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LOCALIZATION OF GLYCOPROTEINS WITHIN ERYTHROCYTE MEMBRANES OF SHEEP

A FREEZE-ETCHING AND BIOCHEMICAL STUDY

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SUMMARY

Freeze-fractured membranes of ghost red cells obtained from sheep blood contain randomly distributed particles which are 80–100 Å in diameter. After treatment of the ghosts with 0.1 M phosphate buffer, pH 7.0, the particles form clusters. Sonication of the ghost membranes with clustered particles leads to the formation of a few vesicles which are formed from membrane areas which were either largely particle free or contained clusters of particles. These two kinds of vesicles were separated by centrifugation on a sucrose density gradient. Glycoprotein analysis of the vesicles showed that vesicles without particles contain less glycoprotein than vesicles with particles. In agreement with ref. 1 (Tillack, T. W., Scott, R. E. and Marchesi, V. T. (1972) J. Exp. Med. 135, 1209–1220), these results suggest that some of the particles exposed in freeze-etched membranes consist of glycoprotein.

INTRODUCTION

The molecular architecture of the erythrocyte membrane has been intensively studied. Recent work has provided evidence that membrane proteins are located asymmetrically within the two faces of the membrane [2] and that several proteins span the thickness of the membrane [3–5]. Some of these proteins which penetrate the membrane are probably glycoproteins. By freeze-etching the erythrocyte membrane these proteins can become visible. Freeze-etching allows one to look into the apolar cores of the membrane [1] and shows particles of approximately 80–100 Å in diameter randomly distributed within the normal membrane. Treating ghosts of sheep erythrocytes with isotonic phosphate buffer causes a lateral shift of these particles and the formation of clusters. Using ghosts pretreated in this manner, we were able to separate parts of the membrane with and without particulate structures. Biochemical analysis of the different membrane fractions revealed a low glycoprotein concentration when the particles are absent, suggesting that the structures observed in freeze-etched erythrocyte ghost membrane may represent glycoproteins.

METHODS

For the preparation of ghosts, red blood cells from sheep were washed and lysed according to Philips and Morrison [2].

Subfractionation of ghost membranes

Ghost membranes obtained from approx. 400 ml of sheep blood were washed five times in a 10-fold volume of 0.1 M phosphate buffer, pH 5.8, and centrifuged for 20 min at 34 000 $\times g$. The sediment was resuspended in 10 ml of phosphate buffer, pH 5.8, and subsequently sonicated four times for 10 s at a 5 A output in a 30-ml Spinco tube using a Branson Sonifier LS 75. 1 ml aliquots of the homogenate were layered over a 12 ml linear sucrose gradient with densities from 1.098 g/ml to 1.161 g/ml at 5 °C in 0.1 M phosphate buffer, pH 5.8. After centrifugation for 60 min at 280 000 $\times g$ in a Spinco SW-40 rotor, the gradient was fractionated into 13 fractions. Fraction 12 from the top of the gradient ($d = 1.08$ g/ml) contained vesicles almost free of particles whereas fraction 5 ($d = 1.141$ g/ml) was enriched with membrane subfractions covered with particles. Sucrose and nonmembrane proteins were removed from the fractions by dialysis against distilled water overnight.

Electrophoresis

The samples were prepared for electrophoresis by incubation at 90 °C for 60 min with an equal volume of the following solution: 10% sodium dodecylsulphate, 100 mM mercaptoethanol, 10 mM EDTA, 100 mM phosphate, pH 7.3, 40% sucrose and pyronin G. Electrophoresis was performed in 7.5% polyacrylamide slab gels with 0.1 M phosphate buffer, pH 7.3, containing 0.1% sodium dodecylsulphate. After the gels had been loaded with 50 μg protein of each membrane fraction, a current of 50 mA per slab gel was applied until the pyronin G marking dye had migrated approximately 7.5 cm. One half of the slab gel was stained with Coomassie brilliant blue for protein and the other half for glycoproteins by the periodate–Schiff reaction [6].

Phospholipid determination

The phosphorus content of the different subfractions of the erythrocyte membrane was determined (after combustion of the samples with 70% perchloric acid) according to Fiske and SubbaRow [7].

Determination of sialic acid

Sialic acid content of the different membrane subfractions of the erythrocyte was determined according to Aminoff [8] after the samples had been washed free of sucrose, and had been incubated for 60 min at 37 °C with 5 units neuraminidase (Behring Werke, Marburg, Germany) in the presence of 2 mM $CaCl_2$ and 0.1 M acetate buffer, pH 5.0. The samples were sonicated before and after incubation for 5 s at an output of 5 A.

Freeze-etching

The specimens were freeze-cleaved, etched and shadowed with platinum–carbon in a Balzers freeze-etching apparatus according to the method of Moor and Mühlethaler. Deep-etching was performed before replication by keeping the cleaved

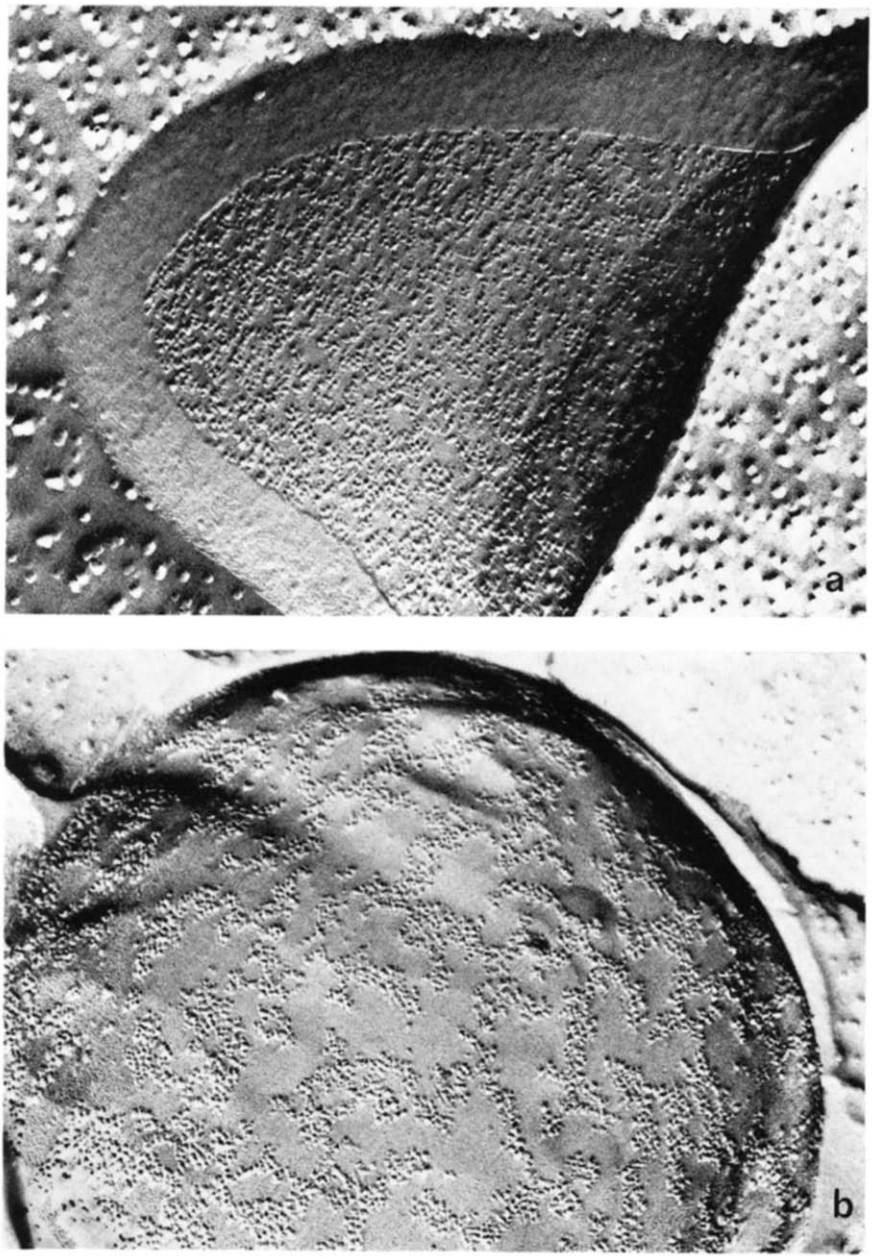

Fig. 1. (a) Freeze-etching of sheep erythrocyte ghost membrane (100 000×); (b) Freeze-etching of sheep erythrocyte ghost membrane after washing with 0.1 M phosphate buffer, pH 7.4. The particles are displaced from their random distribution (cf. Fig. 1a) (100 000×).

specimens at −100 °C for 40 s to 1 min, allowing water to sublime from the fractured face. The platinum–carbon replicas were examined in a Siemens Elmiskop electron microscope.

RESULTS AND DISCUSSION

Lateral diffusion of membrane proteins

Labelling of the membrane proteins from both sides of the membrane by Bretscher [3] and the recent characterization of an isolated glycoprotein by Segrest et al. [5] led to the conclusion that some proteins, especially the glycoproteins, penetrate the membrane from one side to the other.

By the freeze-etching technique, membranes are fractured along the apolar interior zone. Therefore, a protein which extends through the lipid bilayer can be seen when a freeze-fractured membrane is viewed in the electron microscope. Tillack et al. [1] have already provided some evidence that some of the particles seen in freeze-fractured erythrocyte membranes are glycoproteins.

Assuming a normal globular protein, a component with a molecular weight around 100 000 might have dimensions of 50 Å × 50 Å × 100 Å [3]. As shown in Fig. 1a, freeze-etching of the normal sheep erythrocyte ghost membrane exposes particles of approximately 80–100 Å in diameter, which are randomly distributed. When the ghost membranes are washed with 0.1 M phosphate buffer, pH 7.4 or pH 5.8, the particles are displaced from their random distribution and seem to cluster (Fig. 1b). After proteolysis with trypsin [10] or transfer of ghosts from pH 7.4 to pH 5.5 [11, 12] lateral shifting of the particles within erythrocytes has been described. At a constant pH near 7, the shift of the particles in sheep ghosts is observed only at a high ionic strength. For example, when the membranes are washed with 0.01 M phosphate buffer, pH 7.4, the particles remain randomly distributed. This contrasts with the behaviour of particles in human erythrocyte ghosts where Pinto da Silva [11] was able to prevent pH-dependent aggregation by applying buffers of high ionic strength. It is suggested that the different effects are caused by Ca^{2+} which are not present in our experiments.

Subfractionation of erythrocyte ghosts

The dislocation of particles within the ghost membrane causes the formation of areas on the membrane which differ in concentration from these specific proteins. Disruption of the membranes by sonication leads to vesiculation of the membrane. If the particles are unequally distributed, sonication causes the occasional formation of vesicles with different amounts of particles and different densities. Such vesicles can be separated by density gradient centrifugation. Fig. 2 shows the distribution of protein, phospholipid phosphorus and sialic acid in a corresponding density gradient. The top fractions of the gradient (13, 12) contain membranes with a relatively high concentration of phospholipid as compared to the membranes which sediment into fractions of higher density. It is suggested that these fractions additionally contain non-membrane proteins which are lost by subsequent dialysis against distilled water. The concentration of sialic acid based on phospholipid phosphorus in fractions 12 and 13 is about one half of that of the fractions found in regions of higher density.

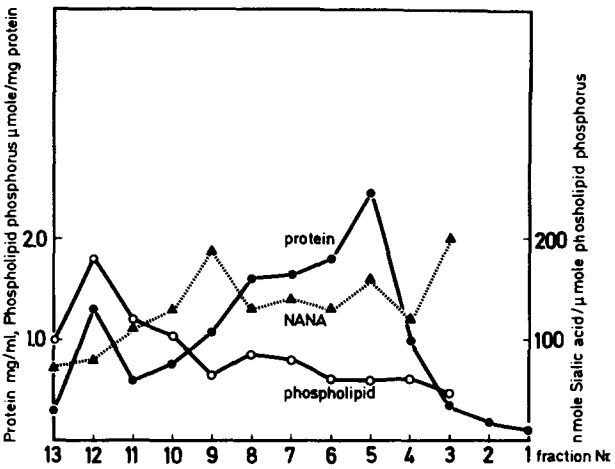

Fig. 2. Distribution of protein, phospholipid phosphorus and sialic acid (NANA) in a linear sucrose gradient (from density 1.098 g/ml to 1.161 g/ml) loaded with sub-fractions of ghost membranes obtained by washing in 0.1 M phosphate buffer and sonication. Fractions 12 and 13 from the top of the gradient contain smooth vesicles whereas the other fractions contain vesicles covered with particles.

This finding corresponds to the electronmicroscopic observation of the different membrane fractions shown in Fig. 3. Fraction 12 obtained from the top of the gradient contains vesicles which are almost free of particles (Fig. 3b), whereas the other fractions contain vesicles with various concentrations of particles (Fig. 3a). The electronmicroscopic observations are documented in Table I. The number of particles per membrane area is significantly lower, by a factor of four, within vesicles obtained from the top of the gradient. On the other hand in vesicles derived from fraction 5, the concentration of particles per area is higher than in the ghosts before subfractionation.

TABLE I

NUMBER OF PARTICLES PER AREA IN GHOST MEMBRANE SUBFRACTIONS OF SHEEP ERYTHROCYTES

The number of particles in each fraction was determined within equal areas of the convex and concave fracture faces. The data are calculated after counting the particles within a total area of $4 \cdot 10^9$ μm^2 per fraction.

	Particles per $6 \cdot 10^{-4}$ μm^2	
Ghosts before sonication	1.467 ± 0.363*	$P > 0.05$
Subfraction No. 12	0.44 ± 0.109**	$P > 0.001$
Subfraction No. 5	1.98 ± 0.995**	$P > 0.001$

* Mean $\pm$S.D. of 8 preparations.
** Mean $\pm$S.D. of 10 preparations.

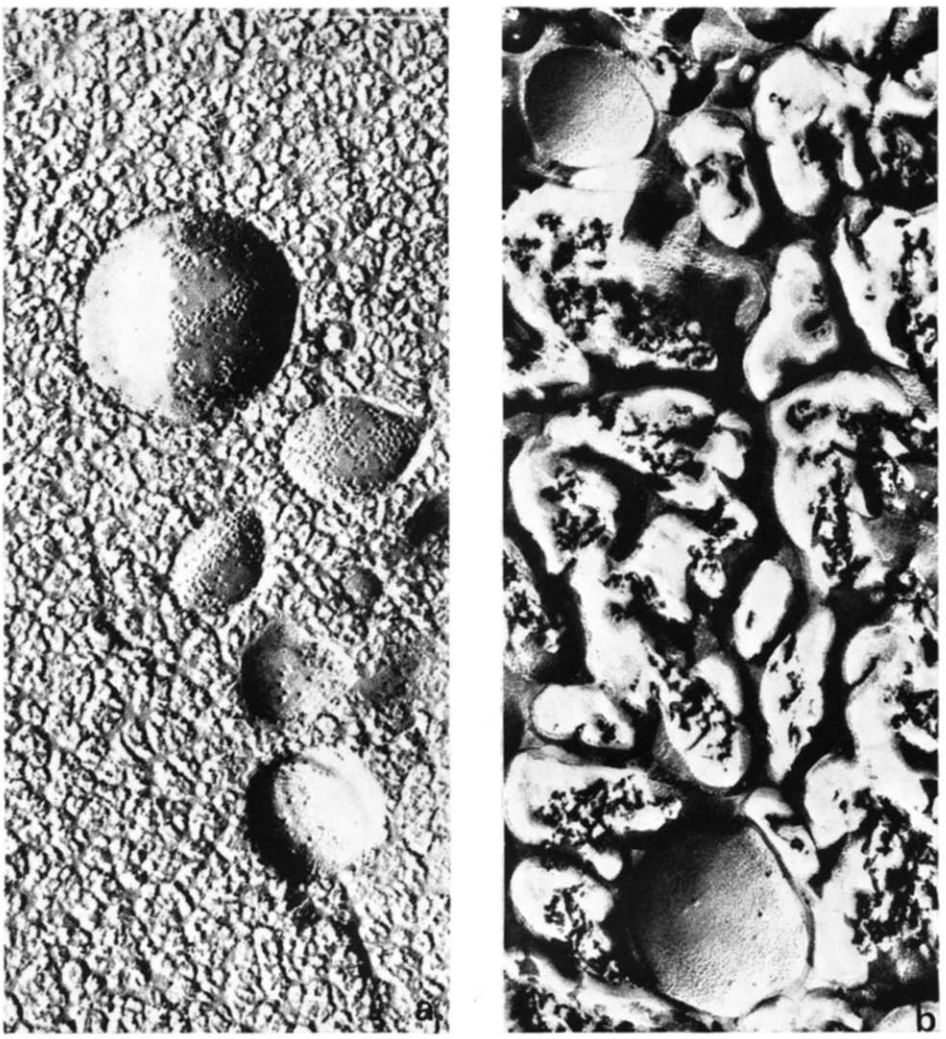

Fig. 3. (a) Freeze-etching of ghost membrane subfractions separated by density gradient centrifugation. Fraction 5, high density (100 000 ×); (b) Freeze-etching of ghost membrane subfractions. Fraction 12, low density (100 000 ×).

Electrophoretic characterization of ghost subfractions

Electrophoresis of the vesicles from the density gradient in 7.5% polyacrylamide gels containing 0.1% sodium dodecylsulphate yields a pattern in which nine bands or groups of bands are resolved (Fig. $4A_1$, B_1 and C_1; Nos 1–9). When stained for carbohydrates with the periodate–Schiff reagent, four well-resolved bands become visible (Fig. $4A_2$, B_2 and C_2; Nos 2, 5–7). In agreement with the findings of Fairbanks et al. [6] on human erythrocytes, some of these bands when stained for protein are very faint or not detectable. Although the fast moving bands contain protein when stained with Coomassie blue, the carbohydrate content can also be attributed to

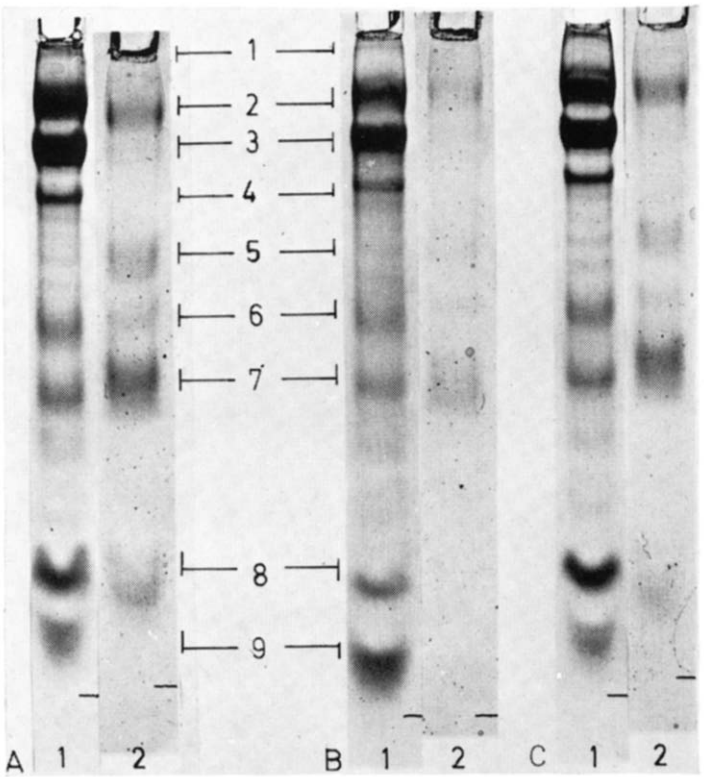

Fig. 4. Electrophoresis of ghost membranes after washing with 0.1 M phosphate buffer and sonication (A_1 and A_2) and of subfractions of ghost membranes (B_1 and B_2, C_1 and C_2). Before electrophoresis all probes were dialysed against distilled water. The gels were loaded with equal amounts of protein. B_1 and B_2, proteins obtained from fraction 12 of the sucrose gradient which contains smooth vesicles. C_1 and C_2, proteins obtained from fraction 5 of the sucrose gradient containing vesicles with many particles. After electrophoresis one part of the slab gel was stained for carbohydrate by the periodate–Schiff reaction (A_2, B_2 and C_2) and the other for protein with Coomassie blue (A_1, B_1 and C_1).

glycolipids. This is suggested because the unstained gels in the corresponding region are turbid. Hence, if we exclude the fast moving bands, erythrocyte membranes from sheep contain four types of glycoproteins.

Erythrocyte ghosts after washing in 0.1 M phosphate buffer and sonication (Fig. $4A_1$ and A_2) and subfractions of the ghost membrane with many particles (Fig. $4C_1$ and C_2) as well as the vesicles with only few particles (Fig. $4B_1$ and B_2) contain all four types of glycoproteins. However, a comparison of the periodate–Schiff staining pattern of the gels loaded with equal amounts of protein demonstrates that the concentration of all four glycoproteins is much lower within the particle-free vesicles.

The fast moving bands 8 and 9 may result from proteolysis during preparation of the samples for electrophoresis. The action of a sodium dodecylsulphate-resistent protease can convert erythrocyte membrane polypeptides into proteins of lower molecular weight. However, it is unlikely that the different content of glyco-

proteins of erythrocyte membrane subfractions is caused by a different activity of proteases within the subfractions. Otherwise a different staining pattern (i.e. faint staining of bands of high molecular weight and strong staining of band 8 or 9) would have been expected especially of carbohydrate-containing bands.

Most of the periodate–Schiff positive material contains a protein which migrates as band No. 7 (Fig. 4). A comparison of the electrophoretic patterns demonstrates that both staining for carbohydrates as well as for protein develops band No. 7 with a lower intensity in vesicles without particles as compared to patterns of vesicles with many particles. Thus, the concentration of carbohydrate containing proteins within the subfractions of the ghost membrane is reduced, corresponding to the reduction of particles seen in freeze-etching. In agreement with Tillack et al. [1] this finding suggests that some of the particles observed within the freeze-fractured erythrocyte membrane represent glycoproteins. However, one has to also consider that structures other than glycoproteins may form particles in freeze-etching, because of the fact that glycoproteins do not exceed 15% of the total protein of the erythrocyte membrane [13].

The data presented above serve to emphasize that it is possible to separate parts of the erythrocyte membrane which are almost free of glycoproteins, and thus may provide a method to study the function of glycoproteins.

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