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Incorporation of the Human Erythrocyte Sialoglycoprotein into Recombined Membranes Containing Cholesterol

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Summary. Glycophorin, the major sialoglycoprotein from the human erythrocyte membrane, has been isolated and recombined with phosphatidylcholine and cholesterol. Sucrose density gradient analysis of the recombinants shows that it is possible not only to recombine this protein with phospholipid, but also with phospholipid-cholesterol mixtures. Surprisingly, by the same analysis, it was possible to make a recombinant with cholesterol and glycophorin, only, in the absence of added phospholipid. The accessibility of the protein to trypsin was tested in each of these recombinants. In all the recombinants which contained either phospholipid, or phospholipid and cholesterol, the protein was protected from extensive hydrolysis. This is consistent with closed vesicles and incorporation of the protein into the recombinant membrane. Extensive hydrolysis of the protein occurred in the cholesterol-glycophorin recombinant indicating some differences in structure. Freeze-fracture electron microscopy of the phospholipid and the phospholipid-cholesterol recombinants showed mostly unilamellar vesicles, 1000 to 5000 Å in diameter. Intramembranous particles were observed on both fracture faces, and the fracture planes were those expected for phospholipid bilayers. The glycophorin-cholesterol recombinants also showed fracture planes consistent with bilayers, and revealed intramembranous particles. Pieces of membrane-like structures as well as apparent vesicular structures were observed. Finally in the recombinants of glycophorin with phospholipid and cholesterol, cholesterol is shown to reduce the population of the motionally restricted phospholipid headgroup environment, in proportion to the mole percent cholesterol content.

Key Words cholesterol · glycophorin · membrane protein · erythrocyte membrane · lipid-protein interactions · membrane structure

Introduction

Glycophorin is the major sialoglycoprotein of the human erythrocyte membrane. This protein has been recombined with phospholipids and the interactions between the phospholipids and glycophorin have been studied (Brulet & McConnell, 1976; van Zoelen et al., 1978; Romans et al., 1979; Utsumi, Tunggal & Stoffel, 1980; Mendelsohn et al., 1981; Ong, Marchesi & Prestegard, 1981; Yeagle & Romans, 1981; Yeagle, 1982). However, glycophorin in the erythrocyte membrane finds itself in an environment consisting of almost as much cholesterol as phospholipid. Therefore to adequately understand the nature of the glycophorin-lipid interactions, glycophorin recombinants containing cholesterol need to be studied.

Recombinant techniques are used in the present study. The results show that not only can glycophorin be recombined with phosphatidylcholine, but also with phosphatidylcholine and cholesterol. Furthermore, in an unexpected development, glycophorin is shown to recombine with cholesterol in the absence of added phospholipid. This is to our knowledge the first such report of a recombinant of a transmembrane protein with cholesterol alone.

Materials and Methods

Trypsin and soybean trypsin inhibitor were purchased from Sigma. Cholesterol and 3,5-diiodosalicylic acid were obtained from Eastman. Cholesterol exhibited a single spot when analyzed on thin-layer chromatography in petroleum ether/diethyl ether/glacial acetic acid (90:10:1, vol/vol) with visualization by acid charring. Ergosterol and bovine serum albumen, fatty acid free, were obtained from Sigma. α-Parinaric acid was obtained from Calbiochem. Egg phosphatidylcholine was purchased from Avanti Biochemicals. Triton X-100 was purchased from Calbiochem. All buffers were thoroughly purged with nitrogen before use and were made from distilled, deionized water.

Preparation of Ergosta-5, 7, 9, 22-tetraen-3-β-ol (dehydroergosterol)

Ergosterol was recrystallized from methanol before use. All reactions were performed in the dark and in a glove box under nitrogen to prevent decomposition. Ergosterol was dehydrated with mercuric acetate in chloroform and acetic acid for 18 hr following previously published procedures (Zurcher et al., 1954). The free alcohol had a melting point of 143° C. The near UV absorption spectrum (250–350 nm) and fluorescence

spectrum from this material were identical with previously published spectra (Rogers, Lee & Wilton, 1977).

Preparation of Lithium Diiodosalicylate (LIS)

3,5-Diiodosalicylic acid was recrystallized from methanol before use. It was then converted to LIS by addition of LiOH in water. The LIS was recrystallized from water.

Preparation of Glycophorin

Freshly out-of-date human red cells were obtained from the local Red Cross Blood Bank. White or slightly pink erythrocyte ghosts were obtained by established procedures (Dodge, Mitchell & Hanahan, 1963). Glycophorin was isolated from the human red cell ghosts by the LIS extraction procedure of Marchesi and Andrews (1971). The material from each preparation was analyzed with SDS polyacrylamide gel electrophoresis on 12% gels as described later. Phosphate analysis did not detect any phospholipid in the glycophorin at a sensitivity level equivalent to 10 phospholipids per protein.

Preparation of Glycophorin/Phosphatidylcholine Recombinants

Glycophorin was recombined with pure egg phosphatidylcholine and with egg phosphatidylcholine and cholesterol. Anhydrous protein and lipid were solubilized in 2-chloroethanol and dried under vacuum. The mixture was hydrated in 1 mM histidine, 1 mM EDTA, 100 mM NaCl, pH 7, overnight. This material was applied to a 0 to 40% continuous sucrose gradient and centrifuged at $130{,}000 \times g$ overnight in an SW 27 rotor at 5° C. Three bands were usually observed: one of unassociated lipid at the top, one of the lipid-protein recombinant at an intermediate position, and one at the bottom of unassociated protein. The gradients were pumped through a UV monitor (Gilson model 111) into a Gilson fraction collector. The individual fractions were analyzed for cholesterol, protein and phospholipid. The lipid-protein complex was harvested, either from the fractions, or directly off the gradient by pipette, washed three times by centrifugation for 30 min at $190{,}000 \times g$ in the above buffer, and analyzed for lipid and protein as described below. These recombinants were then used for the biophysical measurements and for trypsin treatment.

Proteolysis

The recombinants were washed by centrifugation with 150 mM NaCl, 10 mM histidine, 1 mM EDTA, pH 7. They were resuspended in that buffer at 1 mg/ml (protein). Trypsin was then added to a concentration of 40 μg/ml. Incubation for 2 hr at 37° C followed. Soybean trypsin inhibitor was added to 80 μg/ml to terminate the hydrolysis. Aliquots were than subjected to SDS polyacrylamide gel electrophoresis as described below.

Preparation of Liposomes

The appropriate amounts of phospholipids were dissolved in chloroform. This solution was dried under a stream of nitrogen gas and them under vacuum, forming a film on the sides of the flask. Buffer (100 mM NaCl, 1 mM EDTA, 10 mM histidine, pH 7) was added and the solution vortexed to disperse the lipid.

Electrophoresis

SDS gel electrophoresis was performed in tube gels in a water-jacketed Buchler electrophoresis apparatus maintained at 15° C with a Fisher refrigerated water bath. A discontinuous system was employed using a 3% stacking gel at pH 8.8 on top of a running gel of 12% at pH 6.8 (Laemmli, 1970; Smith, Stubbs & Litman, 1975). Gels were stained with Coomassie blue and scanned on a Gilford gel scanner at 550 nm.

To monitor the time course of trypsin hydrolysis of glycophorin in recombinants, slab gels were run in a Biorad slab gel electrophoresis apparatus maintained at 15° C. The same gel system, as above, was used. Gels were stained by the PAS procedure for carbohydrate, and then with Coomassie blue.

UV Spectra

Absorption spectra were recorded on a Cary 17 spectrophotometer. For each absorption spectrum obtained with a probe molecule, an equivalent sample without probe was used in the reference compartment. Spectra were recorded at 23° C.

Assays

Phospholipid concentrations were determined using a modification (Litman, 1973) of the method of Bartlett (1959) in triplicate on each sample. Protein concentrations were determined using the procedure of Lowry et al. (1951) in 3.12% sodium dodecyl sufate (SDS) in triplicate. An 18% correction was applied to this value (Grefrath & Reynolds, 1974) and a molecular weight for the protein part of glycophorin of 14000 was used to calculate the mole ratio of phospholipid/protein. Cholesterol was determined enzymatically (Allain et al., 1974). Thin-layer chromatography demonstrated that the cholesterol is not oxidized.

Freeze-Fracture Electron Microscopy

The methods for temperature-controlled freeze-fracture experiments have been described elsewhere (Stewart et al., 1979). 0.1 μl of the sample was sandwiched between two 75-μm-thick copper foils, equilibrated at 23° C, and then rapidly quenched in liquid propane. Samples were fractured and replicated at −128° C in a Polaron E7500 unit at a vacuum of 5×10^{-7} torr. The replicas were viewed on a Siemens 101 electron microscope.

P-31 NMR Experiments

Most P-31 NMR spectra were obtained at 81 MHz and 30° C with a Bruker WP-200 Fourier transform spectrometer. Some data were also obtained at 40 MHz on a Varian XL 100 spectrometer and at 109 MHz on a JEOL FX 270. Samples were kept in ice and then warmed to 30° C or the temperature of choice and incubated for 10 min before measurements were begun. Samples were introduced to the instrument in 10 mm tubes. To obtain resonance intensities, spectra were accumulated with gated broadband proton decoupling to remove the P-31 (H-1) nuclear Overhauser effect. At 81 MHz 11 μsec ($\pi/2$) pulses with a delay of four to five times the spin-lattice relaxation time T_1 between pulses were used to insure complete relaxation before the next pulse. Data acquisition began 15 μsec after the pulse, using a 50 kHz spectral width and 2048 data points. Volumes of the sample in the tube were identical in all samples and standards, and the position of the sample in the receiver coil was kept constant. In a given series of experiments, the same sample tube was used for all samples.

To standardize the intensity, unsonicated dispersions of egg phosphatidylcholine and membrane extracts with similar resonance shapes and widths to the recombinants being measured, were used. Spectral intensities were determined by cutting and weighing photocopies of the spectra. Multiple measurements produced intensity values $\pm 2\%$. Each sample was measured at least twice and the results averaged.

It was established that standards consisting of egg phosphatidylcholine, total lipid extract of sarcoplasmic reticulum, and total lipid extract of human erythrocyte membranes (containing cholesterol), all described the same standard curve. Standards containing 10 mM buffer and standards containing 100 mM NaCl also described the same standard curve. Standards of unsonicated egg phosphatidylcholine with and without the water-soluble protein, trypsinogen, also described the same standard curve.

The P-31 NMR experiments at 109 MHz were obtained using a CSA echo sequence with multiple phase cycling (kindly provided by Drs. I.C.P. Smith, M. Rance and A. Byrd) and a 50-μsec pulse interval between the $\pi/2$ and the π pulse. No first-order phase corrections were employed.

P-31 Cross Polarization NMR

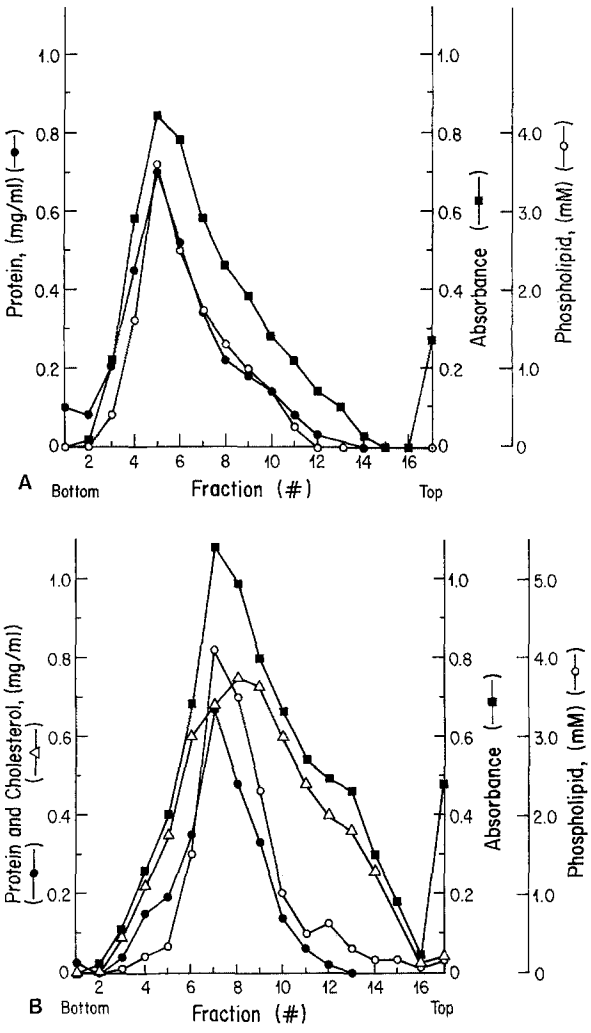
These experiments were performed on a Nicolet NT-150 at the NSF regional facility at Colorado State University. Samples were loaded in the magic angle spinning probe but were not spun. The samples were fully hydrated when measured and were bathed in a stream of nitrogen gas during measurement. Cross polarization P-31 NMR spectra were obtained at 60 MHz.

Results

Glycophorin recombined readily with egg phosphatidylcholine using the procedures described in Materials and Methods, as was reported previously (Yeagle & Romans, 1981). The recombinant was purified on a linear sucrose density gradient. After fractionation the distribution in the gradient of phospholipid and protein was determined. Figure 1 *A* shows the profile obtained when 20 mg dry glycophorin and 30 mg phosphatidylcholine were used to form the recombinant. The majority of the protein and phospholipid comigrate in a fairly narrow band on the gradient (some loss of resolution of the band occurred during fractionation). As shown previously the phospholipid-protein ratio can be varied by changing the starting ratio of the two components (Yeagle & Romans, 1981). The recombinants appear in the linear sucrose gradient at positions which reflect their final phospholipid-protein ratio. At the bottom of the gradient is a small amount of protein which did not recombine. At the top of the gradient, the absorbance increases sharply which may reflect uncombined phospholipid, though not enough to be detected by phosphate assay.

The same approach was used to examine the formation of glycophorin/egg phosphatidylcholine/cholesterol recombinants. The question of importance was whether cholesterol would incorporate into the recombinant along with phospholipid and protein. Because the cholesterol/phospholipid/glycophorin system consists of three components, a complete analysis of the system would be three/dimensional. This was deemed unfeasible. Therefore, a fixed starting phospholipid/protein ratio was used and the starting cholesterol content was varied. A strict relationship between starting ratios and final ratios of components did not obtain, as has been noted in other systems (Hong & Hubbell, 1972). However, in general, if the starting cholesterol content was increased, the final cholesterol content also increased. Cholesterol could be incorporated into these recombinants of glycophorin along with phospholipid, from 0 mole percent cholesterol to 60 mole percent cholesterol. Figure 1 *B* shows the profile obtained from a linear sucrose gradient using 20 mg dry glycophorin, 30 mg egg phosphatidylcholine, and 15 mg cholesterol as the starting material for recombination. As in the egg phosphatidylcholine/glycophorin recombinant, most of the phospholipid, protein and cholesterol comigrated on the gradient. Also similar to the cholesterol-free recombinant is the very small amount of protein on the bottom of the gradient and the small amount of free lipid on the top of the gradient. Even though the fractionation causes some loss of resolution of the bands, it is apparent that in addition to the major phospholipid/cholesterol/glycophorin recombinant, there may be a second band higher on the gradient. If so, this second band is high in cholesterol content, low in phospholipid and protein, consistent with its position higher on the gradient.

The latter observation suggests that it might be possible to form a recombinant from glycophorin and cholesterol alone. Therefore, 20 mg dry glycophorin and 15 mg cholesterol were recombined as described above. The profile of the cholesterol and protein obtained from a density gradient centrifugation of such a recombination appears in Fig. 1 *C*. No phospholipid was added. The cholesterol and glycophorin comigrated on the gradient. Again a small amount of unassociated protein was found at the bottom of the gradient. This recombinant appears on the gradient at a position similar to the possible second band on the sucrose density gradient profile for the glycophorin/cholesterol/phospholipid recombinant. Thus at this point it appears that a complex of glycophorin and cholesterol can be formed that is stable on a sucrose density gradient. For comparison the final compo-



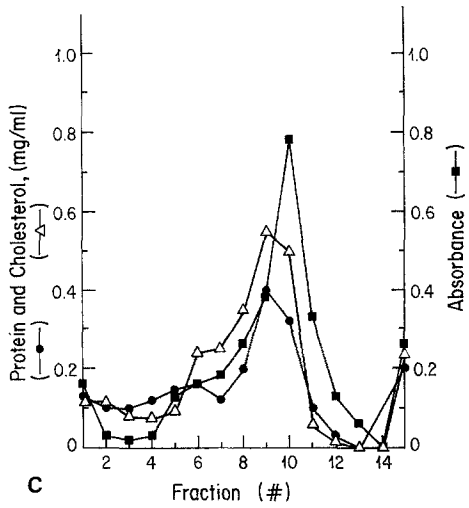


Fig. 1. Sucrose density gradient analysis of glycophorin/lipid recombinants. The recombinants were layered on a 0 to 40% linear sucrose gradient and spun at 100,000 × *g* for 18 hr, and pumped off through a UV monitor and a fraction collector, collecting 2.5 ml fractions. Phospholipid (○), cholesterol (△), protein (●) and light scattering (■) were determined for each fraction. Panel *A* is the glycophorin/egg phosphatidylcholine recombinant; Panel *B* is the glycophorin/egg phosphatidylcholine/cholesterol recombinant; and Panel *C* is the glycophorin/cholesterol recombinant, described in the text. Recombinants formed with different ratios of the protein and lipids banded in a similar fashion on the sucrose gradient, at densities appropriate to their composition

Table. Composition of glycophorin recombinants

Recombinant	Glycophorin (mole %)	Phospholipid (mole %)	Cholesterol (mole %)
Glycophorin/ phosphatidylcholine	2	98	0
Glycophorin/ phosphatidylcholine/ cholesterol	1	56	43
Glycophorin/cholesterol	3	[a]	97

[a] Not detectable by phosphate assay in recombinant.

sitions of the three recombinants analyzed in Fig. 1 are presented in the Table.

The observation of a complex consisting only of glycophorin and cholesterol was surprising. It was important, therefore, to more fully characterize these complexes. Previously, trypsin hydrolysis of the glycophorin/egg phosphatidylcholine recombinant demonstrated protection from extensive proteolysis by the form of the recombinant. This was consistent with a transmembrane incorporation of the protein (Yeagle & Romans, 1981). The same trypsin hydrolysis procedure was applied to the recombinants described above.

In the trypsin proteolysis pattern for glycophorin/egg phosphatidylcholine recombinants, one major and one minor proteolytic fragment of glycophorin are observed, identical to that seen previously (Yeagle & Romans, 1981). No glycophorin is left undigested. Coomassie staining and PAS staining of the same gel produced the same pattern. Therefore, both the products are glycopeptides. Previous experiments showed that glycophorin bound to preformed vesicles, without detergent, was susceptable to extensive proteolysis (Yeagle & Romans, 1981). Because only limited proteolysis occurs when glycophorin is incorporated into the recombinants studied here, the protein enjoys considerable protection from the trypsin hydrolysis.

This protection would be expected if the protein was incorporated in a transmembrane fashion. Since all of the protein molecules are attacked by trypsin, the recombinants likely consist of unilamellar vesicles. This suggestion is consistent with the electron micrography data presented below.

Glycophorin/egg phosphatidylcholine/cholesterol recombinants were examined in the same way. The proteolysis results are identical with the glycophorin/egg phosphatidylcholine recombinants. The form of the recombinant again protects glycophorin from extensive proteolysis. Thus with respect to the protein, the structure of these two recombinants as measured by susceptibility to trypsin, is the same.

The structure of the glycophorin/cholesterol recombinant is different as measured by the susceptibility of glycophorin to trypsin hydrolysis. All of the protein is extensively hydrolyzed by trypsin. There are no bands on the gel except at the dye front. This indicates no protection from proteolysis by the structure of the recombinant. Thus the bulk of this recombinant is probably not in the form of closed vesicles that are stable to partial protein hydrolysis. This is not surprising since there are few, if any, phospholipids in these recombinants.

Freeze-fracture electron micrographs were obtained to gain a better understanding of the structure of these recombinants. In the following the fields shown are representative of all the fields examined. In the case of the phospholipid/glycophorin recombinant, freeze-fracture reveals vesicles 1000 to 5000 Å in diameter that are mostly unilamellar in agreement with the suggestions from the proteolysis results. The fracture planes are consistent with a phospholipid bilayer. The NMR data presented later also indicate the majority of the phospholipids are in a bilayer. The fracture faces contain intramembranous particles (pure lipid exhibited smooth fracture faces) dispersed in the plane of the membrane without obvious pattern. An example is shown in Fig. 2*A*. The intramembranous particles are consistent with a transmembrane incorporation of the protein. The glycophorin/phosphatidylcholine/cholesterol recombinant is similar in morphology to the glycophorin/phosphatidylcholine recombinant, as had been suggested by the proteolysis experiments.

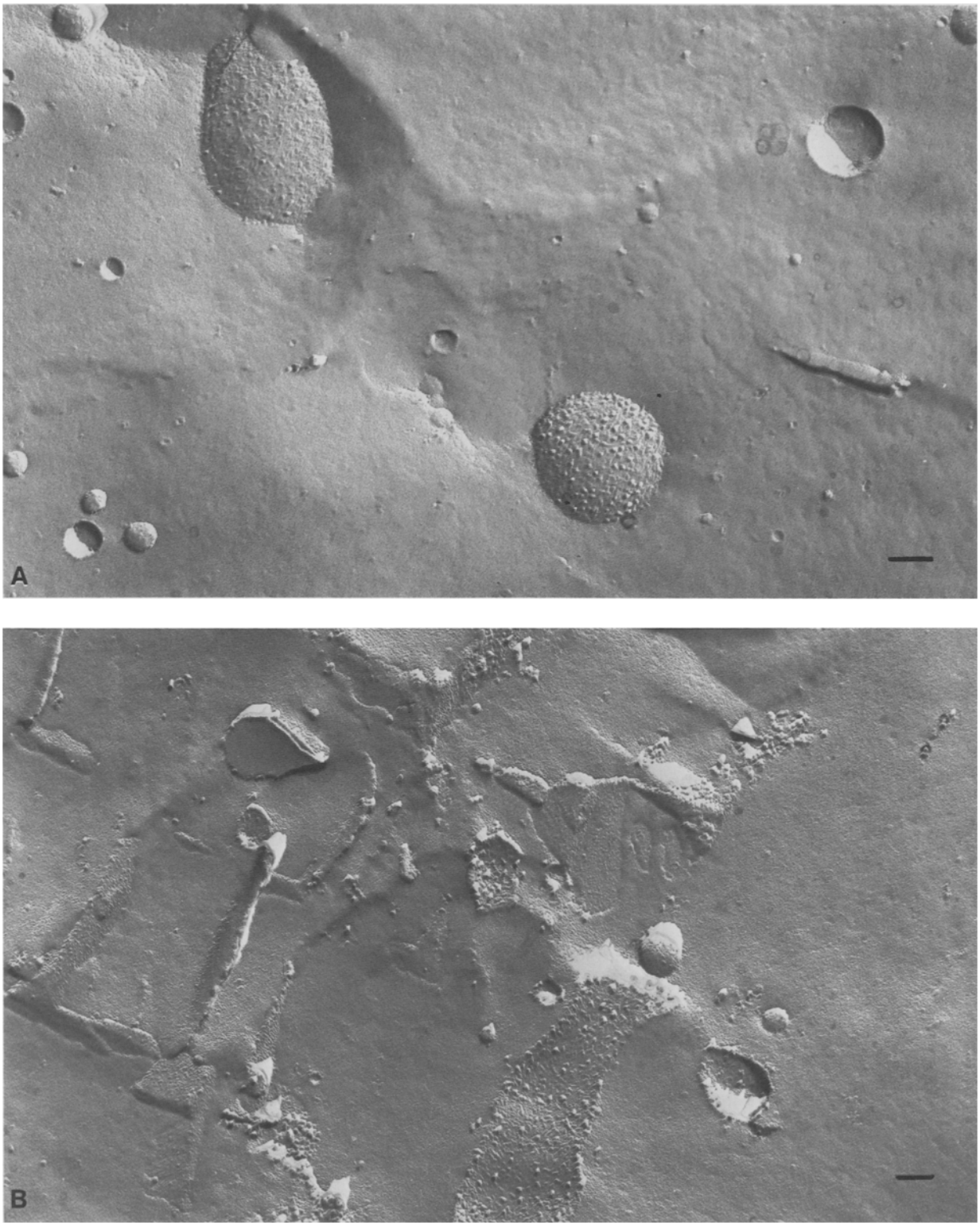
The glycophorin/cholesterol recombinant shows a variety of structures. Figure 2*B* shows some flat membranous sheets containing intramembranous particles. Figure 2*C* shows a vesicular-type structure. These two pictures are representative of the majority of the structures in the sample. It should be remembered that these structures contain both glycophorin and cholesterol in the same ratio, since the recombinant was harvested from a sucrose density gradient. On this gradient unassociated protein is found at the bottom (in a small pellet) and unassociated cholesterol at the top. These glycophorin/cholesterol recombinants may have some of the same bilayer structure found in the phospholipid-containing recombinants because they fracture as would a phospholipid bilayer. Furthermore these glycophorin/cholesterol recombinants do contain protein as shown by the intramembranous particles.

Lipid Binding by Glycophorin

The above results demonstrate that cholesterol as well as phosphatidylcholine can form a complex with pure human erythrocyte glycophorin. This observation was investigated further. The procedure for isolation of glycophorin leaves it in a form, probably aggregated, that is stable in aqueous solution. The relative binding affinity of glycophorin for a sterol (dehydroergosterol) and a fatty acid (α-parinaric acid) were compared. A single mole ratio of approximately 2:1 (protein/lipid) was used. The lipid was introduced in a glass test tube in methanol and dried to a film. A glycophorin solution (in distilled, deionized water) was added to the tube, which was then sonicated for 30 sec in a bath sonicator. The aqueous solution was removed and the absorbance of that solution containing glycophorin was measured in a Cary 17 spectrophotometer, to test for the presence of the probes (dehydroergosterol is not detectably soluble in water under these conditions). This was done by measuring the absorption spectrum in the region where only the probe absorbs. Therefore any probe which was now in solution in the presence of glycophorin could be detected. These experiments demonstrated that under these conditions, glycophorin bound dehydroergosterol but not parinaric acid. However, both lipids bound to fatty-acid free bovine serum albumen under the same conditions. This indicates the association with glycophorin is likely not simply a nonspecific hydrophobic interaction.

P-31 NMR Studies

Figure 3*A* shows the cross-polarization P-31 NMR spectrum of a glycophorin/egg phosphatidylcholine recombinant (1:100 mole ratio). Two overlapping resonances appear to be present: one normal bilayer resonance similar to resonances seen from pure phospholipids; and a second, much broader



resonance. Figure 3*B* shows the computer-generated spectral simulation of a single homogeneous phospholipid environment. The equations used for simulation are those of Seelig (1978). As can be seen the simulation does not contain a broad component. A complete simulation of the experimental spectrum can only be obtained by adding a second, broader component to the "normal" phospholipid

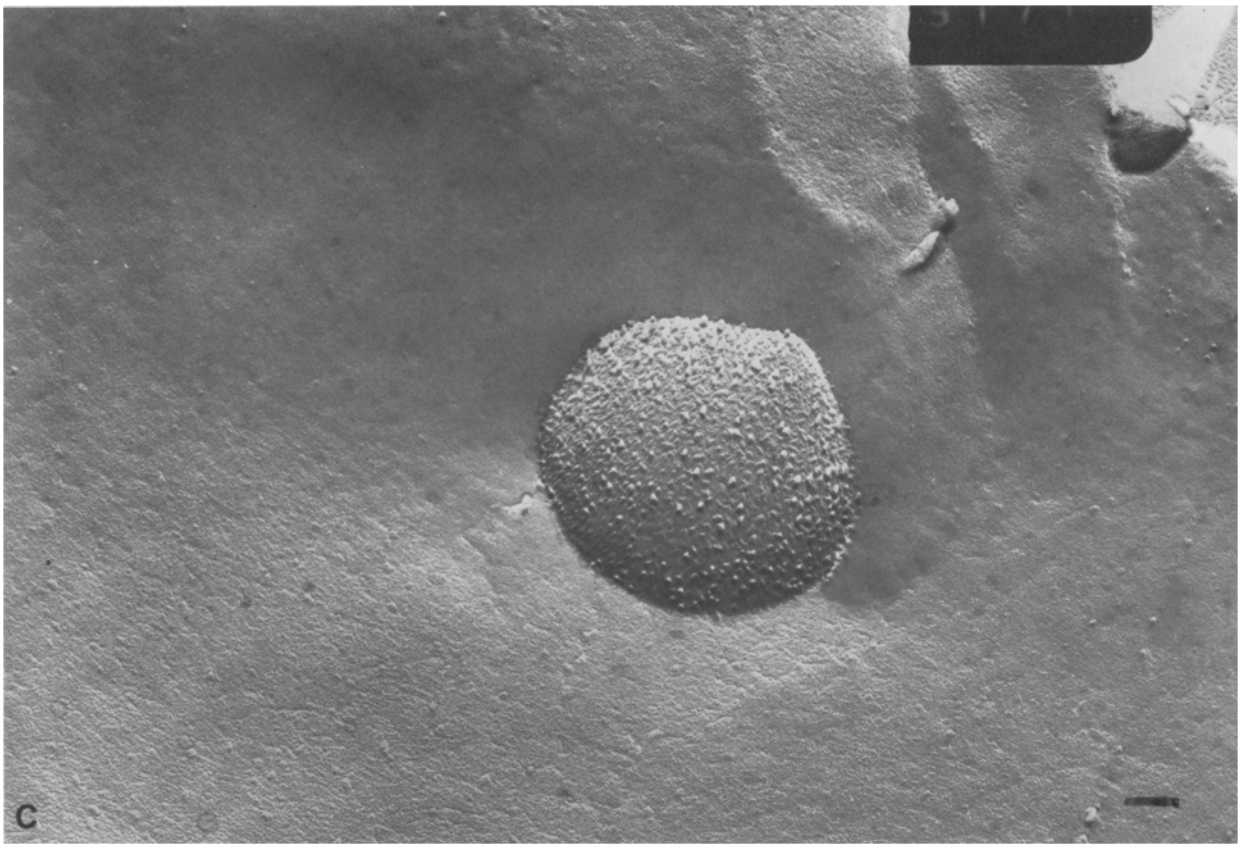


Fig. 2. Freeze-fracture electron micrographs of recombinants. (*A*) Glycophorin/egg phosphatidylcholine. (*B*) Glycophorin/cholesterol. (*C*) Glycophorin/cholesterol. These correspond to samples 1 and 2 in the Table. Bar is 1000 angstroms

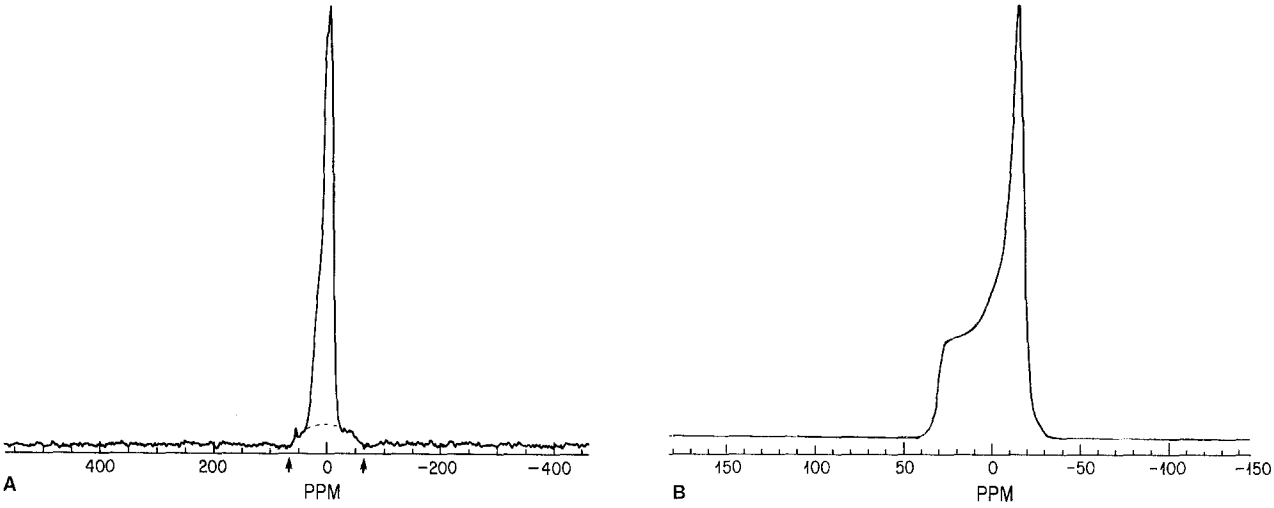


Fig. 3. (*A*) 60 MHz cross-polarization P-31 NMR spectrum of a glycophorin/egg phosphatidylcholine recombinant (100:1 mole ratio) obtained using a 3-msec contact time and 70,000 scans. A broad (160 ppm) component can clearly be seen underlying the "normal bilayer" component, thus indicating two quite different phospholipid headgroup environments in this recombinant. (*B*) Spectral simulation of a normal phospholipid bilayer with a single homogeneous phospholipid environment. Arrows indicate edge of broad peak in *A*

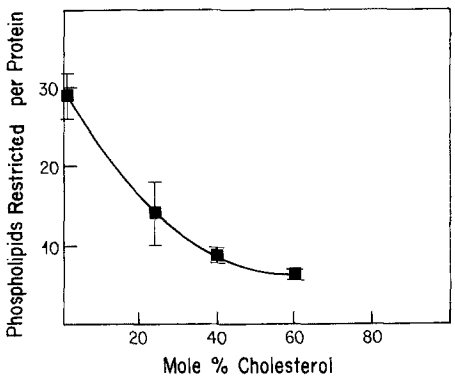


Fig. 4. Number of phospholipid molecules motionally restricted per glycophorin molecule in recombinants containing a varying mole percent cholesterol. Each point represents the mean of three or more determinations on independent preparations of the same composition, and the bars represent the standard deviations of those means

bilayer spectrum. Such a phenomenon was observed previously in recombined membranes containing cytochrome oxidase (Rajan et al., 1981).

The broad component in the membrane containing glycophorin apparently represents a phospholipid environment motionally restricted relative to the former, promoted by the presence of the protein. The broad resonance is not seen in normal Fourier transform spectra of these recombinants because of strong dipolar interactions (Yeagle & Romans, 1981). The cross-polarization experiment is especially suited for seeing motionally restricted molecules because its efficiency is directly proportional to the strength of the dipolar interaction. That dipolar interaction increases as motion is restricted.

Because of differing relative efficiencies in cross-polarization, the population of the two environments is best determined from the "normal bilayer" resonance as described in Materials and Methods. The same results were obtained on three different Fourier transform instruments at 40, 81 and 109 MHz. This was done for a series of cholesterol/egg phosphatidylcholine/glycophorin recombinants, one of which was described in detail above. The population of the two environments were normalized for the mole percent cholesterol in each, and for the protein content. Figure 4 shows that cholesterol causes a significant change in the relative populations of the two environments. Only the population of the motionally restricted environment is plotted here.

Discussion

Glycophorin has been recombined with phosphatidylcholine by several laboratories using chloroform/methanol (MacDonald & MacDonald, 1975), 2-chloroethanol (Romans et al., 1979), octylglucoside (Mimms et al., 1981) and cholate (Ong et al., 1981). In each case, glycophorin seemed to be incorporated in a transmembrane manner, as evidenced by proteolysis experiments or by freeze-fracture electron microscopy. This includes the 2-chloroethanol procedure used here. Freeze-fracture electron microscopy shows intramembranous particles characteristic of transmembrane proteins. The proteolysis pattern seen here resembles that obtained in other recombinants (MacDonald & MacDonald, 1975; Ong et al., 1981) and is consistent with a transmembrane orientation of the protein. Glycophorin in recombinants made with organic solvents binds wheat germ agglutinin and inhibits the hemagglutination promoted by wheat germ agglutinin and Sendai virus (MacDonald & MacDonald, 1975). H-1 NMR (Ong et al., 1981) indicates that the conformation of the protein in the recombinant, to the extent it is sensed by NMR, is similar to the freshly isolated material.

The first observation in this study is that cholesterol can be included along with phosphatidylcholine in a recombinant with glycophorin. According to the measurements reported here, this latter recombinant has a similar structure to the glycophorin/phosphatidylcholine recombinant. This result is not surprising, since the phospholipid bilayer is an important feature of both recombinants (the shape of the P-31 NMR spectrum of both recombinants is consistent with a bilayer organization of the phospholipid, as is the fracturing in the freeze-fracture electron microscopy experiments).

It was then logical to try a recombination of glycophorin and cholesterol without phospholipids. As seen in the previous section, this recombination was also successful. The recombinant banded as a single broad band on a sucrose density gradient, well separated from unassociated cholesterol and unassociated protein. The glycophorin/cholesterol recombinant does not exhibit exactly the same regular vesicular structure seen with the phospholipid-containing recombinants. However, the fracturing observed in the freeze-fracture electron microscopy is similar to that seen for a bilayer of phospholipid and may indicate that a bilayer of cholesterol is present. The isolation of such a recombinant clearly demonstrates the ability of glycophorin to interact strongly and directly with cholesterol. The question then arises whether those components interact in a membrane.

It was shown previously (Yeagle & Romans, 1981) with P-31 NMR that glycophorin motionally restricts about 29 phospholipid headgroups per

protein in these recombinants. Similar results were obtained using C-13 NMR (Utsumi et al., 1980), P-31 NMR (Romans et al., 1979) and H-1 NMR (Ong et al., 1981). This result has been rationalized in terms of one continuous layer of phospholipid around the transmembrane portion of the protein (Ong et al., 1981).

Here the number of motionally restricted phospholipids has been monitored as a function of the cholesterol content of the membrane. Because this is a three-component system, and because resulting lipid/protein ratios are not entirely controllable, the full three-dimensional analysis was not practical. However, it was previously demonstrated that the number of phospholipids motionally restricted per glycophorin was independent of lipid/protein ratio (van Zoelen et al., 1978; Utsumi et al., 1980; Yeagle & Romans, 1981). Therefore it was reasonable to attempt to vary predominantly the cholesterol concentration in this study. It is observed that the number of phospholipids restricted per glycophorin decreases monotonically as the cholesterol content increases. This result could be interpreted as a lateral phase separation of components induced by cholesterol. One piece of evidence which does not support this, however, is that even in pure cholesterol, freeze-fracture electron microscopy indicates that the intramembranous particles are distributed with uniform density. These particles are most likely due to glycophorin, and thus the protein component does not seem to be laterally phase separated. This does not speak for the lipid components, however.

This result can also be interpreted in terms of cholesterol replacement of phospholipids in the motionally restricted environment. If that environment represents phospholipid headgroups motionally restricted at the glycophorin-phospholipid interface, then the data in Fig. 4 may represent replacement of phospholipid by cholesterol at that interface. We cannot unambiguously differentiate between these two possibilities.

If an interaction between glycophorin and cholesterol can be considered as one possible explanation for these data, then it is interesting that recent monolayer data suggest band 3 of the human erythrocyte membrane interacts strongly with cholesterol (Klappauf & Schubert, 1977). Data on human erythrocyte Na^+,K^+-ATPase show inhibition by cholesterol which may also reflect cholesterol-protein interactions in that membrane (Yeagle, 1983). Finally, cholesterol may modulate transferrin binding to reticulocytes (Nunez & Glass, 1982).

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Abbreviations

LIS, lithium diiodosalicylate
NMR, nuclear magnetic resonance
SDS, sodium dodecylsulfate

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