Journal of Supramolecular Structure 7:121–134 (1977)
Cell Surface Carbohydrates and Biological Recognition 201–214

Structural Analysis of a Membrane Glycoprotein: Glycophorin A

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Glycophorin A is the major sialoglycoprotein of the human erythrocyte membrane. Structural studies indicate that this molecule is made up of 3 domains composed of 2 hydrophilic segments which are separated by a region of 22 nonpolar amino acids. The N-terminal half of the molecule contains all the carbohydrate associated with this protein.

Glycophorin A forms high-molecular-weight complexes which can be dissociated only under certain conditions. The site of subunit interaction is located within the hydrophobic segment, which serves both to mediate protein-protein and protein-lipid interactions within the bilayer membrane. Glycophorin A spans the membrane presumably as a dimeric complex with the carboxyterminal ends extending into the cytoplasm of the red cell. The transmembrane nature of the polypeptide chains finds strong support from the use of specific antibody-ferritin conjugates applied to thin sections of fixed and frozen intact cells.

Preliminary information on the analysis of human red cell variants which may lack some or all of the sialoglycopeptides are consistent with the presence in normal cells of a second sialoglycoprotein, provisionally labeled glycophorin B.

Key words: erythrocyte, plasma membrane, glycoproteins, amino acid sequence

The human erythrocyte membrane has been studied for many years to probe questions of membrane structure and function. A fairly well established model for this membrane system has been elaborated (1, 2). It seems that the bulk of the membrane proteins are located at the cytoplasmic side of the lipid bilayer and are linked in unknown ways to each other and to the membrane (3, 4). Only a few major proteins are associated with the membrane in a more intimate way by interacting with the hydrocarbon interior of the bilayer. These are considered to be major transmembrane proteins (5, 6). In addition to these there seem to be other proteins embedded in the membrane and protruding from the surface, which are not easily detectable by current standard methods because of the small number of copies per cell (7, 8).

Most of the data in suport of this general model have been obtained by analytical techniques such as proteolytic treatment and radiolabeling of intact cells and "leaky" or "sealed" ghosts (1, 2). Detailed structural information is largely missing since only a few of the major protein components have been isolated and purified enough to permit such studies. Particular difficulties have been encountered with the class of membrane proteins

Received May 10, 1977; accepted May 20, 1977.

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which associate directly with lipid. Only recently have techniques become available which allow their isolation and separation from other membrane components (2, 6, 9).

Glycophorin A obtained from human erythrocyte membranes is the first membrane glycoprotein for which the primary structure is known (10). This knowledge not only makes it possible to test and corroborate earlier data on its orientation in the membrane, but also to study the genetics of its expression on the cell surface and to design experiments on its role in differentiation and in the mature cells.

SIALOGLYCOPROTEINS AS VIRUS RECEPTORS AND BLOOD GROUP SUBSTANCES

In the late 1940s Winzler and his colleagues isolated material from human red cell membranes by hot phenol extraction, chloroform-methanol treatment, and ultracentrifugation, which was able to inhibit myxovirus hemagglutination (11). Treatment with neuraminidase and tryptic digestion destroyed the activity suggesting that the virus receptor was a sialoglycoprotein. It was realized early that the isolated molecule tended to aggregate in aqueous buffers and that treatment with detergents, organic solvents, or acids produced smaller proteins with a molecular weight estimated at 30,000 daltons (12).

This viral receptor substance apparently is related to molecules which carry MN blood group activity (13). The MN antigens were detected by Landsteiner and Levine in 1927 by using antisera obtained by immunization of rabbits with red blood cells (14) and subsequently by plant lectins, such as that from Vicia graminea, which possesses anti-N activity (15). The first insight into the chemical nature of the MN antigens came from observations by Springer, that neuraminidase from influenza virus and from Vibrio cholerae abolishes the MN activities in human erythrocyte in addition to destroying their influenza virus receptor activity (16). Treatment of intact cells with proteolytic enzymes releases a considerable amount of the cell surface carbohydrates in the form of glycopeptides which also results in the loss of MN determinants from the cell. It was concluded that sialic acid residues serve not only as receptor sites for influenza virus, but are also essential for MN antigens, and that these activities are linked to a glycoprotein. Isolation of the MN substances resulted in proteins of very high molecular weight and studies on the antigenic structures were severely hampered by the fact that activity depended largely on the state of aggregation, being higher for the highest molecular weight aggregates. Small glycopeptides have only low activity and isolated oligosaccharides completely lack M or N activity (16). However the purity and homogeneity of these isolated products have never been rigorously established. Subsequently other methods for the isolation of these sialoglycoproteins were developed such as the use of aqueous pyridine, chloroform/methanol, detergents, affinity chromatography, and combinations of the various methods (2). The yields were usually low and the purity of the preparations was difficult to assess. In addition, a wide range of molecular weights was reported. The use of sodium dodecyl sulfate gel (SDS-gel) polyacrylamide electrophoresis added still more confusion, since a major sialoglycopeptide was found to have an apparent molecular weight of about 85,000, but the migration of the protein varied with the acrylamide concentration (17). In addition, more complex gel patterns were obtained when other electrophoresis systems were employed (18, 19).

Fairbanks et al. in their gel electrophoresis system described 4 bands for red cell membranes which were stainable with periodic acid-Schiff's reagent (20). The staining property on the gel of these peptides depends largely on the presence of sialic acid, but chemical labeling of sialic acid in intact cells or membranes and other labeling techniques

confirmed these results (21). The sialoglycoproteins bands were termed PAS-1, PAS-2, PAS-3, and PAS-4. This complex pattern on SDS-gels raises several questions: What is the chemical nature of these glycopeptides, do individual bands correspond to unique glycopeptides, and what is their relationship to each other?

GLYCOPHORIN A AND GLYCOPHORIN B

Isolation of the sialoglycoprotein fraction from human red cell membranes gives a preparation with a gel pattern after PAS-staining identical to that of the total membrane (Fig. 1). Further fractionation by gel filtration in detergents (22) resolved 3 protein containing peaks (Fig. 2), and 2 of these (peaks B and C) apparently contain the same peptide (glycophorin B), which is different from the protein component in the main peak A (glycophorin A). Although both peptides appear to be related to each other and contain approximately the same amount of carbohydrate and similar, yet distinct, amino acid compositions, gel filtration and SDS-gel analysis showed that glycophorin A corresponded to the PAS-1 and glycophorin B to the PAS-3 band while the peptides eluted from the PAS-2 position appeared to be a mixture of both proteins (23). Surprisingly, reelectrophoresis of the isolated peptides did not result in single bands but revealed a tendency to aggregate to multiple-molecular-weight forms (Fig. 3). Similar observations have been made by other groups (24). Glycophorin A and glycophorin B isolated by gel filtration also do not appear as homogeneous peptides on SDS-gels (22, Fig. 4). Glycophorin B isolated from both regions of the chromatogram, although apparently homogeneous (unpublished), gives at least 4

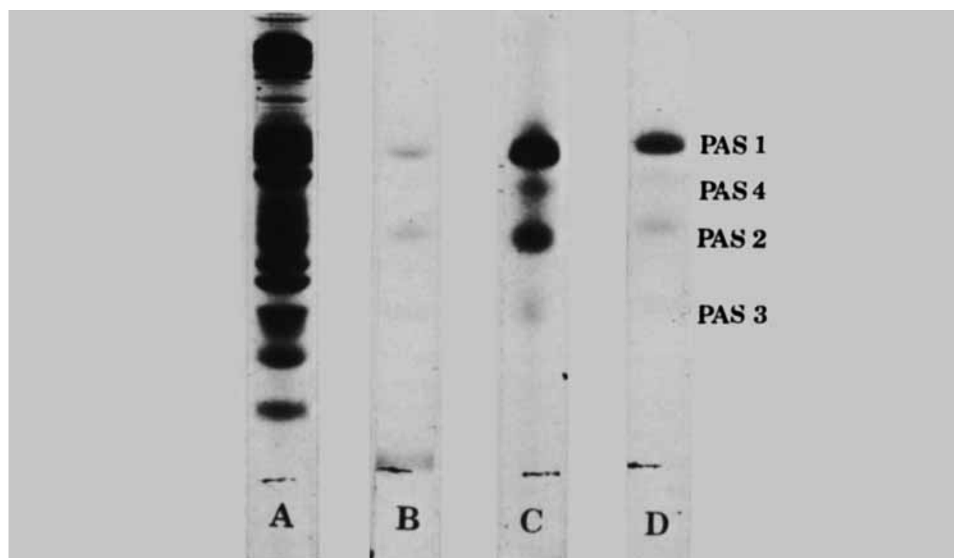

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) of human red cell membranes (A,B) or the isolated sialoglycoprotein fraction (C,D), stained with Coomassie blue (A,C) or periodic acid-Schiff's reagent (B,D).

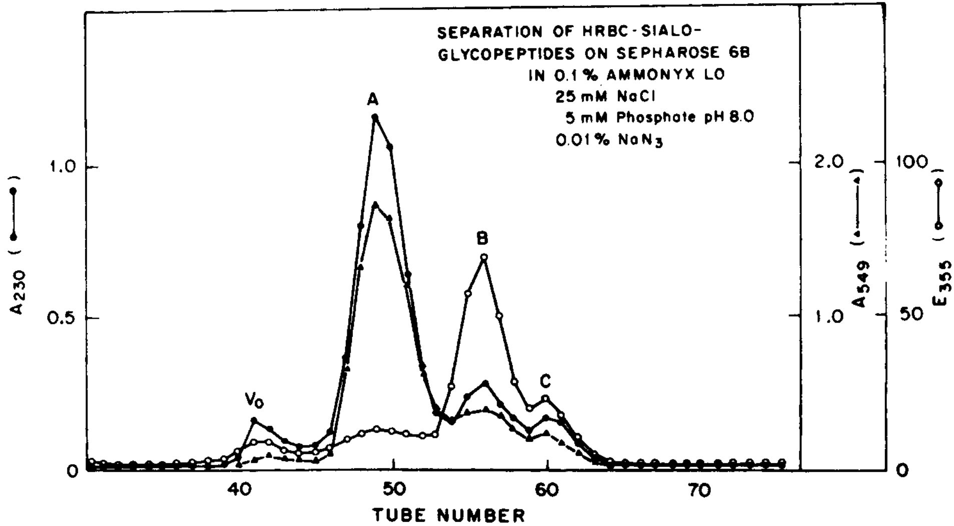

Fig. 2. Gel filtration of Biorad A 1.5 in detergent of human erythrocyte membrane sialoglycoproteins isolated by the LIS-phenol procedure. The effluent was monitored for protein (●-●-●), sialic acid (▲-▲-▲), and tryptophan fluorescence (excitation 290 nm, emission 355 nm, o-o-o). Peak A contains glycophorin A and peaks B and C, glycophorin B. Vo is the void volume. (Reprinted with permission from Biochem Biophys Res Commun 65:113–121, 1975.)

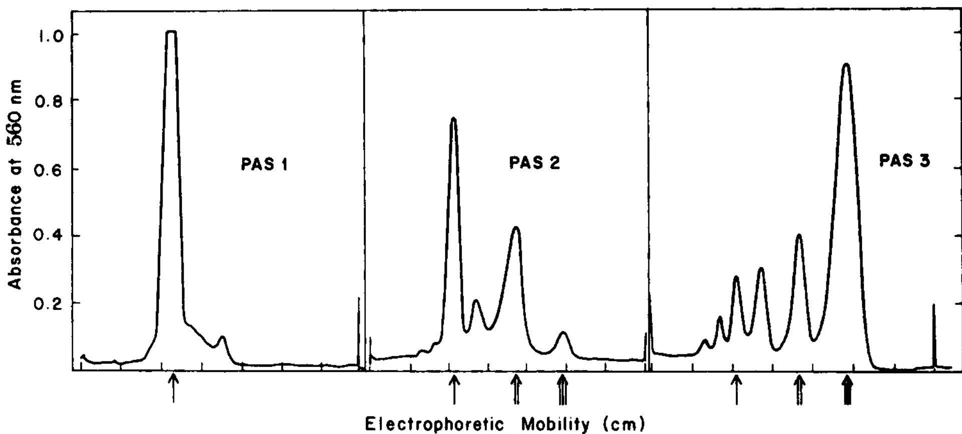

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) of sialoglycopeptides isolated from regions of the original unfixed and unstained SDS-polyacrylamide gels corresponding to PAS-1, PAS-2, and PAS-3 (cf. Fig. 1C, D). Gel slices containing individual glycopeptides were cut from the tube gels, which had been run under standard conditions, with a razor blade and were crushed into small pieces with a spatula. After extraction of the protein by incubation overnight at room temperature in excess buffer, containing 50 mM ammonium bicarbonate and 0.05% sodium dodecyl sulfate, the gel particles were removed by centrifugation. The extract was extensively dialyzed against distilled water and lyophilized. After an additional extraction with 95% ethanol at ice temperature, the protein precipitate was dissolved in water and lyophilized again. Gel electrophoresis was done on 20 to 50 µg aliquots of the isolated proteins. The gels were stained with periodic acid-Schiff's reagent and scanned at 560 nm. The arrows indicate the positions of PAS-1 (↑), PAS-2 (↑↑), and PAS-3 (↑↑↑) on a control gel run in parallel (not shown).

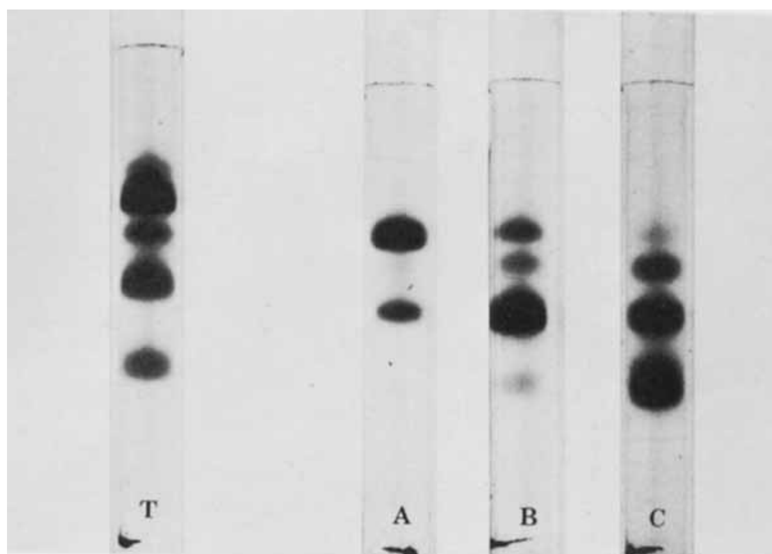

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions A, B, and C from Fig. 2, after extensive dialysis against distilled water and lyophilization. The gels were stained with periodic acid-Schiff's reagent. Gel T is obtained for the sialoglycoprotein mixture before fractionation.

bands comigrating with the PAS-1, -2, -3, -4 bands of the original unseparated mixture. To explain this complexity, it is assumed that these sialoglycopeptides have various preferred states of association in the presence of detergents (or in the membrane), which are not disrupted by weak detergents or even SDS under certain conditions.

Glycophorin A has been shown to be homogeneous (see structural studies discussed below) and yet it migrates in both the PAS-1 and PAS-2 position on SDS-polyacrylamide gels. The structural information available for glycophorin A allows us to interpret some of the complexities mentioned above and to exclude heterogeneity of the protein as a likely basis for these results.

When glycoprotein samples are heated in SDS before electrophoretic separation, conversion of the PAS-1 form into the PAS-2 form can be demonstrated (Fig. 5). This conversion is dependent on protein and detergent concentration, temperature, time of incubation, and furthermore is reversible (25–27, 52). At least for glycophorin A, the gel patterns are thus entirely dependent on a variety of conditions, which predictably will influence the amount of the low- or high-molecular-weight forms observed for any given experiment. These observations provided the basis for the idea that glycophorin A is an oligomeric molecule composed of low-molecular-weight subunits which can undergo reversible dissociation and reassociation reactions. Although the simplest explanation proposes dissociation of the dimeric form PAS-1 into monomers under these conditions, the absolute number of subunits in the undissociated complex is not known. Molecular weight estimates for glycoproteins on SDS-gels are not reliable and do not allow the determination of true molecular weights for the various products observed on the gels (28). In sedimentation equilibrium studies, however, a single molecular weight of 29,000 has been determined for the subunit of glycophorin A (29) in the presence of SDS, which correlates well with data

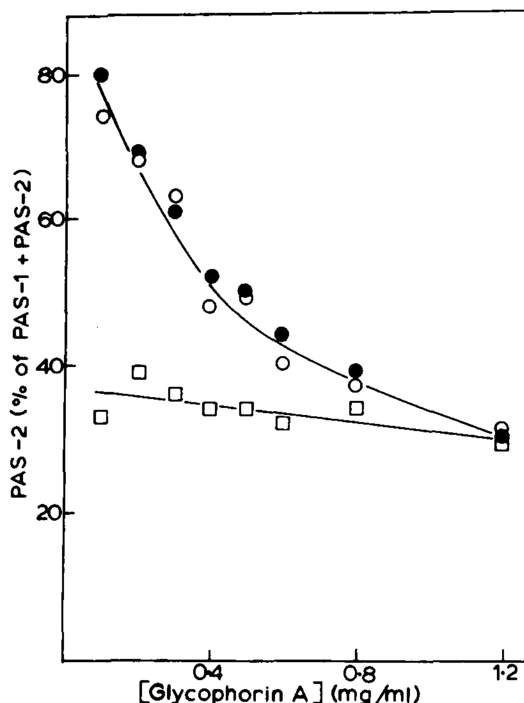

Fig. 5. Effect of incubation conditions on the distribution of glycopeptides migrating in the position of PAS-1 or PAS-2 on sodium dodecyl sulfate-polyacrylamide gels. Glycophorin A was dissolved in gel loading buffer and heated at 80°C for 15 min before electrophoresis. The gels were stained with periodic acid-Schiff's reagent, scanned at 560 nm and the peak areas determined by triangulation. The amount of PAS-2, expressed as percentage of the total PAS-1 and PAS-2, is plotted against the concentration of glycoprotein in the sample loaded onto the gel. ○) Protein diluted before heating to the concentrations shown; 50 μl of sample per gel. ●) Protein diluted before heating; 100 μl of sample per gel. □) Protein heated at 1.25 mg/ml and then diluted to the concentrations shown; 50 μl of sample per gel. (Reprinted with permission from Biochemistry 15:1448–1454, 1976. Copyright by the American Chemical Society.)

calculated from the amino acid and carbohydrate contents. The finding of a single molecular-weight species is clearly in contrast with the conclusions drawn above and the reasons for this discrepancy are not clear at present.

STRUCTURE OF GLYCOPHORIN A

The entire amino acid sequence of glycophorin A is now known and is given in Fig. 6 (30, 31). The polypeptide chain contains 131 amino acid residues and as suggested previously (32), these are arranged into 3 domains. An amino-terminal segment is the carbohydrate-containing portion rich in hydroxy amino acids, to which 15 oligosaccharide units are attached O-glycosidically and which contains one asparagine, to which a more complex carbohydrate chain is linked N-glycosidically (for a more extensive discussion of the carbohydrate structure see recent review, Ref. 2). Residues 73 through 95 do not contain any charged side chains, but have instead a high number of hydrophobic residues in addition to 3 hydroxy amino acids and most of the glycyl residues found in the poly-

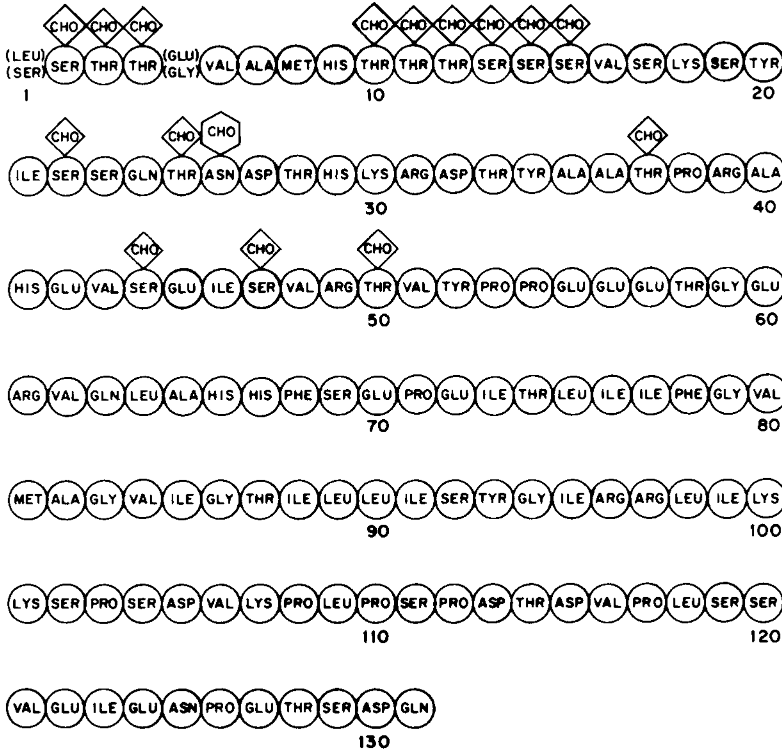

Fig. 6. Amino acid sequence of glycophorin A. Boxes above certain residues indicate attachment of oligosaccharides at these sites.

peptide chain. This region conveys properties to the protein and a tryptic fragment derived from it which made its characterization fairly difficult (31). The hydrophobic region is probably responsible for aggregation of the intact molecule in aqueous salt solutions, for its binding of high amounts of SDS, and for its association with lipids (10). The C-terminal sequence of 36 amino acid residues contains a high number of imino acids and predominantly acid amino acids.

With the exception of 2 positions of the amino acid sequence (positions 1 and 5) no evidence has been obtained suggesting additional heterogeneity of the polypeptide structure. In most of these studies pooled blood from several individuals has been used. It is conceivable that products of different genes, e.g. genes coding for the proteins which carry M or N specificities, are distinguished by a few important differences in their amino acid sequences. The heterogeneity described above could be due to such different gene products or differences could have been overlooked due to technical difficulties in the sequence determinations of multiple glycosylated peptides. Preliminary studies on peptides isolated from glycophorin A obtained from individual donors, however, do not indicate that this is the case, since no differences were seen in amino acid analysis of purified peptides. Likewise, there was no indication for heterogeneity in the nonglycosylated carboxy-terminal portion of glycophorin A (33).

In all glycophorin A preparations studied, whether from pooled or individual samples, a different type of heterogeneity however, was apparent (Fig. 7). Proteolytic cleavage using trypsin or chymotrypsin was incomplete in about one-third to one-half of the polypeptide chains at a few sites resulting in multiple peptides from regions identical in amino acid sequence, but which were different with respect to carbohydrate content (34). Cleavage at these insensitive peptide bonds in the larger peptide fragments (e.g., T1 or CH1 in Fig. 7) was only obtained after removal of terminal sialic acid residues, suggesting either heterogeneity of attachment sites of oligosaccharide chains or interaction of terminal oligosaccharide structures with the polypeptide backbone, or both. Both possibilities would have the effect of preventing cleavage by these enzymes at certain sites. It is possible that heterogeneity of this type, namely variability in the distribution as well as length of the oligosaccharide at various locations along the polypeptide backbone, determine characteristic antigenic features.

THE INTRAMEMBRANOUS SEGMENT OF GLYCOPHORIN A

Molecules which are amphiphilic, with an ionic part soluble in water and a second part repelled from water as a result of hydrophobicity, will be forced to adopt unique orientations and to adopt certain organized structures. This is the case in biological membranes which can form spontaneously and in which individual lipids are arranged in a bilayer structure. It is clear, that some of the membrane proteins interact with the hydrocarbon interior of the lipid bilayer, but the structural basis for this interaction is largely unknown.

Some membrane proteins have been found to contain a peptide or peptide region which is very much enriched in hydrophobic amino acid residues. Such hydrophobic peptides have been isolated from cytochrome b_5 (35), the coat protein of filamentous bacteriophages (36), the glycoproteins of an arborvirus (37), and glycophorin A. Amino acid sequence data are available only for some of these and even less is known about the conformation of these particular regions (38, 39). However, there are some suggestions that an α-helical structure is the preferred mode of organization of hydrophobic polypeptide regions in the apolar lipid environment (40, 41), although other arrangements have been postulated (38, 42).

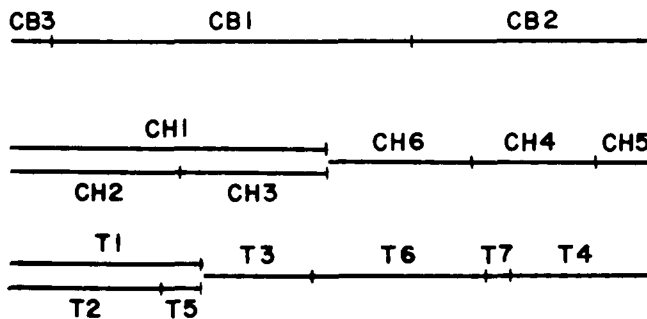

Fig. 7. Schematic representation of cyanogen bromide (CB), tryptic (T), and chymotryptic (CH) peptides of glycophorin A, drawn to scale. Total length of the polypeptide chain is 131 amino acid residues. The amino-terminal end of the molecule is to the left.

As mentioned above, glycophorin A apparently forms relatively stable complexes which can dissociate and reassociate in the presence of detergents. Two different observations suggest a role for the hydrophobic region of the glycophorin A polypeptide chain in the interactions between subunits. The first indication came from studies on the reversibility of the effect described above (26), namely that dissociated subunits can reassociate to form the original PAS-1 complex. If glycophorin A subunits can reassociate even in sodium dodecyl sulfate, then smaller peptides may inhibit complex formation of the larger subunits. Binding studies with peptides prepared from labeled glycophorin A in fact demonstrated that a tryptic peptide which contains the entire hydrophobic segment, not only binds to the smaller molecular weight form PAS-2, but also prevents reassociation of larger subunits. Binding was only observed after dissociation of the original glycophorin A complex by heat treatment, but not to the undissociated form PAS-1 (Fig. 8). Under these conditions a hybrid molecule was generated, which is composed of large, intact glycophorin A polypeptides and small hydrophobic peptides. This hybrid structure can be distinguished by SDS-gel electrophoresis from the glycophorin A complex PAS-1, but not from the dissociated form PAS-2.

In agreement with the role of the hydrophobic region for the interaction between subunits are studies on the chemical modification of glycophorin A. Alkylation with iodoacetic acid under conditions which selectively modify methionyl side chains, will result in complete conversion to the low-molecular-weight form. Modification of methionine 81 located within the hydrophobic amino acid sequence, is essential. Sodium dodecyl sulfate will prevent alkylation of this amino acid residue and thus conversion, but will not

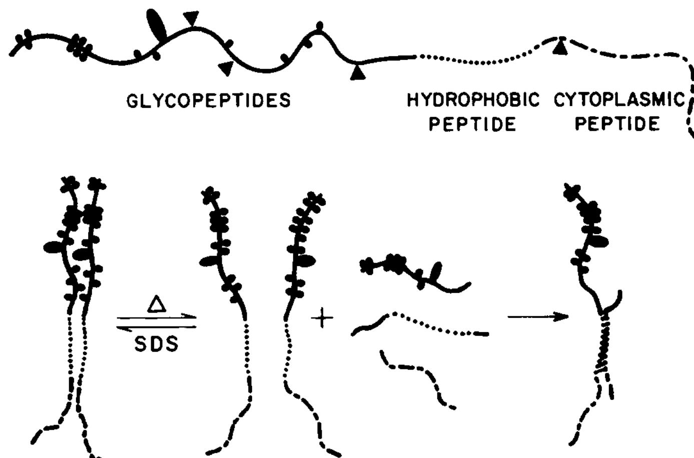

Fig. 8. Model of the glycophorin A subunit and its interaction with the hydrophobic tryptic peptide T6. The model is derived from the observation that incubation of glycophorin A together with a tryptic peptide derived from the hydrophobic segment of glycophorin A (residues 62 through 96) in sodium dodecyl sulfate solutions at 100°C generates hybrid molecules, which are composed of intact glycophorin A subunits and the short hydrophobic peptide, when analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis. Binding of the hydrophobic peptide to the glycophorin A complex (migrating in the PAS-1 position) is not observed, when incubation was done at 37°C, suggesting that dissociation of the complex is required for binding. ▲) Tryptic cleavage sites; ●) location of oligosaccharide units. (Reprinted with permission from Biochemistry 15:1137–1144, 1976. Copyright by the American Chemical Society.)

protect against alkylation of the only other methionyl group in position 8 of the amino acid sequence (Fig. 9).

More information is clearly needed to describe the interaction of this part of glycophorin A with neighboring polypeptides and with membrane lipids. If the favored conformation in the bilayer is an α-helical structure, it is conceivable that separate parts of the helix are in association with the hydrocarbon chains of fatty acids and with other subunits or proteins, and that the stability of these structures is determined to a large extent by the primary structure.

It is difficult at present to extrapolate from these experiments and to make predictions about the stability of glycophorin A complexes within the natural membrane environment. An intriguing possibility exists that glycoprotein complexes are not preformed, but are generated transiently as a result of interaction with a ligand at the exterior or interior of the cell. The other alternative that some ligands require multivalent associations with receptor complexes with each subunit contributing to binding is equally attractive.

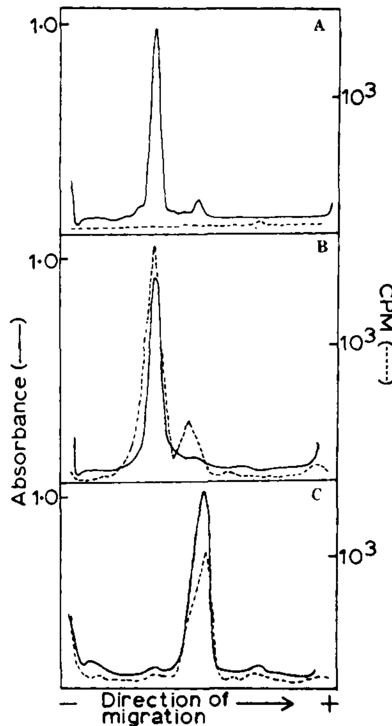

Fig. 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of unmodified (A) and carboxymethylated glycophorin A (B,C). B) Glycophorin A was carboxymethylated with [1-^{14}C] iodoacetic acid for 4 h at 37°C in the presence of 2% sodium dodecyl sulfate; C) glycophorin A was carboxymethylated as in (B) with unlabeled reagent and recarboxymethylated with [1-^{14}C] iodoacetic acid in the presence of 7 M guanidine hydrochloride. For further explanations see text. (Reprinted with permission from Biochemistry 15:1448–1454, 1976. Copyright by the American Chemical Society.)

ORIENTATION OF GLYCOPHORIN A IN THE MEMBRANE

The transmembrane orientation of the major sialoglycoprotein has been demonstrated repeatedly in many laboratories primarily on the basis of differential labeling of molecules in intact cells versus leaky ghost membranes (2, 43). Objections have been raised to this approach for various reasons. Enzyme-catalyzed iodination experiments have produced variable results, and some laboratories have failed to label the C-terminal segment of glycophorin at the cytoplasmic side of the membrane. Recently it has been possible to analyze this question in a more direct way for intact cells. Antibodies specific for a small region on the cytoplasmic portion of glycophorin A (Fig. 10) were coupled to ferritin and these conjugates were applied to frozen thin sections of fixed intact erythrocytes (44, Fig. 11). The electron-dense ferritin particles are found inside the cytoplasmic density regularly distributed throughout the circumference of the cell and at equal distances from the membrane. This distribution seems to reflect the linear mode of insertion of the peptide porition of glycophorin into the membrane. Since the interaction between glycophorin A and the membrane depends upon and is fixed by the hydrophobic region, one may expect that the C-terminal region has a similar fixed location within the cell.

VARIANT RED CELLS

The antigenic determinants of the MNSs and possibly Uu blood group systems are located on the sialoglycoproteins, which can be isolated from human erythrocyte membranes (45, 46). Although the antibody reagents and lectins, which are being used for blood typing have proven to be reliable, when applied carefully, they do not seem to provide markers specific enough for individual glycoproteins. Preliminary studies on rare

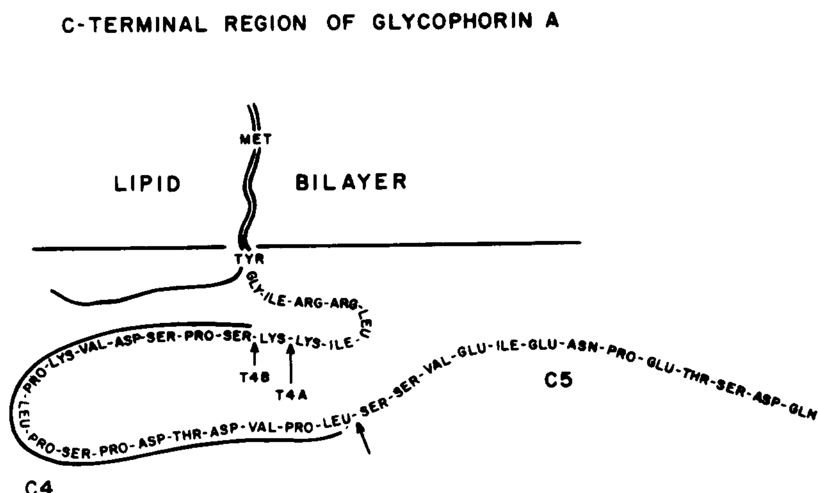

Fig. 10. The C-terminal cytoplasmic region of glycophorin A. The solid line above 17 residues within the C-terminal amino acid sequence denotes the maximum size of an antigenic determinant to which rabbit antibodies are directed. Arrows denote cleavage sites for trypsin (T) and chymotrypsin (C) to yield the fragments indicated.

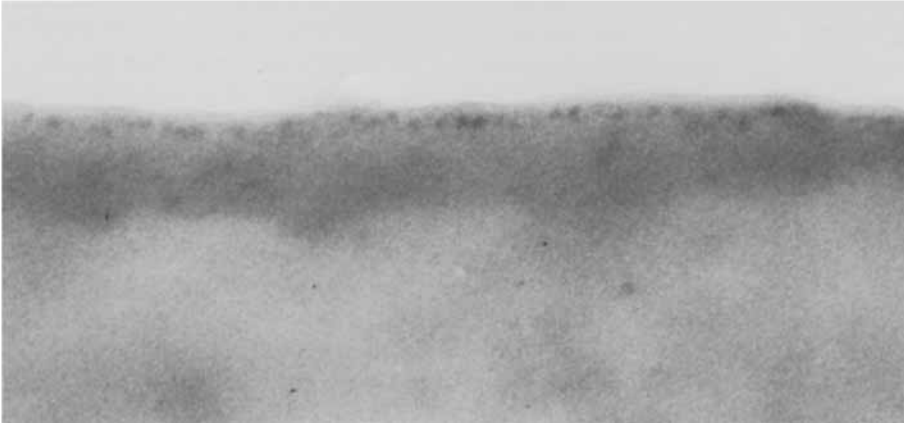

Fig. 11. Frozen thin sections of intact human red blood cells incubated with ferritin-antibody conjugates, specific for the C-terminal antigenic determinant indicated in Fig. 10 (magnification 249,900 X). Electron-dense ferritin particles are seen at the cytoplasmic side of the membrane over the cytoplasmic density, but not at the exterior of the cell.

blood types which lack some of these determinants have shown that there may be a correlation between glycophorin A and the MN active material on the one hand and glycophorin B (or PAS-3) and Ss activity on the other hand (47, 48). En (a–) individuals who do not express MN determinants, seem to completely lack glycophorin A (49–51). On the other hand, anti-N reagents such as human or rabbit antibodies or the lectin from Vicia graminea will also react with the isolated PAS-3 glycopeptides or even with N-negative cells after trypsinization (15). Individuals who do not express Ss lack this N receptor and apparently also glycophorin B (48). There are numerous other examples of rare blood types, in which the normal expression of MNSs antigens is affected. The structure of these antigenic determinants is not known, but there is evidence for participation of terminal sialic acid residues and other carbohydrates. In view of the involvement of carbohydrate structures in antigenic activity and the known changes in sialic acid content and other carbohydrates in these variant cells, it becomes essential to develop methods which allow the quantitative determination of the amount of these glycoproteins per cell. Analysis of membranes by SDS-gel electrophoresis in combination with PAS staining of the gels or cell-surface labeling may not be tools reliable enough to study the presence or absence of these membrane proteins. It would be unwise to draw too many conclusions with respect to the genetic relationships and expression of these different proteins until such data are available (15). These deletion types, if proven, are however of considerable interest and will receive attention in attempts to study the function of glycophorins in differentiation, cell maturation, and on their effect on the mature cell.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 5 R01 GM-19929, Membrane Center Grant P17-GM 21714, a grant from the American Cancer Society, ACS-BC 102C, and Anna Fuller Fund No. 470. The dedicated technical assistance of Mr. John Albert for obtaining frozen thin sections and electronmicroscopy is appreciated.

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