid compared with species containing stearic acid in the phosphatidylserine bound to glycophorin is very striking. While only 2% of the total erythrocyte phosphatidylserine is found in the phenol extract bound to glycophorin, no less than 15% of all spe-

TABLE V

PERCENTAGE COMPOSITION OF THE PHOSPHOLIPIDS BOUND TO GLYCOPHORIN AFTER PHENOL EXTRACTION (I) AND TREATMENT WITH ETHANOL AND CHLOROFORM/METHANOL, 2 : 1 (II) COMPARED WITH THE COMPOSITION OF THE MAJOR PHOSPHOLIPIDS OF THE HUMAN ERYTHROCYTE MEMBRANE

	Ghosts	I	II
Phosphatidylcholine	28.3	6.5	0.4
Sphingomyelin	25.8	5.3	0.2
Phosphatidylethanolamine	26.7	10.3	5.0
Phosphatidylserine	12.7	74.2	90.4
Phosphatidic acid	1.0	3.7	4.0

cies of phosphatidylserine containing palmitic acid is present in this extract. Although no such conclusion could be drawn with respect to the poly-unsaturated fatty acids, since they may be degraded during the prolonged dialysis procedures, this still may indicate that glycophorin is surrounded by a lipid environment, which differs from the bulk of the human erythrocyte membrane. Also, when glycophorin is extracted from the membrane with chloroform/methanol using the method of Hamaguchi and Cleve [12], the protein contains bound phospholipid consisting predominantly of phosphatidylserine (data not shown).

Fully delipidated glycophorin contains 0.20 μmol phosphate/mg protein, which is due to the presence of phosphorylated amino acids at the C-terminal side of the protein (Marchesi, V.T., personal communication). Using a molecular weight of 31 500 (ref. 13), this gives rise to about 2.5 phosphate groups per monomer of glycophorin.

Recombination experiments of a delipidated protein with lipids can inform us about the nature of the lipid-protein interaction and show us what is required for such an interaction. The monolayer penetration technique used in this study is a very simple one, but is only suited to show whether there is an interaction or not. No data can be obtained about the stoichiometry of the interaction.

The monolayer experiments clearly show that asialoglycophorin preferentially binds to negatively-charged phospholipids like phosphatidylserine, phosphatidylglycerol or phosphatidic acid; this in spite of the fact that the protein itself also has a net negative charge under the conditions used. However, evidence is presented that hydrophobic interaction between the protein and the lipid also plays a role. This is indicated by the effect of cholesterol in mixed monolayers and by the results of the tryptic digestion.

Our results favour the idea that a salt bridge between the negatively-charged polar head group of the phospholipids and the positively-charged amino acids at the cytoplasmic side of glycophorin, next to the hydrophobic region of the protein (-Arg-Arg-Leu-Ile-Lys-Lys-; see ref. 13), will give the correct orientation for obtaining a hydrophobic interaction. The fact that asialoglycophorin treated with trypsin is not able to give an interaction with a monolayer of phosphatidylserine supports this idea, because trypsin splits the protein at these positively-charged amino acids.

It should be emphasized that the preferential binding of glycophorin to negatively-charged phospholipids is only shown in this study under conditions where the protein is dissolved in a water solution. Under these conditions the protein will inevitably have a conformation different from the native one, which may influence the lipid binding capacities of the protein.

It has been suggested by Bretscher [32] that in the erythrocyte membrane glycophorin is tightly associated with phosphatidylserine, because of the combined electrostatic and hydrophobic interaction. Provided that glycophorin in vivo is surrounded by a lipid bilayer and that the lipids around the protein show the same asymmetry over the two sides of the bilayer as the lipids of the bulk of the membrane, it is reasonable to presume however that other phospholipids besides phosphatidylserine are also involved in the binding of glycophorin with the lipid matrix.

The system investigated in this study may bear some resemblance to the situation where glycophorin is incorporated in the membrane during maturation of the cell, a process which occurs in a very early state of cell development [33,34].

It has been suggested by Lodish and Small [35] that extrinsic membrane proteins are synthesized on free ribosomes and intrinsic membrane proteins on membrane-bound ribosomes. In this second situation the ribosome will have an interaction with the membrane.

Our finding that water-dissolved glycophorin shows a high affinity for monolayers containing negatively-charged phospholipids does not exclude the possibility that synthesis occurs on free ribosomes. In this case the protein would be released first in the cytoplasm and would then spontaneously bind to that side of the membrane that contains phosphatidylserine, prior to incorporation in the membrane. In this respect it is of interest to note that in this stage of cell maturation glycoproteins are only partially glycosylated and do not contain sialic acid [36], while the membrane contains only low amounts of cholesterol. Both of these factors would stimulate the binding of the protein to the membrane.

From the results of De Pierre and Dallner [37] it can be concluded that in rat liver microsomes, membrane-bound ribosomes are bound to that side of the membrane that is rich in phosphatidylethanolamine and phosphatidylserine. Assuming that glycophorin is synthesized on membrane-bound ribosomes in a similar way, not only the ribosome itself but also the produced protein will give an interaction with the membrane, as can be seen from the results of this study. Probably the N-terminal side of the protein, which is synthesized first, is inserted in the membrane and subsequently glycosylated at the luminal side of endoplasmic reticulum and Golgi complex. This sequence of events will lead to the observed conformation of glycophorin in the erythrocyte membrane.

Acknowledgements

The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

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