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Surface proteins of erythrocyte membranes*

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SUMMARY

Diazotized sulfanilic acid labeling and trypsin digestion studies of human and bovine erythrocytes indicate that the glycoprotein of each species is the only major membrane protein which is readily accessible at the cell surface. Trypsin digestion of isolated human erythrocyte membranes under conditions identical to the erythrocyte digestion results in extensive cleavage of the major membrane proteins, indicating that they are readily susceptible to digestion in the ghost but not in the intact cell.

Surface proteins of erythrocyte membranes

Virtually all of the models for biological membranes which have been presented to date show a considerable fraction of the membrane protein present at the exterior surface[1]. Experiments with human erythrocyte membranes suggest that the distribution of the proteins of the membrane is not as uniform as most models portray. Using diazotized sulfanilic acid, a protein reagent which penetrates slowly the membrane barrier of intact erythrocytes, Berg[2] was able to obtain an enhanced specificity of labeling of certain proteins of human erythrocyte membranes but noted no labeling of the major high molecular weight protein. Phillips and Morrison[3] have recently used the enzyme lactoperoxidase to label the exterior proteins of the erythrocyte. Only one significant band of radioactivity was noted when the membrane proteins were fractionated by acrylamide electrophoresis in sodium dodecyl sulfate. To investigate this problem further we have used two different methods for modifying surface proteins of human and bovine erythrocytes. These two species were chosen for the following reasons: (1) the protein patterns are essentially identical for the major bands of proteins observed by sodium dodecyl sulfate electrophoresis, and these patterns are stable to a range of isolation conditions (D. Kobylka,

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unpublished observations); (2) the molecular weights of the glycoproteins* of the two species are quite different as determined by sodium dodecyl sulfate–acrylamide electrophoresis[4]; and (3) the bovine glycoprotein, because of its high molecular weight, is cleanly separated on electrophoresis from the other major membrane proteins, a phenomenon that is not observed for the erythrocyte glycoprotein of any other species we have studied. The carbohydrate staining profile for bovine erythrocyte membranes shows only two peaks, one near the origin (glycoprotein) and one at the sodium dodecyl sulfate front (lipid).

Human and bovine erythrocytes were treated with diazotized sulfanilic acid according to the procedure of Berg[2] in isotonic phosphate buffer at pH 7.4. The reagent concentration was reduced to 0.3 mM in an attempt to enhance the specificity of the reaction by reducing the penetration of the reagent into the cell. Membranes were isolated by the procedure of Dodge *et al.*[6], and subjected to electrophoresis using the sodium dodecyl sulfate–acrylamide procedure[4]. The protein patterns observed by Coomassie Blue staining were essentially identical for the two species. The species variation in glycoproteins does not alter this observation, since the membrane glycoproteins do not stain as readily as the other membrane proteins with Coomassie Blue. For detection of radioactive labeling patterns gels were sliced[7], extracted and counted[8]. Fig. 1 shows the radioactivity profile, a tracing of the gel scan from the periodate–Schiff-treated gel and a diagram of the gel standard for protein, obtained from identical 6% gels of labeled human erythrocyte membranes. One major area of radioactivity is noted, a broad band with a peak at a molecular weight of 85 000 similar to that observed by Phillips and Morrison[3] after lactoperoxidase labeling. Two areas of carbohydrate stain are present in the gel, a glycoprotein band centered at 85 000 molecular weight and a band corresponding to the sodium dodecyl sulfate front which contains the membrane lipid. The radioactive band corresponds rather closely to the glycoprotein, but the broadness of the band suggests that other proteins in this area may also be labeled. This question was resolved by experiments on the bovine erythrocytes. The radioactivity profile of the bovine erythrocyte membrane on 6% acrylamide gels shows two peaks, a major one at the origin corresponding to the glycoprotein and a smaller, broad peak in the 100 000 molecular weight range, which does not stain for carbohydrate. A clearer picture of the labeling was obtained using 4% gels (Fig. 2), a technique which permits clean separation of the bovine membrane glycoprotein from the origin and from the other membrane proteins by virtue of its high molecular weight (> 200 000). The labeling and staining patterns of 4% gels show that the major band of radioactivity is coincident with the glycoprotein and that the smaller band corresponds to the protein of molecular weight 108 000. To rule out the possibility that the specificity of labeling was due to a decreased reactivity of certain membrane proteins rather than their relationship to the permeability barrier, similar experiments were performed on isolated membrane ghosts, which are more freely permeable to the reagent. In these cases the

*Erythrocyte membranes from all species examined show a single glycoprotein or related family of glycoproteins when stained under appropriate conditions. Earlier reports of other major[5] or minor[4] glycoproteins apparently result from nonspecific staining of negatively charged sodium dodecyl sulfate–protein complexes by the positively charged Schiff reagent. A procedure for circumventing this difficulty has been kindly communicated to us by Dr. H. Glossman (manuscript submitted for publication).

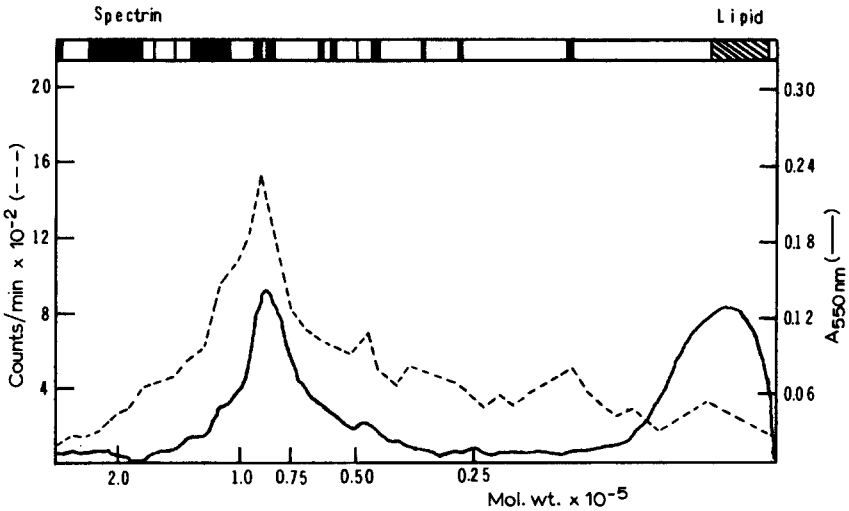


Fig. 1. Labeling patterns for membrane proteins of human erythrocytes labeled with ^{35}S-diazotized sulfanilic acid. Membranes were solubilized in 3% sodium dodecyl sulfate and subjected to electrophoresis on 6% acrylamide gels[4]. The radioactivity distribution across the gel (- - -) and the densitometer tracing of the identical gel stained for carbohydrate (—) are shown. The bar graph at the top of the figure represents the protein staining pattern with Coomassie Blue.

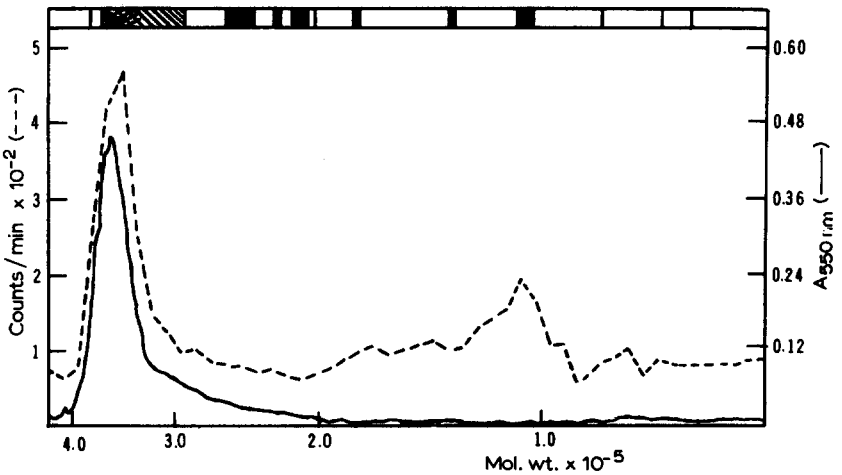


Fig. 2. Labeling patterns for membrane proteins of bovine erythrocytes labeled with ^{35}S-diazotized sulfanilic acid. Electrophoresis was performed on 4% gels.

radioactivity was spread over the entire range of proteins, including the high molecular weight protein doublet Spectrin[9]. In addition, experiments performed with acetic anhydride and iodoacetic acid, reagents which readily penetrate the membrane, have shown that all membrane proteins are accessible to reaction and that their relative reactivities are not greatly altered in going from erythrocyte to ghost[10].

Further studies of the structural aspects of erythrocyte membranes were performed by trypsin digestion of erythrocytes and ghosts. Although it is well known that trypsin

releases glycopeptides from erythrocytes[11], the accessibility of other membrane proteins to trypsin digestion cannot be ascertained in this manner, since their cleavage products may not be released from the membrane. The effect of trypsin treatment can be determined from the protein patterns on sodium dodecyl sulfate electrophoresis, because the cleavage of any protein in the membrane will result in a shift in its position on the gel toward the area of lower molecular weight proteins. Erythrocytes were treated with a range of trypsin concentrations in isotonic phosphate–saline, and the membranes were isolated after hypotonic hemolysis[6]. Sodium dodecyl sulfate–acrylamide electrophoresis was used to monitor changes in the protein (Coomassie Blue stain) and glycoprotein (periodate–Schiff stain) components. Glycoprotein content was estimated by densitometric analysis of periodate–Schiff-stained gels from the areas under the curves. For trypsin-treated samples only the peak corresponding to the original glycoprotein was included in the area calculations, so that staining of cleavage products does not contribute to the glycoprotein determination results. Acetylcholinesterase was determined on either intact erythrocytes or isolated ghosts[12], and sialic acid[13] and cholesterol[14] were determined on the isolated ghosts. Fig. 3 shows the changes in sialic acid, acetylcholinesterase activity and glycoprotein of the isolated ghosts from trypsin-treated erythrocytes as a function of trypsin concentration. Approximately 60% of both the sialic acid and acetylcholinesterase activity were lost, and virtually all of the glycoprotein molecules were cleaved. Since only about 30% of the red cell sialic acid is released during trypsin digestion, these results suggest that the sialoglycopeptides produced by trypsin cleavage are differentially released from the membrane (30% of the sialic released from the red cell, 30% released during membrane preparation, and 40% not released). The fate of the other membrane proteins can be shown by staining the electrophoretic gels with Coomassie Blue and is shown in Fig. 4. Examination of the gels shows that the patterns are not visibly changed for the treated sample either by reduction of the number of bands or a significant decrease in the relative intensity of any band. This indicates that none of the major proteins of the erythrocyte membrane are readily

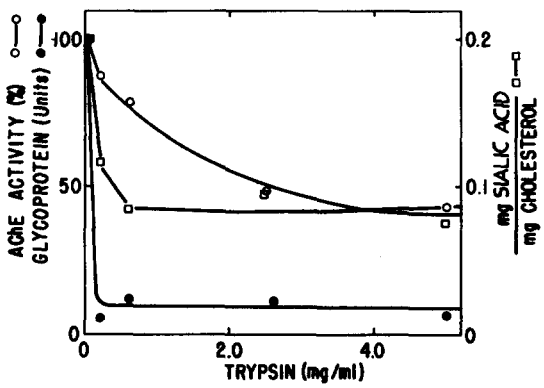
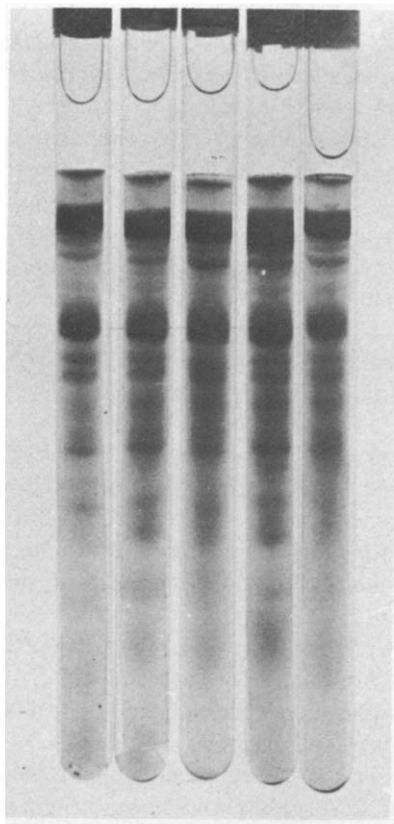


Fig. 3. Alteration in acetylcholinesterase (AChE) activity[12], sialic acid content[13] and glycoprotein content of membranes isolated from trypsin-treated human erythrocytes. Washed human erythrocytes (25% suspension) were treated with trypsin in isotonic phosphate–saline at pH 7.4 for 1 h at room temperature. Trypsin inhibitor was added to stop the reaction, cells were washed in cold isotonic phosphate and membranes were isolated by the procedure of Dodge *et al.*[6]. Membranes were assayed for acetylcholinesterase, sialic acid and cholesterol and were subjected to gel electrophoresis. Glycoprotein was estimated from gel scans using Gilford Model 2000 after periodate–Schiff staining.



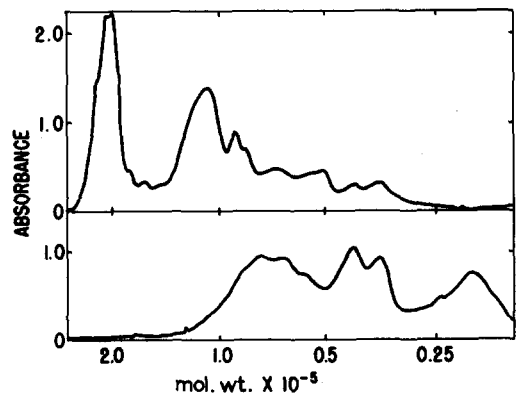


Fig. 4. Polyacrylamide gel electrophoresis patterns of erythrocyte membranes from trypsin-treated human erythrocytes. Trypsin concentrations (left to right) were 0, 0.25, 0.50, 2.5 and 5.0 mg/ml. Gels were stained with Coomassie Blue in 7% acetic acid after 1 h fixation in 10% trichloroacetic acid. Because of the experimental procedure slightly different amounts of solubilized membrane were loaded onto the different gels. Therefore the intensity of the bands increases from Gel 1 to Gel 4, but the effect is uniform throughout each gel.

Fig. 5. Scanning patterns of isolated erythrocyte membranes treated with trypsin at 25 μg/ml for 1 h at room temperature. Membrane concentration was equivalent to that in 25% suspension of erythrocytes. Reaction was stopped by addition of trypsin inhibitor, and membranes were washed with cold 20 ideal mosM phosphate before electrophoresis. Top, untreated control; bottom, treated sample.

accessible to trypsin treatment of the intact cell except the glycoprotein, and that the glycoprotein is not represented by any of the Coomassie Blue bands.

To show that erythrocyte membrane proteins are not inherently resistant to proteolysis, isolated erythrocyte ghosts were subjected to trypsin digestion in isotonic phosphate. Fig. 5 shows scanning profiles of untreated and treated ghosts digested with 25 μg/ml of trypsin. All higher molecular weight proteins have been cleaved and displaced toward lower values. At the higher trypsin concentrations comparable to those used with the intact erythrocytes all membrane polypeptide chains were cleaved and the gels were virtually blank. Therefore, the resistance of the proteins of the membrane of the intact red cell to proteolysis appears to result from their arrangement in the membrane rather than their individual structural features.

The foregoing experiments have several important implications concerning the structure of erythrocyte membranes. The evidence from both labeling and trypsin digestion studies suggest that glycoprotein is the only major membrane protein which is readily accessible at the surface of the cell and outside of the membrane permeability barrier. This is the major species labeled by both the methods of Berg[2] and of Phillips and Morrison[3]. A second polypeptide (molecular weight 108 000) also appears to be outside the permeability barrier based on the labeling results, but it is apparently less accessible than the glycoprotein. The other major membrane proteins apparently exist as part of the barrier or inside the barrier with little or none of their structures available at the surface. The enhanced susceptibility of proteins of the ghost to trypsin digestion indicates that the membrane of the ghost must differ in its structure or stability from that of the intact red cell or that it must be grossly permeable to the enzyme during the course of the digestion. Similar conclusions can be reached from the recent results of Phillips and Morrison on lactoperoxidase labeling of intact erythrocytes and isolated membranes[15] and of Zwaal *et al.*[16] on treatment of erythrocytes and ghosts with purified phospholipase C. In all of these cases gross differences were noted between the intact erythrocyte and the ghost in their susceptibility to attack by enzymes. The recent report by Zwaal *et al.*[16] is of particular interest concerning other components of the erythrocyte surface. They found the phospholipids of intact erythrocytes to be resistant to cleavage by highly purified phospholipase C of *Bacillus cereus*, whereas the phospholipids of the isolated ghost were cleaved to the same extent as in dispersions of the isolated membrane lipids. These studies on proteins and lipids of the erythrocyte place strong restrictions on the nature of the substances which can be present at the erythrocyte surface. If phospholipids or proteins (other than those mentioned above) are present at the erythrocyte surface, they must be protected by the carbohydrate of the glycoprotein or by interactions which repress their reactivity or availability. This type of model would also require that these repressive interactions be removed by structural alterations in going from erythrocyte to ghost. An alternate explanation of the differences between intact erythrocytes and ghosts would require that the ghost be permeable to all reagents and enzymes used in these studies. Although it is not possible to answer these questions conclusively at present, it is interesting to note that in previous chemical studies we have found differences in the reactivities of membrane lipid groups between the intact erythrocyte and the ghost which are apparently not related to reagent permeability[10]. Further studies are obviously needed to determine more accurately the nature of the components at the erythrocyte surface and the differences which exist between the membrane of the intact erythrocyte and the ghost. However, the present investigations do emphasize the need for caution in interpreting studies on isolated membranes in terms of the structure of the membrane of the intact cell.

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