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Distribution of Glycophorin on the Surface of Human Erythrocyte Membranes and Its Association With Intramembrane Particles: An Immunochemical and Freeze-Fracture Study of Normal and En(a−) Erythrocytes

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Human erythrocyte membranes of the En(a−) blood group lack the major sialoglycoprotein (glycophorin). By absorption of a crude antiglycophorin antiserum with En(a−) membranes a specific antiglycophorin antiserum was obtained. By immune electron microscopy we showed that glycophorin is randomly distributed on the surface of normal erythrocytes. When polycationized ferritin, which mainly binds to glycophorin, was used as a marker a similar even labeling of normal erythrocyte membranes was seen. En(a−) membranes bound much less of this marker. In freeze-fracturing the intramembrane particles of both membrane types had a similar distribution and appeared in equal amounts. However, partial removal of spectrin from these membranes, followed by incubation at pH 6 resulted in more extensive aggregation of the particles in En(a−) membranes than in normal membranes. The results may be interpreted as glycophorin contributing by electrostatic repulsion to the random distribution of the intramembrane particles in normal cells. This repulsion is weakened in En(a−) cells by the lack of glycophorin.

Key words: erythrocyte membranes, glycophorin, intramembrane particles

The human erythrocyte membrane has been extensively studied and serves as a model for plasma membranes in general [1–3]. It contains four major integral glycoproteins and many minor glycoproteins [4, 5], which have their carbohydrate located only on the outside [6–8] and many if not all of the glycoproteins penetrate the membrane [9, 10]. The major sialoglycoprotein, PAS 1 [4] or glycophorin [11], contains 60%

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carbohydrate, including a large proportion of the sialic acid of the cell. Though its primary structure has been determined [12] its physiologic function remains unclear.

The exact arrangement of glycophorin in the membrane is unknown. Attempts to localize glycophorin have been made using polycationized ferritin [13], influenza virus [14], and phytohemagglutinin [11, 14], but the results were complicated by the possible binding of these reagents to surface molecules other than glycophorin.

The major integral glycoprotein, band 3 [4], contains a few percent carbohydrate with little or no sialic acid [15–17]. It probably exists as a dimer in the membrane [18] and constitutes 20–25% of the membrane protein. It is probably involved in transport of anions [16, 19] and possibly in glucose transport through the membrane [20].

When membranes are freeze-fractured along their hydrophobic interior, characteristic intramembrane particles (IMP) are seen [21]. These are probably formed by integral glycoproteins and in the erythrocyte membrane mainly band 3 and glycophorin have been claimed to constitute the particles [22, 23].

We and others recently found that the membrane of the rare human blood group En(a–) [24, 25] lacks glycophorin [17, 26, 27]. We have taken advantage of this fact to obtain a specific antiglycophorin antiserum by absorption of a crude antiserum with En(a–) membranes. We here report that glycophorin is randomly distributed on the cell surface both when studied by an immunoferritin technique and with polycationized ferritin. In addition we have found that the distribution of the IMPs of En(a–) cells and normal cells is identical in isolated ghosts, but when spectrin [28] is partially removed, the IMPs more easily form aggregates in En(a–) membranes than in normal membranes.

METHODS

Cells

Normal human erythrocytes (AB Rh+) and En(a–) erythrocytes (AB Rh+) were obtained through the Finnish Red Cross Blood Transfusion Service, Helsinki. The cells were processed within two weeks of blood donation.

Surface Labeling of Cells and Isolation of Membranes

Normal and En(a–) erythrocytes were labeled by the neuraminidase-galactose oxidase/NaB^3H_4 technique [7, 17]. Membranes were isolated and aliquots counted for radioactivity as described previously [7].

Production of Antiglycophorin Antiserum

A glycophorin preparation was isolated from normal erythrocytes by chloroform-methanol extraction [29]. One major band was present on gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) with an apparent molecular weight of 85,000. Rabbits were immunized three times at two-week intervals subcutaneously with 0.5 mg of this preparation in 0.5 ml of PBS (0.15 M NaCl–0.01 M sodium phosphate, pH 7.4) emulsified in 1 ml of Freund's adjuvant (Difco). Ten days after the last injection the rabbits were bled. One milliliter of the serum was absorbed two times with 1 ml of packed En(a–) membranes at 4°C for 24 h. After absorption the serum was centrifuged at 100,000g for 1 h and stored at 4°C with 0.02% sodium azide.

Immunoprecipitations With Protein A-Containing Staphylococcus aureus Strain Cowan I

Normal and En(a−) erythrocyte membranes, labeled by the neuraminidase-galactose oxidase/NaB^3H_4 technique, were solubilized in PBS containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (Sigma), and 1% ethanol. The samples were centrifuged at 10,000g for 30 min and the supernatants recovered. To 0.2 ml of these were added 5 μg mouse IgG and 5 μl of rabbit anti-mouse IgG antiserum, prepared by standard techniques. All subsequent incubations were done at 0°C. After 1 h, 100 μl of a 10% suspension of Staphylococcus aureus Cowan I strain (obtained from Dr P. Landwall, Stockholm) [30] was added and the tubes incubated for 30 min. The samples were then centrifuged at 3,000 rpm for 10 min and the supernatants recovered. Five μl of the antiglycophorin antiserum or preimmunization serum was added and the tubes incubated for 2 h. Then 0.2 ml of the staphylococcal suspension was added and the incubation continued for 1 h. The bacteria were then washed three times in the Triton X-100-containing buffer, and the proteins eluted from the staphylococci by boiling in 1% SDS for 1 min.

Polyacrylamide Gel Electrophoresis

Membrane or protein samples were dissolved in electrophoresis sample buffer, boiled for 2 min, and electrophoresed on 8% cylindrical acrylamide gels according to Laemmli [31] The cylindrical gels were sliced and counted after NCS solubilization [7] or stained according to Weber and Osborn [32].

Immunoelectron Microscopy

Sheep anti-rabbit IgG, purified by affinity chromatography on Sepharose 4 B-coupled rabbit IgG [33], was conjugated to ferritin (Sigma) using the two-step method of Otto, Takamiya, and Vogt [34] as described earlier [35]. For immunoelectron microscopy the erythrocytes and the membranes were first reacted with the antiglycophorin antiserum (10 μl/0.1 ml packed cells or membranes) for 60 min at 0°C. The specimens were then washed three times in PBS and incubated with ferritin-conjugated anti-rabbit IgG for 60 min at 0°C. After washing three times in PBS, the samples were fixed at 0°C for 60 min in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2. After dehydration in ethanol the samples were embedded in Epon 812, thin-sectioned, poststained with uranyl acetate and lead citrate, and examined in a Jeol 100 B electron microscope.

Labeling of Cells With Polycationized Ferritin

The cells were labeled with polycationized ferritin (Miles-Yeda, Rehovoth, Israel) at a ferritin concentration of 0.1 mg/ml in PBS at 22°C for 60 min. Thereafter the cells were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) for 60 min and processed for thin sectioning as described above.

Freeze-Fracturing Studies

The following samples of membranes were studied by freeze-fracturing: a) isolated membranes of normal and En(a−) cells left after isolation at 0°C; and b) membranes which were depleted of spectrin. Two different methods were used to achieve spectrin removal: 1) Normal and En(a−) membranes were incubated in a 50-fold volume of 0.1 mM ethylenediaminetetraacetate (EDTA), pH 8.0, at 4°C for 16 h [28]. After incubation the membranes

were pelleted by centrifugation in a Sorvall centrifuge at 12,500 rpm for 30 min. The membranes were then incubated in PBS at 37°C for 30 min to achieve possible redistribution of the IMPs. 2) Membranes of both cell types were incubated for 16 h at 37°C in 20 mM sodium phosphate (pH 8.5) containing 0.02% sodium azide [36]. Thereafter the membranes were pelleted by centrifugation. The samples were pretreated for electron microscopy by incubation in 0.05 M sodium phosphate (pH 6.0) for 30 min. The membranes were fixed in 2.5% glutaraldehyde as above, washed twice with PBS, and impregnated with 30% glycerol in PBS at 4°C for 16 h. The samples of membrane suspensions were rapidly frozen in liquid Freon 22 cooled to its freezing point by liquid nitrogen. The freeze-fracture replicas were prepared in a Balzers 360 M apparatus by electron gun evaporation of Pt-C and C.

Protein Determinations

Protein was determined according to Lowry et al [37] with bovine serum albumin as a standard.

RESULTS

Surface Labeling of Normal and En(a–) Erythrocytes

Normal and En(a–) erythrocytes were surface-labeled and the membranes isolated and studied on cylindrical polyacrylamide gels. In normal cells PAS 1 (glycophorin dimer [38]) and PAS 2 (mainly glycophorin monomer) were strongly labeled (Fig 1 A), whereas these peaks were absent from En(a–) membranes (Fig 1 B). The mobility of band 3 from En(a–) membranes was slower and it was more strongly labeled than in normal membranes.

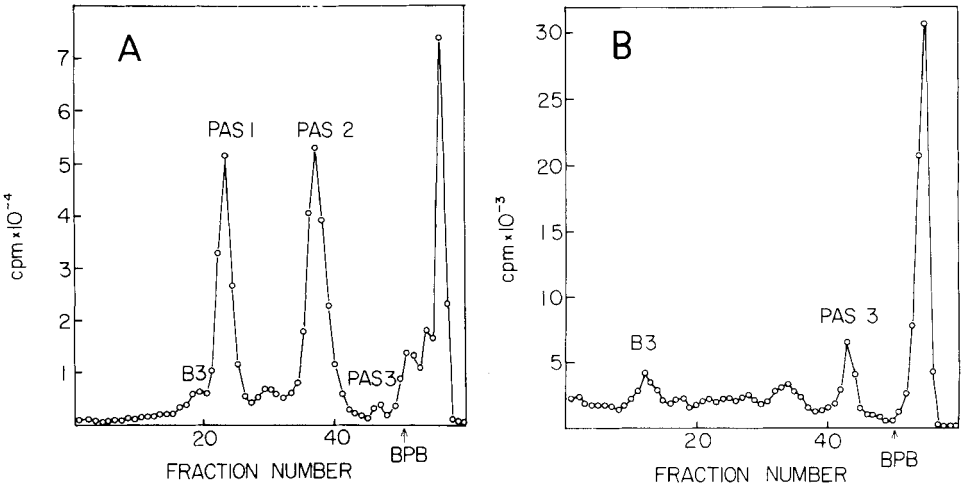


Fig 1. Polyacrylamide gel patterns of surface-labeled normal and En(a–) erythrocyte membranes. A) Pattern of normal membranes; B) pattern of En(a–) membranes. B3) band 3; PAS 1–3) the periodic acid-Schiff-positive sialoglycoproteins (PAS 1 = glycophorin dimer, PAS2 = glycophorin monomer (predominantly). BPB) position of bromophenol blue marker dye.

Distribution of Glycophorin in Normal Erythrocytes as Revealed by Immunoferritin Technique

Glycophorin was visualized by an indirect immunoferritin method using specific antiglycophorin antiserum. The specificity of the antiserum was shown as follows: When normal erythrocyte membranes were solubilized and treated with the antiglycophorin antiserum and were immune-precipitated with protein A-containing staphylococci and studied by polyacrylamide gel electrophoresis, the only peak observed corresponded to the monomer of glycophorin, or PAS 2 (Fig 2 A). No peak was obtained from labeled En(a–) membranes (Fig 2 B) nor did rabbit preimmunization serum precipitate any antigen from normal membranes (not shown). When normal erythrocytes were stained by the immunoferritin technique, ferritin particles were seen along the entire cell surface (Fig 3 A). Notably, when isolated membranes from normal erythrocytes were similarly labeled, a distinct asymmetric labeling of the membranes was observed on the external surface (Fig 3 B), judged from the fibrillar material marking the cytoplasmic face of the membranes.

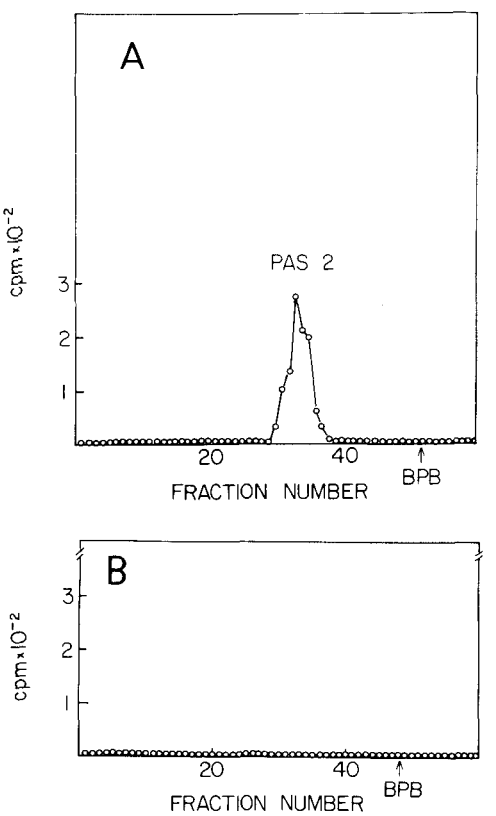


Fig 2. Polyacrylamide gel patterns of surface-labeled normal erythrocyte membranes precipitated with antiglycophorin antiserum and preimmunization serum. A) Pattern obtained from normal membranes after treatment with antiglycophorin antiserum and protein A-containing staphylococci; B) pattern obtained from normal membranes after treatment with preimmunization serum and staphylococci.

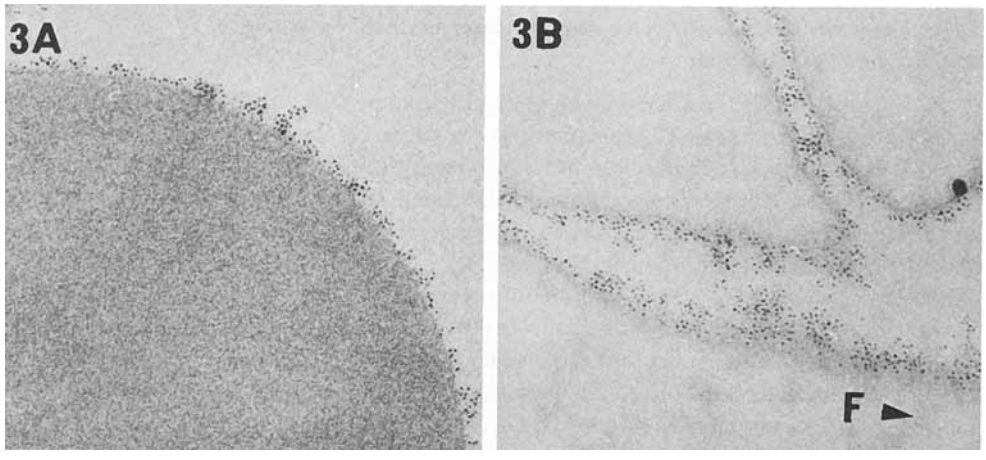


Fig 3. Visualization of glycophorin distribution by indirect immunoferritin technique. A) Normal erythrocyte surfaces are densely labeled with ferritin; B) isolated membranes of normal erythrocytes show label on the external membrane surface. Note the fibrillar material (F) indicating the cytoplasmic surface of the membranes. (Magnification 70,000 ×).

Distribution of Anionic Sites on Normal and En(a−) Membranes as Seen With Polycationized Ferritin

When normal erythrocytes were incubated with polycationized ferritin, a dense, uniform distribution of ferritin particles was seen on the cell surface (Fig 4). In contrast to this, En(a−) cells showed less binding and more uneven binding of the marker (Fig 5). The difference in the labeling of the two cell types was consistent and evident both in tangential (Fig 4 A, 5 A) and transverse (Fig 4 B, 5 B) sections of the membranes.

Distribution of Intramembrane Particles in Freeze-Fractured Membranes of Normal and En(a−) Erythrocytes

When erythrocyte membranes were freeze-fractured, a random distribution of intramembrane particles was seen both in normal (Fig 6A) and in En(a−) (Fig 6B) membranes. When spectrin was partially removed by incubation of the isolated membranes at 37°C in 20 mM sodium phosphate (pH 8.5) and the membranes were then transferred to pH 6.0 before freeze-fracturing, normal membranes showed a slight and variable aggregation of the intramembrane particles (Fig 6C), whereas the particles on En(a−) membranes were clearly and consistently more aggregated (Fig 6D). When membranes were depleted of spectrin by incubation with EDTA, they formed small vesicles. With this treatment there was an extensive aggregation of intramembrane particles in both normal (Fig 6E) and in En(a−) (Fig 6F) membranes.

DISCUSSION

The structure of glycophorin is remarkably well known. The primary structure of the polypeptide has been determined [12]. It contains three major regions, including a hydrophobic amino acid stretch, which probably is located within the lipid bilayer. The NH_2-terminal portion shows amino acid acid substitutions which depend on the MN antigen activity [39], and it carries the carbohydrate of the protein.

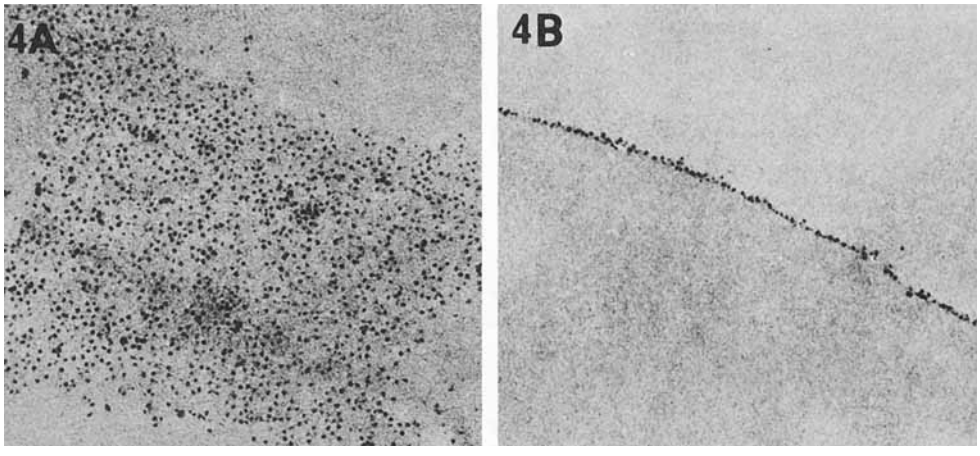


Fig 4. Normal erythrocyte membranes labeled with polycationized ferritin. A) In tangential section a dense and uniform labeling is seen; B) in transverse section a continuous labeling of the cell surface is evident. (Magnification 70,000 ×.)

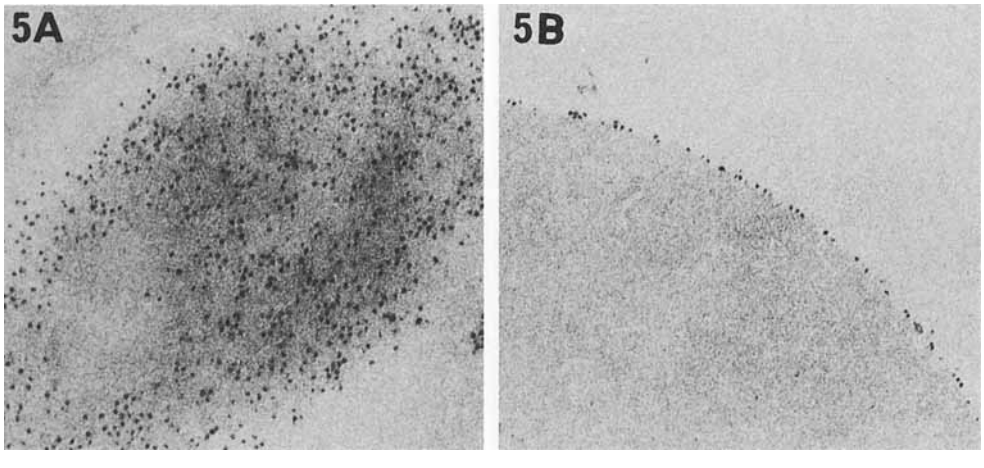
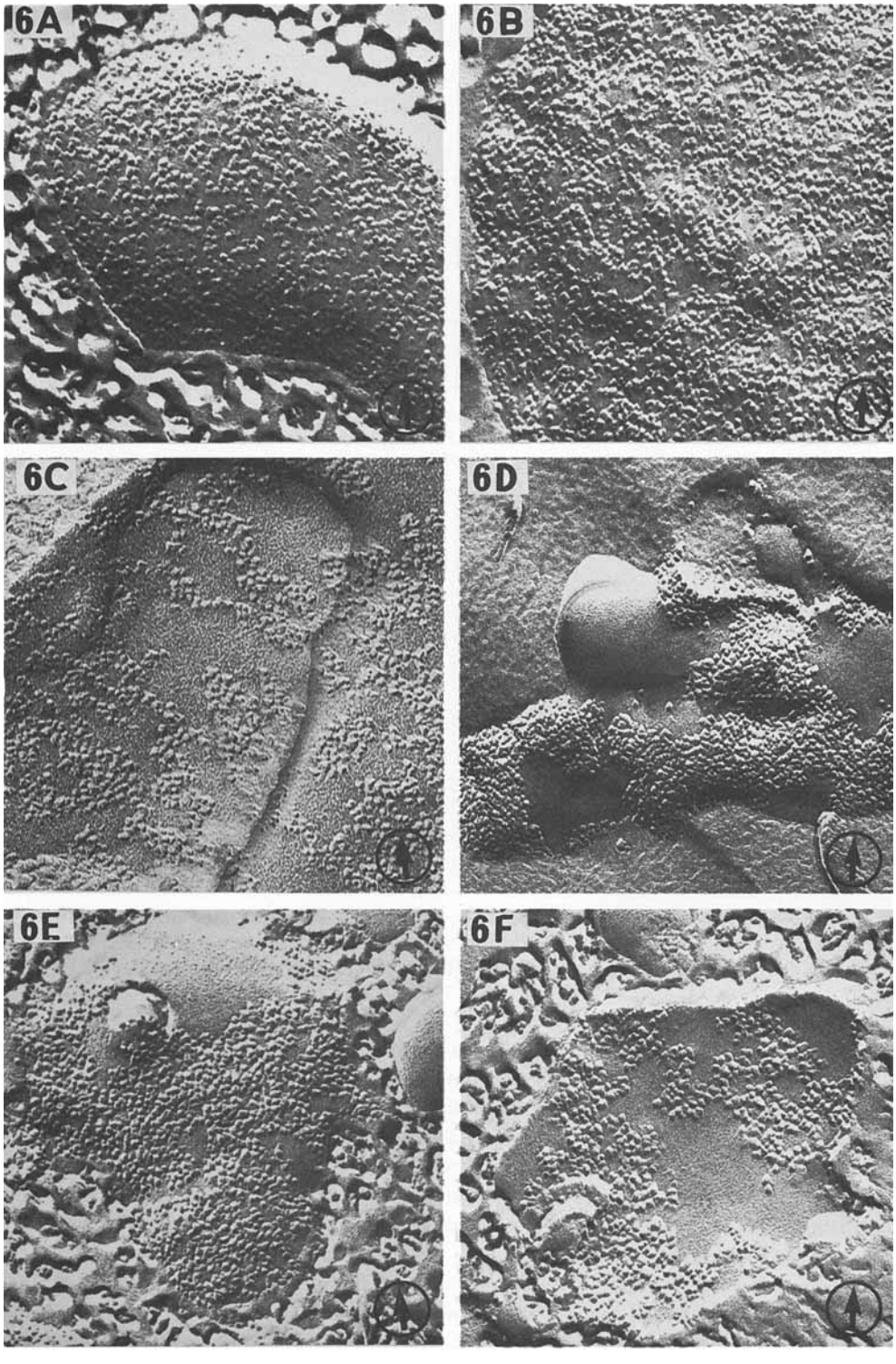


Fig 5. En(a−) erythrocytes labeled with polycationized ferritin. A) In tangential section a relatively sparse and more uneven labeling than in Figure 4A is evident; B) in transverse section a clearly discontinuous staining of the cell surface is seen. (Magnification 70,000 ×.)

The precise location of glycophorin in the membrane has been difficult to determine. We have approached this problem by the use of erythrocytes of the rare blood group En(a−). Erythrocyte membranes of this blood group lack glycophorin. This was first shown by protein staining and radioactive labeling techniques [17, 26, 27] and recently Furthmayr arrived at the same conclusion by use of a radioimmunologic method [39].

In the present experiments the polycationized ferritin marker was more sparsely distributed on the surface of En(a−) membranes than on normal membranes. The difference in the patterns of normal and En(a−) membranes should be due to glycophorin and it can be concluded that its surface distribution seems to be random. A similar result was obtained with the indirect immunoferritin technique using antiglycophorin antiserum and ferritin-conjugated sheep anti-rabbit IgG antiserum. It should be noted that the antibodies reacted exclusively with the outer surface of the membranes and thus with the NH_2-


6A
6B
6C
6D
6E
6F

terminal portion. This is in contrast to the antibodies produced by Cotmore, Furthmayr, and Marchesi [40], which were raised against the COOH-terminal fragment of glycophorin and actually only reacted with the cytoplasmic surface of the membrane.

By use of En(a−) membranes we have approached the problem whether glycophorin is a component of the IMPs seen in freeze-fracturing of the membranes. We have been able to confirm the results of Bächi et al [41] that untreated En(a−) membranes contain IMPs evidently of normal size, distribution, and number. Therefore glycophorin is not an essential or a major component of these particles. Both band 3 and glycophorin preparations have been reconstituted into lipid vesicles, and particles resembling IMPs have been observed [22, 42, 43], but the significance of these results is still unclear.

It is obvious that the IMPs somehow are associated with spectrin. After removal of spectrin the IMPs can be aggregated [36], and we have recently shown with selective cross-linking of spectrin by periodate, that the IMPs are aggregated [44]. It is also known that antibodies against spectrin redistribute surface anionic sites which mainly are due to glycophorin [45], and recently Shotton et al [46] showed that antispectrin antibodies aggregate the IMPs. It is evident, however, that the attachment of IMPs to spectrin cannot be mediated solely by glycophorin, because then one would expect a nonrandom distribution of IMPs in En(a−) cells even under conditions when spectrin is not removed. We favor the hypothesis that this connection is mainly formed by band 3.

After treatment with 20 mM phosphate followed by incubation at pH 6 there was a more extensive aggregation of IMPs in En(a−) membranes than in normal membranes. This further indicates that glycophorin normally is a constituent of the particles and is involved in part in keeping the particles dispersed. This assumption is supported by the fact that neuraminidase treatment enhances the aggregation of the IMPs in normal cells [36], and Shotton et al [46] recently published evidence that most major surface proteins are involved in the formation of the IMPs.

A schematic model of the structure of IMPs and their relation to band 3, glycophorin, and spectrin is shown in Figure 7. The major part of the particles is formed by band 3 proteins. Both band 3 and glycophorin exist as dimers and penetrate the membrane, but only band 3 is more closely associated with spectrin, perhaps by intercalation between spectrin fibers [47]. Our results are only suggestive, and only when IMPs are isolated in pure form can definite knowledge of their composition be obtained.

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Fig 6. Cleaved fracture faces of normal and En(a−) erythrocytes. Both in membranes from normal (A) and En(a−) (B) erythrocytes a similar even distribution of the intramembrane particles is seen. When membranes of normal cells were depleted of spectrin by incubation in 20 mM sodium phosphate (pH 8.5) at 37°C for 16h and then transferred to 0.05 M sodium phosphate (pH 6.0) and incubated at 37°C for 30 min, a slight aggregation of IMPs is seen (C). After a similar treatment of En(a−) membranes a clear aggregation is seen (D). When membranes were depleted of spectrin by incubation in 0.1 mM EDTA (pH 8.0) at 4°C for 16 h and then incubated in PBS at 37°C for 30 min, an extensive aggregation of the IMPs occurred both in normal (E) and En(a−) (F) membranes. Shadowing direction is indicated by circled arrow.(Magnification 75,000 ×.)

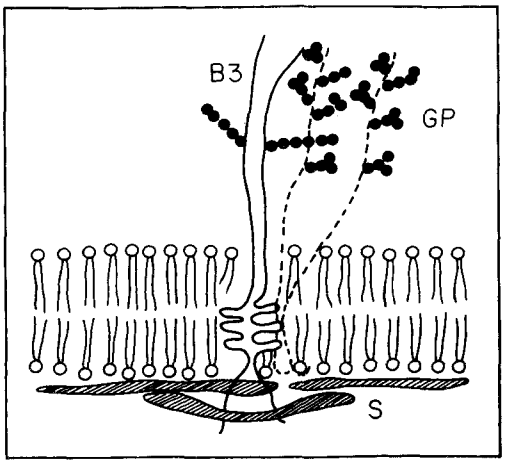

Fig 7. Schematic drawing of the normal erythrocyte membrane showing the band 3 (B3) and glycophorin (GP) dimers forming the intramembrane particles, and the intercalation of band 3 between the spectrin fibers (S).

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