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Interactions between a Membrane Sialoglycoprotein and Planar Lipid Bilayers

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Summary. Bilayer membranes formed from lipids dissolved in decane were exposed to glycophorin, a sialoglycoprotein which had been extracted from human red cell membranes. The interaction with the bilayer produced an increase in the steady state electrical conductance of the membrane proportional to the amount added. Fluctuations in membrane current when the electrical potential difference was constant were observed concommitantly with this increase in membrane conductance. The minimum size of the fluctuations corresponds to a conductance of 10^{-10} mho. The increase in conductance as well as the current fluctuations persisted after extensive washout of the chamber containing the protein (cis-side). Subsequent addition of lectins (wheat germ agglutinin and phytohemoagglutinin) to the cis-side produced rupture of the membranes, whilst these hemoagglutinins added to the trans-side failed to produce an effect. Measurements of changes in surface potential using K^+ nonactin as a probe indicated that glycophorin induces a negative surface charge. At high protein concentrations, the magnitude of the induced surface potential became independent of glycophorin concentration. The maximum number of charges introduced onto the membrane under these conditions was $1.4 \times 10^5/\mu m^2$. Cis (but not trans)-side addition of neuraminidase abolished these charges, indicating that they can be ascribed to the sialic acid residues that the protein bears. These results suggest that glycophorin incorporates into bilayer membranes with its N-terminal end (where the sialic acid and carbohydrates are located) facing the cis-side. Spectrin reversibly lowered the glycophorin-induced membrane conductance when added to the trans-side. Cis-side additions failed to produce an effect. Trypsin present on the trans-side irreversibly lowered the membrane conductance. These results indicate that parts of the glycophorin molecule, probably the C-terminal end, are accessible to reagents in the solution bathing the trans-side of the membrane. Thus glycophorin spans the planar bilayer in much the same way as it spans the red cell membrane.

During recent years, investigations in many laboratories have been directed toward illuminating the relationship between function and molecular architecture of biological membranes. The concept has emerged

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that biological membranes are lipid bilayers into and through which integral membrane proteins are inserted and on which peripheral membrane proteins are absorbed (*cf.* [25]). One approach to the investigation of this problem is to study the interaction between membrane proteins and model systems such as planar lipid bilayers or lipid vesicles. We have previously shown that an integral membrane protein, glycophorin, can be incorporated irreversibly into planar lipid bilayers [30]. Glycophorin is a sialoglycoprotein from the red blood cell membrane which carries in its carbohydrate moiety the receptors for some plant lectins, MN blood groups and influenza virus [17]. The glycoprotein-bilayer interaction was followed by measuring the changes in the electrical conductance of the bilayer produced by the addition of protein to one of the aqueous phases bathing the membrane. We found that glycophorin increased the membrane conductance and that this increase persisted after removal of the protein from the aqueous phases. Furthermore, when the bilayer containing glycophorin was exposed to Concanavalin A in the presence of calcium, there was a further increase in membrane conductance. Bretscher, Segrest *et al.*, and others [5, 18, 24, 27] have proposed that glycophorin penetrates the erythrocyte membrane from side to side, with its C-terminal end exposed at the inner surface and its N-terminal end, bearing the majority of the carbohydrates, exposed at the outer surface. Furthermore, it has been proposed that glycophorin is associated with the 85 Å membrane particles seen by freeze-fracture electron microscopy and is connected in some way to the spectrin network at the inner surface of the membrane [9, 18].

Recently, Grant and McConnell [13], MacDonald and MacDonald [16] and Redwood *et al.* [23] reported the incorporation of glycophorin into liposomes. Freeze-fracture electron microscopy revealed that the incorporated glycoprotein forms particles which have a tendency to cluster in groups [13]. Furthermore, it was shown that glycoprotein-containing vesicles fluoresced upon exposure to fluorescent wheat germ agglutinin [16]. This interaction with lectins produced aggregation of unfused vesicles, very much like the agglutination of red cells produced by lectins [23]. Thus the N-terminal carbohydrate-bearing end of some of the glycophorin molecules incorporated into vesicles are exposed to the external solution and able to react with lectins in a way similar to that observed in intact red cells.

The studies described in this paper were undertaken to determine the orientation of glycophorin molecules incorporated asymmetrically into planar lipid bilayers. To this end, we studied the reactions that

took place when such protein-containing membranes were subsequently allowed to react with lectins (i.e., wheat germ agglutinin and phytohemoagglutinin), enzymes (i.e., neuraminidase and trypsin) and spectrin through additions of these substances to one of the aqueous solutions bathing the bilayer. Furthermore, using the K^+-nonactin system as a probe for changes in surface potential, it was possible to estimate the number of glycoprotein molecules adsorbed onto the membrane.

Materials and Methods

Membranes were formed from sheep red cell lipids (RBC lipids) extracted as described previously by Andreoli *et al.* [3] or from bacterial phosphatidylethanolamine (PE) purchased from Supelco, Inc. To form membranes the lipids were dissolved in decane (Eastman Organic Chemicals) to concentrations of 14 mg/ml (red cell lipids) or 25 mg/ml (PE). All inorganic salts were reagent grade. Lithium 3,5 diiodo salicylate (LIS) was purchased from Eastman Kodak Co. Trypsin (twice crystallized) from bovine pancreas and neuraminidase from *Clostridium perfringens* (Type VI) were both obtained from Sigma. Nonactin was a gift of Barbara Stearns (Squibb). The human red cell glycoprotein preparations (glycophorin) were kindly supplied by Drs. F. Lau (Duke University), V. Marchesi (Yale University), and J.P. Segrest (University of Alabama). These preparations were extracted from the membranes using 0.3 M LIS, as first described by Marchesi and Andrews and were supplied lyophilized [17]. These samples were dissolved in the appropriate buffered solutions to a concentration of 5–10 mg/ml and subsequently kept frozen. The amount of free LIS in these samples was determined by absorbance at 330 nm and was found to be less than 10^{-5} M in solutions where the protein concentration was 1 mg/ml. Dr. S. Grefrath (Duke University) kindly provided a sample of glycoprotein (human red cells) extracted with chloroform/methanol followed by passage through a sodium dodecyl sulfate (SDS) column and then heated to 80 °C. Following removal of SDS the sample was lyophilized to make a protein concentration approximately 6 mg/ml in sodium phosphate buffer [14]. This sample was used as provided and stored frozen. Lens Culinaris phytohemoagglutinin (PHA) and wheat germ agglutinin (WGA) were purified and kindly provided by Dr. H. Sage (Duke University). Spectrin was a gift from Dr. J. Singer (University of California at San Diego).

Bilayer membranes were formed with a brush across an aperture (1–3 mm^2 in area) on a polyethylene or a Teflon partition separating two chambers. Both chambers were stirred with magnetic stirrers. Unless otherwise stated, the aqueous solutions were 0.1 M NaCl or 0.1 M KCl buffered to pH 7 with phosphate (1 mM) and the experiments were conducted at room temperature (approx. 21 °C). Perfusion of the chambers was carried out using a pair of matched, mechanically coupled syringes. The membrane conductance (G_m) was determined using a four electrode system. One pair was connected to a high input impedance differential amplifier to measure membrane potential (V_m). A third electrode was connected to the voltage source, and the fourth was used to measure the current flowing across the membrane (I_m). The sign of the electrical potential difference across the membrane is defined $V_m = V_{cis} - V_{trans}$ where V_{cis} is the potential of the protein containing bathing solution and V_{trans} is the potential of the opposite protein-free solution. Positive charge flowing from the cis to the trans-side is plotted as positive current. All measurements of steady state G_m were taken 20 to 30 min after addition of protein to a bathing solution and using step potentials to values not exceeding ± 30 mV. In this region of applied poten-

tials, the current vs. voltage (*I-V*) curves were found to be linear. Determinations of G_m at different glycophorin concentrations were done by successive additions of aliquots of concentrated solutions of protein to either one or both sides of the membrane. Control experiments were performed to measure the effect of LIS, the reagent used to solubilize the sialoglycoprotein from the red cell membrane, on membrane conductance and zero-current membrane potential. Aliquots of a concentrated LIS solution were added sequentially to the solutions bathing one or both sides of the membrane. Unilateral LIS (10^{-3} M) produced no zero-current potential and bilateral LIS (10^{-3} M) produced no change in membrane conductance even after 30 to 40 min of exposure to the bilayer. Bilayers formed from oxidized cholesterol or phosphatidylcholine did not change their electrical properties upon addition of glycophorin to one or both aqueous phases.

For studies on the effect of lectins, bilayers were exposed unilaterally to glycophorin. Once G_m reached a constant value, the aqueous compartment where it had been added (cis) was perfused with ca. 12 ml of the solution (protein-free) in which the membrane was formed and the electrical characteristics determined again. This was followed by addition of an aliquot of a concentrated aqueous solution of lectin to either the cis or the trans compartment.

For experiments designed to measure changes in surface potential, bilayers were formed in 0.1 M KCl or 1 M KCl, at pH 7.0 in the presence of 10^{-6} M nonactin. After the membrane conductance had reached a steady value, aliquots of glycoprotein were added to both sides of the membrane and the new value of the membrane conductance determined for small applied potentials (<30 mV). To test the effect of neuraminidase on the surface potential, glycophorin was added to one bathing solution of a membrane formed in the presence of nonactin. After determination of the new value of G_m, the cis compartment was perfused with a nonactin containing solution (protein-free) and G_m determined again. This was followed by addition of neuraminidase to the cis-side.

Results

Effect of Glycophorin on the Steady-State Membrane Conductance (G_m)

Fig. 1 *A* shows the effect of unilateral and bilateral addition of glycophorin on the steady state conductance of membranes formed from sheep red cell lipids. Not all preparations available were tested at all concentrations indicated, but no significant difference could be observed among the preparations. Membrane conductance increased progressively with increasing concentrations of the sialoglycoprotein. Fig. 1 *B* shows that the same effects were observed when membranes were formed using PE. Since the membrane conductance was found to be the same for NaCl as for KCl in PE as well as in red cell lipid membranes, the incorporation of glycoprotein produces little or no selectivity between Na^+ and K^+. Furthermore, measurements of zero current potentials in the presence of a 10-fold gradient in salt concentration indicate that the selectivity for cations over anions is very small (e.g., the transference number for K^+ in a KCl system was about 0.6).

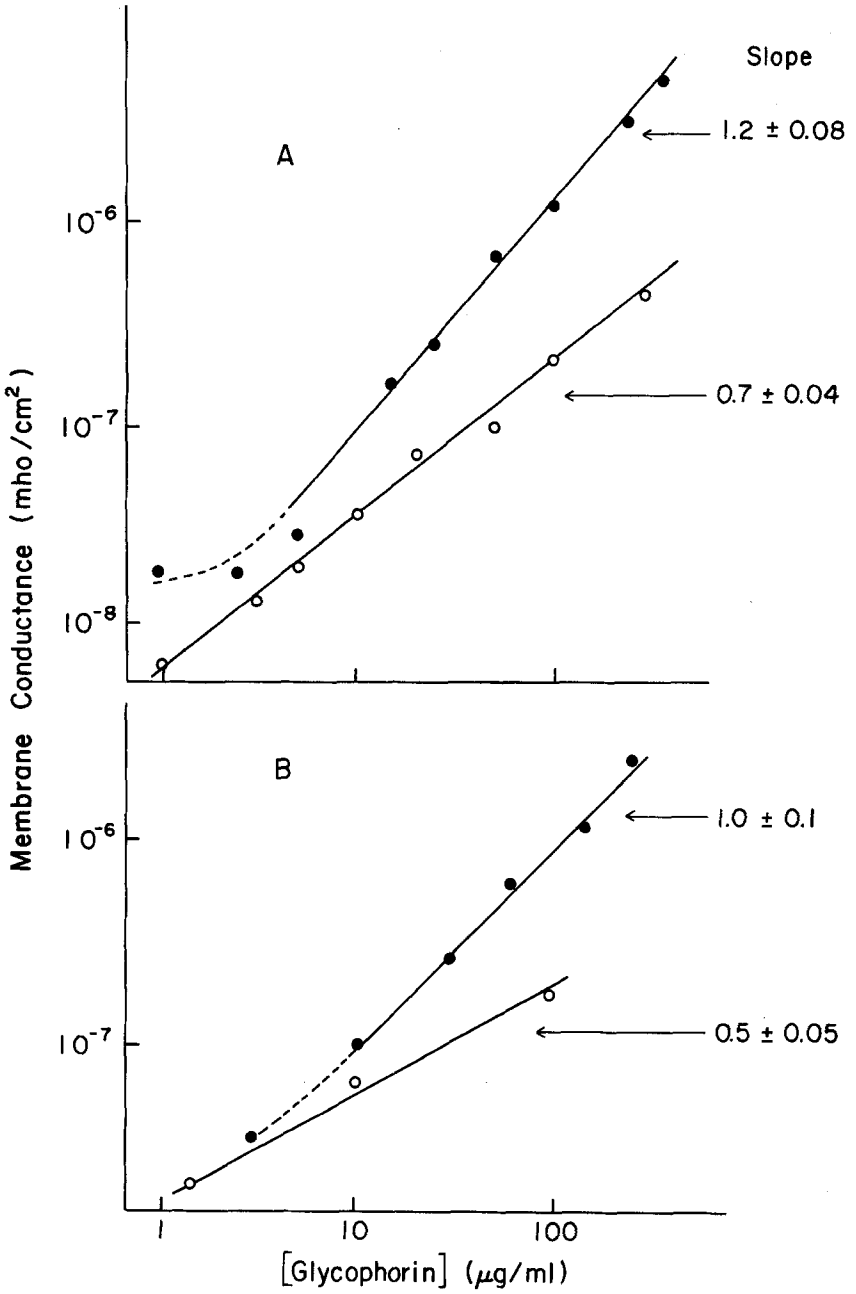

Fig. 1. Dependence of the zero-current, zero-voltage steady state conductance (G_m) of the sheep red cell lipid (*A*) and PE (*B*) bilayers on the concentration of glycophorin. The protein was present in one (open symbols) or both (closed symbols) chambers. The solutions contained 10^{-1} M KCl or 10^{-1} M NaCl, buffered to pH 7.0. The lines were calculated by least squares. Numbers correspond to the slopes ± the standard deviation of the estimate. Experimental points correspond to mean values obtained in at least 3 different membranes

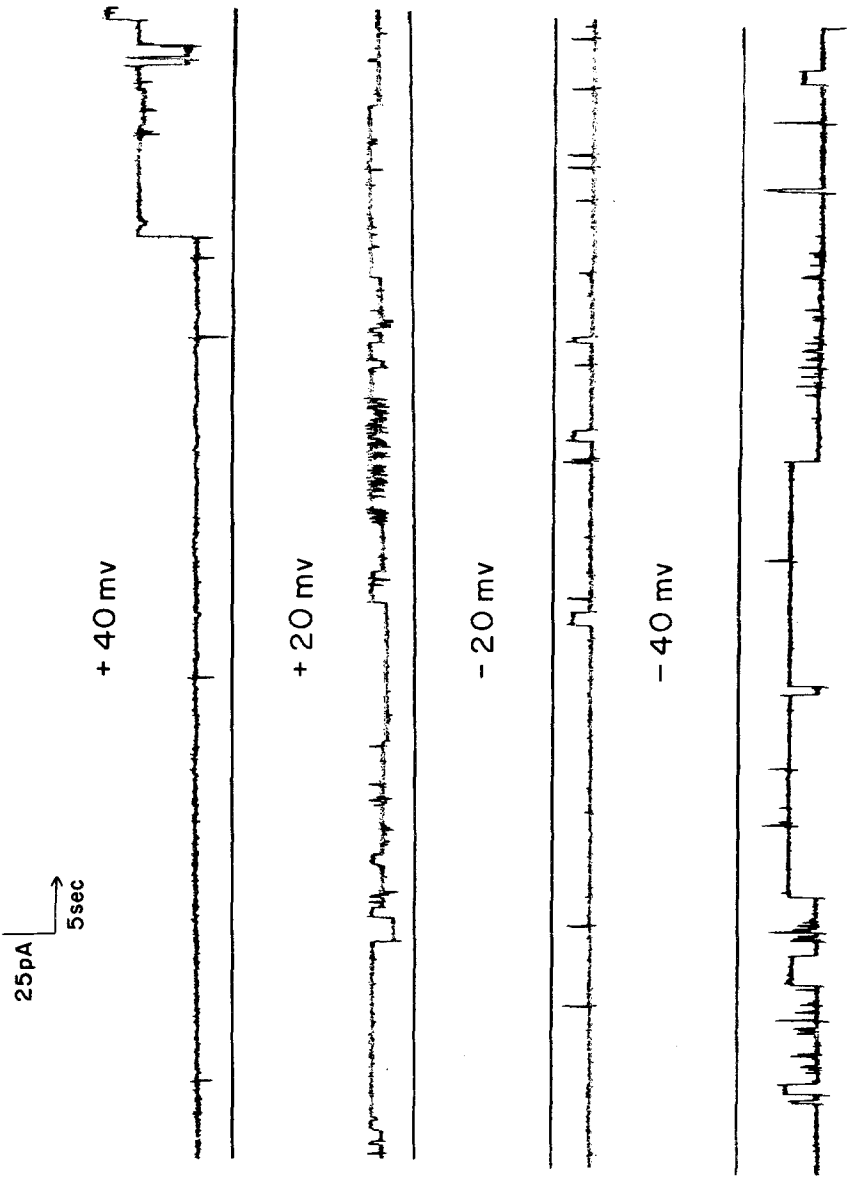

Fig. 2. Recorder trace showing membrane current for different applied potentials as a function of time. PE membrane, 10^{-1} M NaCl, pH 7.0, and 50 μg/ml glycophorin on one side

The slopes of the double logarithmic plots of G_m as a function of glycophorin concentration are significantly different for unilateral and bilateral additions of the protein. The slope for bilateral addition is 1.2 (1.0 for PE), approximately twice the value of 0.7 (0.6) for unilateral addition. This suggests that there are cooperative interactions among

glycoprotein molecules when added to both sides of the bilayer. This type of nonindependent behavior of the monolayers of lipid membranes was also observed by Cass *et al.* for membranes exposed to nystatin or Amphotericin *B* [6]. Although in the case reported by these authors the slopes of the double logarithmic plots of G_m vs. antibiotic concentration were greater than unity, the slope obtained for bilateral addition was twice that corresponding to unilateral addition (the latter measured with a large excess of antibiotic on the opposite side).

While measuring steady state G_m in bilayers exposed to glycophorin, fluctuations in membrane current were observed when membrane voltage was maintained constant. These fluctuations were not seen before the increase in G_m was observed. Some examples are shown in Fig. 2. This behavior is characteristic of bilayers in which ion-conducting channels are opening and closing [2, 4, 8, 12]. We are currently investigating the properties of these fluctuations to determine the extent to which they can account for all of the effects of glycophorin on the electrical properties of bilayers.

Surface Charge Induced by Glycophorin

Since it is known that glycophorin has charged groups (sialic acid and amino acid residues such as lysine and glutamate) [29], incorporation of the protein into the bilayer should alter the surface charge on the membrane. We have used this effect to estimate the density of glycophorin molecules in planar bilayers. The change in interfacial potential produced by glycophorin was computed from the change in conductance of bilayers containing K^+-nonactin [19]. The concentration of nonactin used (10^{-6} M) was high enough to insure that membrane current was carried almost entirely by K^+-nonactin complexes. Under these conditions, the change in interfacial potential at each concentration of glycophorin will be given by [15]:

$$\Delta\psi = -(RT/zF) \ln (G_{N+GP}/G_N) \tag{1}$$

where G_N is the steady state conductance in the limit of zero applied potential for a bilayer exposed to K^+-nonactin; G_{N+GP} is the membrane conductance measured in the presence of K^+-nonactin and glycophorin; $\Delta\psi$ is the change in interfacial potential and z, F, R, and T are the charge of the current carrying species, the Faraday, the gas constant and the absolute temperature, respectively.

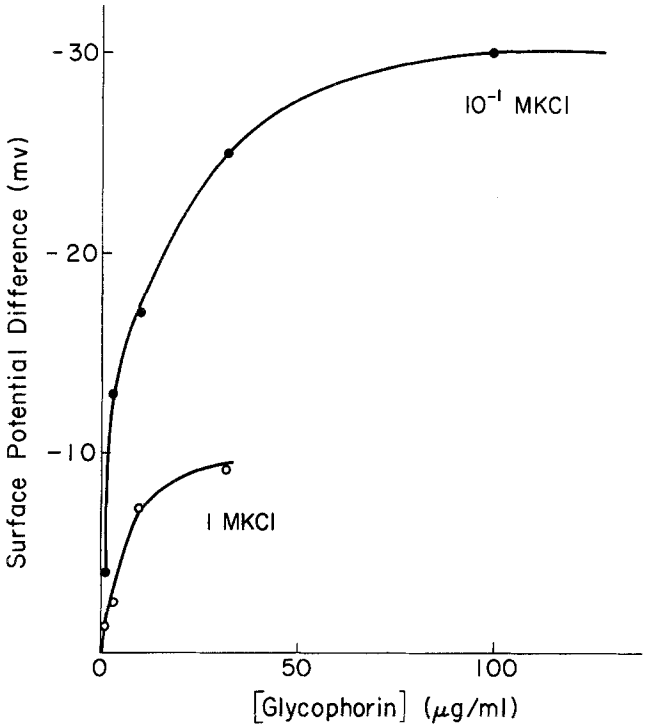

Fig. 3. Surface potential ($\Delta\psi$) as a function of glycophorin concentration. PE membranes were formed in 10^{-1} M KCl+10^{-6} M nonactin, pH 7.0 (●) or 1 M KCl+10^{-6} M nonactin, pH 7.0, (○). Glycophorin was added to both sides of the membrane. $\Delta\psi$ was calculated as indicated in text

If the change in interfacial potential arises from a change in surface potential due to a change in surface charge, it is then possible to estimate the surface charge density using the Gouy equation:

$$\sigma = (C^{1/2}/136) \sinh (F\Delta\psi/2RT) \quad (2)$$

where σ is the surface ion density (charge/Å^2) and C is the molar electrolyte concentration. PE membranes were formed in 0.1 M KCl or 1 M KCl, both at pH 7.0 and in the presence of 10^{-6} M nonactin, and the change in interfacial potential produced by addition of glycophorin determined as outlined in Materials and Methods. The results of these experiments shown in Fig. 3 strongly suggest that the measured change in K^+-nonactin conductance is due to a change in surface potential, since $\Delta\psi$ was changed with a change in ionic strength as predicted by Eq. (2).

Table 1. Surface charge induced by glycophorin

Glycophorin (aqueous) (μg/ml)	$G_{N\text{-}GP}/G_N$	$-\Delta\psi$ (mV)	σ (esu/Å$^2 \times 10^4$)	Glycophorin (surface) (molec/Å$^2 \times 10^6$)
a) 1 M KCl				
1	1.05	1.2	1.8	6.0
3	1.1	2.4	3.5	11.7
10	1.3	6.8	10.0	33.3
30	1.43	9.0	13.3	44.3
b) 0.1 M KCl				
1	1.18	4.0	2.0	6.7
3.6	1.68	13.0	6.0	20.0
10	1.98	17.0	8.0	26.7
33	2.72	25.0	12.0	40.0
100	3.32	30.0	14.0	46.7
330	3.46	31.0	14.0	46.7

PE membranes were formed in the presence of 10^{-6} M nonactin (pH 7.0). Glycophorin was added to both sides at the concentrations indicated. $\Delta\psi$ and σ were calculated as indicated in the text.

Table 1 shows the results of calculation of σ for progressively higher protein concentrations in the aqueous phases bathing the membrane. The number of charges introduced onto the membrane by glycophorin appears to saturate at a value of about 14×10^{-4} charges/Å^2 at high protein concentrations.

From the values of σ, it is possible to make a rough estimate of the number of glycoprotein molecules present in the bilayer provided that the number of charges per molecule that contribute to the surface charge is known. We have estimated this number from the action of neuraminidase on the system. Table 2 shows the results of experiments in which glycophorin and/or neuraminidase were added to one side of a bilayer exposed bilaterally to nonactin (10^{-6} M). Changes in surface potential were estimated from changes in the steady state conductance produced by adding the proteins to these bilayers. Neuraminidase alone caused no change in surface potential but abolished the effect of glycophorin when added to the cis-side at pH 5.0, the pH optimum for this enzyme [7]. Neuraminidase did not abolish the effect of glycophorin on surface potential either at pH 7.0 when added to the cis-side or at pH 5.0 when added to the trans-side. These results suggest that all of the charges on glycophorin which contribute to the change in surface

Table 2. Effect of neuraminidase on the surface charge induced by glycophorin

Cis-side	Trans-side	$\Delta\psi$ (mV)	
		pH 5.0	pH 7.0
GP		−20	−23
GP+N		0	−21
N		0	0
N+GP		0	−20
	N	0	
GP	N	−28	
GP+N	N	−2	

PE membranes were formed in 0.1 M KCl and 10^{-6} M nonactin, pH as indicated. Glycophorin (GP) and neuraminidase (N) concentrations were 30 and 25 μg/ml, respectively. Each condition was tested in a minimum of three different membranes.

potential of the bilayer are due to the sialic acid residues. Since there are in the order of 30 sialic acid residues per glycophorin molecule, this is the maximum change in σ which the incorporation of one glycophorin molecule could produce. Assuming this figure, we may make a provisional estimate of the density of glycophorin molecules in the bilayer at high protein in the aqueous solutions of 1 molecule/$2 \times 10^4 Å^2$ or a lipid to protein ratio of about 300.

Effects of Lectins and Neuraminidase

In order to test for the presence and availability of specific receptors present in the glycophorin molecule, use was made of phytohemoagglutinin (PHA) and wheat germ agglutinin (WGA). These two plant lectins were chosen since it has been shown that their receptors are present at the N-terminal end of the glycophorin [1].

Table 3 shows that the addition of lectins to the cis-side of a bilayer led to its rupture with a time lag which was found to be a function both of the initial aqueous concentration of glycoprotein and of the lectin concentration. Experiments where lectins were added to the trans-side failed to produce an effect, even though the concentration of lectins used was 500 μg/ml. These results suggest that glycophorin incorporates into bilayer membranes with its N-terminal, carbohydrate-containing end facing the side of the bilayer exposed to the protein. This contention is further supported by experiments designed to test the effect of neurami-

Table 3. Effects of lectins on glycophorin treated membranes

[GP] (μg/ml)	[WGA] (μg/ml)	[PHA] (μg/ml)	t_d (min)	
100	–	–	≧120	(50)
30	50		19–29	(8)
100	50		7–10	(4)
100	100		1– 3	(5)
30		50	≧120	(4)
30		100	5– 9	(4)
100		150	≦1	(8)

Sheep red cell lipid bilayers formed in 10^{-1} M NaCl or 10^{-1} M KCl, pH. 7.0, were exposed unilaterally to glycophorin (GP). After removal of nonbound protein from the aqueous phase, wheat germ agglutinin (WGA) or phytohemoagglutinin (PHA) was added to the same compartment. t_d values: range of survival time of bilayers after lectin addition; numbers in parenthesis indicate number of membranes tested.

nidase on the surface potential induced by glycoprotein. The results of these experiments, summarized in Table 2, indicate that neuraminidase removed sialic acid residues from membrane-bound glycophorin when it was added to the cis, but not to the trans-side of the membrane.

Effects of Trypsin and Spectrin

Having established that the N-terminal end of membrane-bound glycophorin is accessible only to the cis-side from which it entered the membrane, we next explored the question of whether other parts of the protein could be reached by reagents in the solution bathing the trans-side. We found that two compounds, trypsin and spectrin, altered the electrical properties of glycophorin containing membranes when added to the trans-side.

Tomita and Marchesi have recently determined the complete sequence of glycophorin [29]. They have shown that the COOH-terminal segment could be cleaved by trypsin to produce one peptide. Furthermore, it has been shown by Steck that trypsin cannot act across the red cell membrane since addition of the enzyme to the outside of inside-out resealed red cell ghosts did not release sugar and sialic acid from the inside surface of these ghosts [26]. Fig. 4 shows the effect of trypsin addition to the trans-side of a bilayer containing glycophorin. Fig. 5 shows that trypsin not only reduced the steady state membrane conductance, but also greatly reduced the frequency of current fluctuations. Clearly some

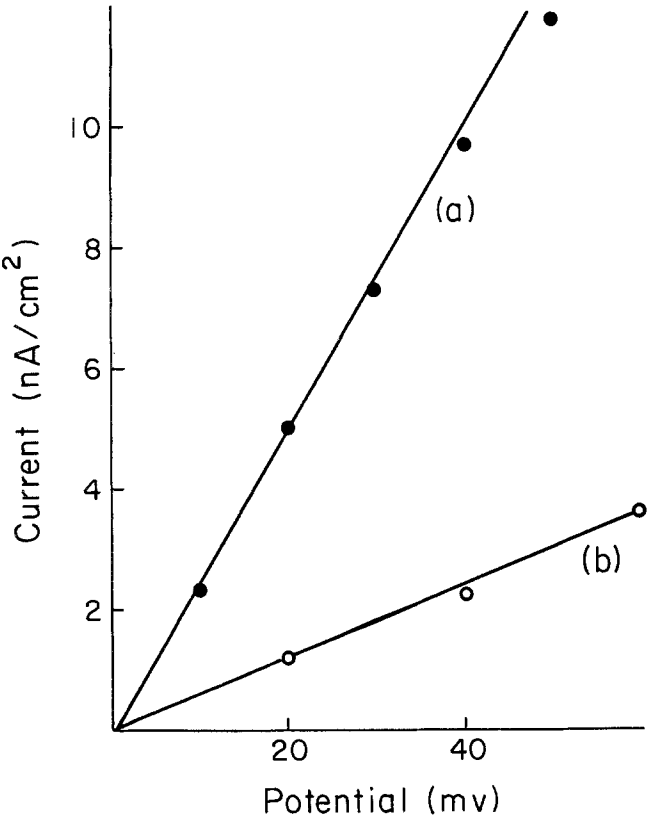

Fig. 4. Current-voltage curves for a PE membrane formed in 10^{-1} M KCl, pH 7.0. (*a*): After cis-addition of 100 μg/ml glycophorin. (*b*): After treatment with trypsin (30 μg/ml) in the trans-compartment. 20 min after addition of trypsin, the trans-compartment was perfused with trypsin-free solution and the electrical parameters were determined

part of the glycophorin molecules in the bilayer, presumably the C-terminal end, can be attacked by trypsin present in the trans-side.

Spectrin is a fibrous, peripheral protein which constitutes about 20% of the total red cell membrane protein and is entirely located at the inner surface [18]. In the past several years, evidence has accumulated to support the view that glycophorin as well as Band 3 (another major glycoprotein present in the erythrocyte membrane) are the components of the red cell membrane which constitute the 85 Å intramembrane particles seen in electron micrographs and that they interact with spectrin [26]. For instance, Nicolson and Painter have shown that addition of antispectrin to the inside of red cell ghosts produces aggregation of sialic acid residues on the outer surface [21]. Similarly, Elgsaeter *et al.* have shown that a gentle perturbation of spectrin in ghosts is associated

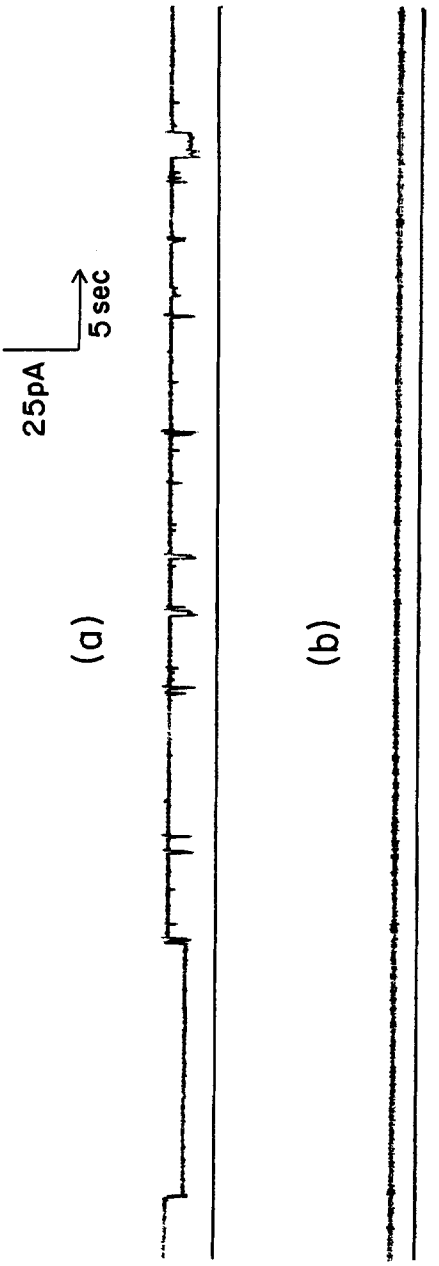

Fig. 5. Recorder trace showing membrane current as a function of time, $V_m = 20$ mV before (*a*) and after (*b*) exposure of the trans-side of a glycophorin containing bilayer to trypsin. Same experimental conditions as Fig. 4

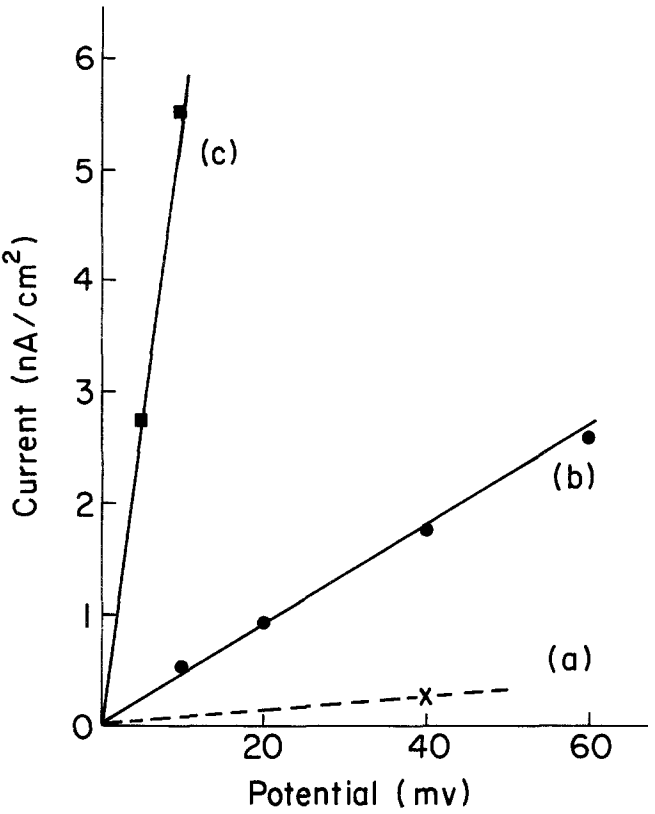

Fig. 6. Current-voltage curves for a red cell lipid membrane formed in 10^{-1} M NaCl, pH 7.0. (*a*): After trans-addition of 15 μg/ml spectrin (only I_m for ± 40 mV indicated in graph; no difference with control values). (*b*): After 20 min of cis-addition of 100 μg/ml glycophorin (*c*): 20 min after perfusion of trans-compartment with spectrin-free 10^{-1} M NaCl

with increased clustering of the intramembrane particles during subsequent exposure to media favoring particle (and spectrin) aggregation [10]. Fig. 6 shows steady state current-voltage curves for a bilayer formed from red blood cell lipids and subsequently exposed first to spectrin and then to glycophorin. Addition of spectrin alone to the trans-side did not affect membrane conductance (curve *a*). Subsequent addition of glycophorin to the cis-side in the presence of spectrin on the other side produced only a modest increase in G_m (curve *b*). Removal of spectrin from the trans-side in the presence of glycophorin on the cis-side resulted in a marked increase in membrane conductance (curve *c*). Thus, spectrin substantially and reversibly inhibits the increase in G_m produced by glycophorin. Fig. 7*a* shows the current fluctuations observed in a bilayer

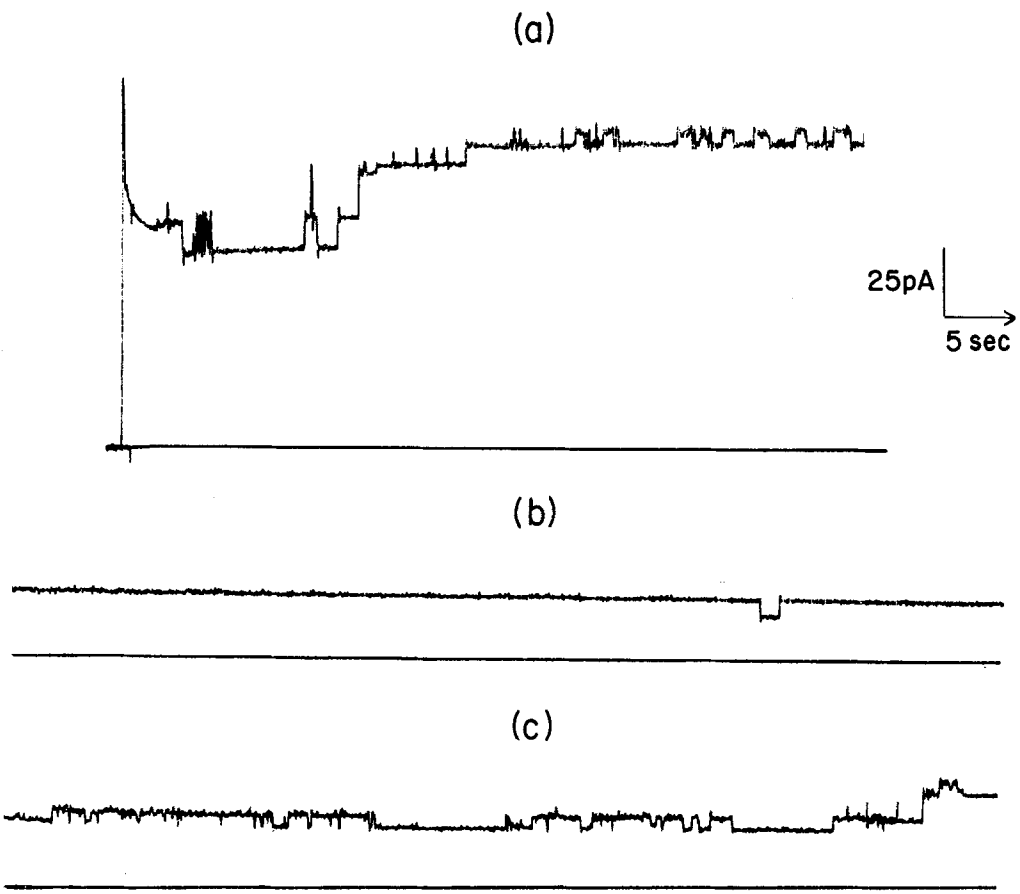

Fig. 7. Recorder trace showing membrane current as a function of time at a constant electrical potential difference of +40 mV. PE membrane formed in 10^{-1} M KCl, pH 7.0. Experimental conditions as follows: (*a*): After exposure of cis-side to glycophorin (100 μg/ml for 20 min) and subsequent perfusion of the cis-compartment with glycophorin-free solution. (*b*): 2 min after addition of 15 μg/ml spectrin to the trans-side. (*c*): 1 min after perfusion of trans-compartment with spectrin-free solution

into which glycophorin had been incorporated from the cis-side. Prior to the measurement, excess free glycophorin was removed from the cis bathing solution. Addition of spectrin to the trans-side reduced the steady state conductance and the frequency of the fluctuations as shown in Fig. 7*b*. Fig. 7*c* shows that within one min after washout of spectrin the current fluctuations reappeared. The steady state membrane conductance also increased after removal of spectrin but more slowly, reaching a steady value 10 to 20 min after washout (not shown). Addition of spectrin to the cis-side of a glycophorin containing bilayer produced

no electrical effects (not shown). These results are consistent with the hypothesis that spectrin interacts with the C-terminal end of glycophorin molecules at the trans-side of the membrane.

Discussion

The data presented in this paper are consistent with the idea that glycophorin incorporates into planar bilayer membranes in very much the way it is thought to be oriented in the erythrocyte membrane. Evidence from several different laboratories indicates that glycophorin spans the human red cell membrane with its N-terminal end on the outside surface and its C-terminal end located at the cytoplasmic side, interacting with spectrin [5, 10, 18, 20, 24, 27]. The evidence shown in Tables 2 and 3 strongly suggests that the N-terminal end of glycophorin molecules incorporated into planar bilayers lies exclusively on the cis-side, from which the protein entered the membrane. The information provided in Figs. 4–7 indicates that some other part(s) of the glycophorin molecules, probably including the C-terminal end, are available to trypsin and spectrin present in the bathing solution on the trans-side. In planar bilayers, the side of addition is sufficient to determine the sidedness of orientation of glycophorin in the membrane. No asymmetries in composition of bilayer or bathing solutions are required since the systems used in our experiments were symmetrical in these respects. This result seems reasonable in view of the large amount of work that would be required to move the many hydrophylic sugar residues and charged groups on the N-terminal end of glycophorin through the hydrophobic membrane interior. Furthermore, the penetration of glycophorin into planar bilayers, that is, its incorporation as an "integral" membrane protein, appears to be a spontaneous event tending toward equilibrium and not to depend on the continuing performance of work on the system (e.g., an imposed potential across the bilayer). Whether similar considerations apply to the sidedness and "integral" location of glycophorin in red cell membranes is not yet clear. The relative stability of the orientation of glycophorin in the red cell membranes to such drastic manipulations as the formation of vesicles [27] can be interpreted to favor the view that the protein is also at equilibrium in this system. It is possible that the orientation of glycophorin in the erythrocyte membrane also depends only on the exposure of just one side to the protein during membrane formation.

The maximum number of glycophorin molecules that were adsorbed to planar bilayers in our experiments agrees well with the density of this protein in human red cell membranes. Measurements of the change in surface potential produced by addition of glycophorin to planar bilayers lead to the conclusion that a maximum increase in surface charge of about 1.4×10^5 esu/μm^2 can be conferred by adsorption of the protein on the membrane (Table 1). This value is almost identical to that obtained by Eylar *et al.* for the surface charge of the human red cell membrane [11]. These authors found that this number corresponds to the number of molecules of sialic acid released when these cells are exposed to neuraminidase. Our results indicate that the surface charge induced by glycophorin on planar bilayer membranes could also be totally removed by exposure to neuraminidase and, therefore, attributable to sialic acid residues on the protein (*cf.* Table 2). Furthermore, the calculated density of protein molecules ($4 \times 10^3/\mu m^2$, Table 1) corresponds to that of the density of 85 Å particles seen in the sum of both freeze-fracture faces of the erythrocyte membrane [28].

From some of the evidence reported in this paper, we surmise that only a small fraction of the glycophorin molecules on planar bilayers span the membrane. When the concentration of glycophorin was 100 μg/ml, the steady state membrane conductance was 3×10^{-7} ohm^{-1}/cm^2 or 3×10^{-15} $ohm^{-1}/\mu m^2$. If this membrane conductance resulted from the formation of glycophorin channels, each with a conductance of 10^{-10} ohm^{-1}/channel, the channel density in this membrane would be $3 \times 10^{-5}/\mu m^2$. If each channel was formed through one protein molecule, then the density of glycophorin molecules spanning the membrane was $3 \times 10^{-5}/\mu m^2$, about 10^{-8} times the number of glycophorin molecules absorbed on the membrane as estimated from changes in surface potential (Table 1). Furthermore, the change in surface potential produced on bilayers occurred immediately after addition of glycophorin, whereas the conductance increase and appearance of channels required 20–30 min of exposure to protein. Therefore, it seems reasonable to conclude that glycophorin absorbs rapidly onto bilayers, but that penetration of the C-terminal end of the molecule through the membrane is slow and improbable.

Glycophorin produces channels in planar bilayers, as demonstrated in Figs. 2, 5 and 7. It is likely but not yet proved that the increase in steady state conductance produced by glycophorin in bilayers is entirely due to the formation of these channels. Thus, glycophorin is one of a growing family of proteins known to produce channel formation in

bilayers, such as EIM [4, 8], hemocyanin [2], black widow toxin [12], and bacterial ATPase [22].

Addition of either trypsin or spectrin to the trans-side of a bilayer containing glycophorin markedly reduced channel formation (Figs. 5 and 7). In the former case, trypsin has been shown to digest the C-terminal end of glycophorin up to the beginning of the "hydrophobic" segment [29]. Perhaps partial hydrolysis of the C-terminal end of glycophorin molecules spanning the bilayer made them assume a conformation unsuitable for channel formation. In the case of spectrin, it is attractive to speculate that this peripheral protein interacts with the C-terminal end of the glycophorin molecules to reduce their lateral diffusion and channel formation. Such an interpretation presumes that more than one glycophorin molecule is required to form a channel, a point which is not established and requires further investigation.

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