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Glycophorins A, B, and C: A Family of Sialoglycoproteins. Isolation and Preliminary Characterization of Trypsin Derived Peptides

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Two new sialoglycoproteins, glycophorin B and glycophorin C, were isolated from erythrocyte membranes by extraction with lithium diiodosalicylate, partition in aqueous phenol, gel filtration in detergent, and preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The two proteins were characterized by amino acid and carbohydrate analysis, separation of tryptic peptides, and isolation and purification of the amino terminal glycopeptide from each polypeptide chain. Glycophorin B is found in two forms in electrophoretograms of normal erythrocyte membranes corresponding to monomer and dimer, as has been similarly described for glycophorin A. By using antibodies to a carboxy terminal determinant of glycophorin A, and direct staining of gels with antibodies and ^{125}I-protein A from Staph. aureus, as well as by two-dimensional immunoelectrophoreis, only the two known forms of glycophorin A are detectable. The data confirm and extend the notion that the sialoglycoproteins in human red cells are dimeric molecules which are either preformed in the membrane or which can readily be generated in vitro. Only glycophorin A and glycophorin C are sensitive to trypsin while in situ in the intact red blood cells.

Key words: glycophorins A, B, and C, isolation, membrane protein, glycopeptides, erythrocyte

The sialoglycoproteins of the erythrocyte membrane have been under extensive investigation for many years in different laboratories [1–3]. First isolated and described as a myxovirus receptor substance capable of inhibiting the aggregation of human erythrocytes by influenza virus, then as a blood group substance carrying MN blood group activity, the major sialoglycoprotein became known as glycophorin A. Based on considerable structural work [4–7] it was possible to firmly establish earlier claims [8] that the protein

Abbreviations: ESR – electron spin resonance; SDS – sodium dodecyl sulfate; PAS – periodic acid-Schiff's reagent; Tos-PheCH_2Cl or TPCK – L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone; LIS – lithium diiodosalicylate; AMLO – Ammonyx-LO, N,N-dimethyl laurylamine-N-oxide; Tos-LysCH_2Cl – N-p-α-tosyllysyl chloromethyl ketone.

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spans the lipid bilayer and extends into the cytoplasm of the cell with a short carboxy terminal segment of about 35 amino acid residues [9]. As calculated from compositional data, about 80% of the total mass of the glycophorin A subunit is located at the surface of the cell, of which 75% is contributed by a large number of oligosaccharide chains, linked covalently to threonine, serine, and asparagine. It has been suggested that the protein exists in the membrane as a complex due to protein-protein interaction within the hydrophobic region of two or more subunits [10]. Despite the considerable knowledge of its structure, the functional role of glycophorin A for the cell or in membrane organization is virtually unknown. The protein in more or less pure form has been incorporated into artificial lipid bilayer systems and as a result, presumably of interaction with the bilayer, changes in electrical conductance [11] and in translocation of phospholipids across these membranes have been observed [12]. Ideas of implicating the cytoplasmic segment of glycophorin A in the organization and/or anchoring of the submembranous cytoskeleton have not found an experimental basis [3]. Yet while such experiments may well tell us about perturbation of a lipid bilayer by introducing a segment of a membrane protein into its interior, it is questionable that they describe the major functions of this heavily glycosylated protein. Based on ESR spectroscopy data of phosphate-linked, spin-labeled gangliosides and glycophorin incorporated into lipid bilayer vesicles, Sharon and Grant recently suggested that noncovalent, carbohydrate-based interactions between sialic acid bearing molecules may be responsible for the formation of a structural coat at the cell surface [13]. This coat is envisaged to carry a variety of receptors, to be highly deformable, and to allow alterations in the expression of membrane receptors.

Relatively little is yet known about the chemistry of cell surface glycoproteins, their conformation, or the relationship of glycosylated regions to the polar head groups and other structures on the membrane. This study deals with the description of two further species of sialoglycoproteins, glycophorin B and glycophorin C, of which at least one is closely related in a structural sense to glycophorin A.

MATERIALS AND METHODS

Materials

Reagent grade chemicals were used throughout. Dialysis tubing was boiled in EDTA at pH 8–9 and thoroughly rinsed in distilled water before use. The lithium salt of diiodosalicylic acid was prepared from the free acid obtained from Eastman Kodak after 2 X recrystallization from anhydrous methanol. Phenyl methane sulfonyl fluoride was purchased from Calbiochem. Sodium dodecyl sulfate was from Alcolac Chemical Corporation, Ammonyx-LO was obtained from Onyx Chemical Company. Chemicals used to prepare polyacrylamide gels were acrylamide, N,N′-methylene bisacrylamide from Biorad; ammonium persulfate from Canalco; N,N,N′N′-tetramethylethylenediamine from Eastman; Tris and urea, ultrapure, from Schwarz/Mann. Reagents for labeling were dansylchloride from Pierce and fluorescamine from Hoffman-LaRoche.

Methods

Isolation of sialoglycoprotein fractions. Membranes were prepared from human red cells as described by Furthmayr and Marchesi [10] except that for the most part outdated blood (about 20 units) was used. After lyophilization of the white ghosts, the crude glycoprotein fraction was prepared by suspension in a solution of LIS and

partition in a phenol-water mixture according to the procedure of Marchesi and Andrews [14]. After extensive dialysis at 4°C against distilled water, the water phase obtained from the two-phase mixture was lyophilized. The dry material was then suspended in ice-cold ethanol and kept stirring for 30 minutes on ice. After centrifugation for 30 minutes at 4,000 rpm in a Sorvall RC-3 centrifuge, the ethanol extraction of the precipate was repeated one more time. The final precipitate was dissolved in water and lyophilized. Glycophorin A was separated from the other sialoglycoproteins by gel filtration in AMLO on Biorad A 1.5 as described [6, 15]. The sialoglycoprotein fraction (80–100 mg) was well resolved into three peaks on a column 100 × 5 cm. After extensive dialysis against distilled water, the three proteins containing pools (AMLO-pool A, B, and C) were lyophilized.

Labeling of sialoglycoprotein fractions. Glycopeptides contained in AMLO-pools A, B, and C (see above) were labeled with dansylchloride or fluorescamine in the presence of SDS. Usually, 20 mg of glycoprotein was dissolved in 1 ml of 0.02 M sodium phosphate, pH 8.0 containing 1% SDS, and 100 μl of fluorescamine at a concentration of 20 mg/ml acetone was added slowly while stirring. After a brief incubation at room temperature, 1 ml of a buffer was added containing 20 mM Tris-HCl, pH 6.8, 4 M urea, 6% SDS, 2 mM EDTA, and the sample was applied onto a polyacrylamide slab gel as described below. Alternatively, 20 mg of glycoprotein was dissolved in 2 ml 0.02 M sodium phosphate, pH 8.0, and 1% SDS and labeled by addition of 1 ml of freshly prepared dansylchloride at a concentration of 0.5% in acetone. After incubation at 37°C for 30 minutes, acetone was added and the precipitate collected by centrifugation. The precipitate was dissolved at a concentration of 10 mg/ml of the buffer given above, mixed with unlabeled protein at a ratio of 1 part labeled to 9 parts unlabeled and was subjected to electrophoresis.

Polyacrylamide gel electrophoresis in dodecyl sulfate. Gels were prepared as described by Furthmayr and Marchesi [10] with slight modifications of the system of Fairbanks et al [16]. Preparative slab gel electrophoresis was done using the gel and buffer system described by Laemmli [17]. Electrophoretic separation was done on polyacrylamide gel slabs (17 × 15 cm or 23 × 15 cm, 5 mm thickness) originally using stacking gels and running times of about 12 hours at 50 mA with or without cooling of the plate. In later experiments the stacking gel was omitted without sacrifice of resolution in separating the various sialoglycopeptide species. Samples for electrophoresis were incubated at 37°C for 30 minutes and 2 minutes at 100°C. Protein loads per slab gel were between 20 mg and 40 mg depending on size. Electrophoresis was stopped after pyronin Y, which was included in the sample before loading, had moved 9–10 cm into the gel bed.

Isolation of labeled sialoglycopeptides from polyacrylamide gels. The labeled protein bands were visualized under UV light and the fluorescent gel strips were cut with razor blades. After thorough homogenization with a Teflon pestle, excess buffer was added to the gel particles containing 50 mM sodium bicarbonate, 0.05% SDS, 0.02% sodium azide, and the protein was extracted by shaking at 37°C overnight. Gel particles were removed by filtration on nylon filters and washed by several additions of water. After dialysis against water, the solution was centrifuged for 30 minutes at 5,000 rpm in a Sorvall RC-3 centrifuge and then lyophilized. Since it was found difficult to remove excess glycine and acrylamide polymers by dialysis, the lyophilized material was subjected to column chromatography on LKB Ultrogel AcA 54 in 0.05% SDS, 5 mM sodium phosphate, pH 8.0 (column dimensions 2.5 × 90 cm). The fractions containing protein were pooled, dialyzed against distilled water, and lyophilized. To remove residual SDS, the lyophilized material was extracted twice with acetone, solubilized in distilled water, and lyophilized.

Proteolytic digestion. Sialoglycoproteins were dissolved at a concentration of 10

mg/ml in 50 mM Tris-HCl, pH 8.0 and after addition of sufficient trypsin (TPCK-treated, Worthington) in 0.001 N HCl, 1 mM $CaCl_2$ to give an enzyme:substrate ratio of 1:30, the sample was incubated at 37°C for 20 hours. Digestion was stopped by acidifying the solution with glacial acetic acid to pH 4.5. A precipitate formed during this step was removed by centrifugation and was found to contain hydrophobic peptide material [cf 7]. The soluble peptides were separated by column chromatography on LKB Ultrogel AcA 54, equilibrated in 50 mM ammonium acetate, pH 6.1, 0.02% sodium azide (2.5 × 150 cm or 1.5 × 182 cm). Column effluents were monitored continuously at 230 nm. Appropriately pooled fractions were concentrated by evaporation and desalted on Biogel P2 columns equilibrated with 0.05 N acetic acid. After lyophilization, some of the peptides were rechromatographed by ion-exchange chromatography on DEAE-cellulose (Whatman DE52), and equilibrated with 50 mM sodium formate, pH 6.1. The peptides were eluted from the column (1.5 × 15 cm) by using a linear gradient from 0 to 0.3 M sodium chloride over a total volume of 400 ml.

Digestion of intact red blood cells with trypsin. Freshly drawn blood was washed several times with excess isotonic phosphate buffered saline, pH 7.2. To 400 ml of packed cells was added 400 ml of 0.2 M sodium phosphate buffer, pH 8.0, containing 0.2 M NaCl as described by Jackson et al [34]. The cells were divided into two parts, and trypsin hydrolysis was initiated by addition of 30 mg and 200 mg of Tos-PheCH_2Cl-treated trypsin (Worthington), respectively, and the solutions were shaken gently at 37° for 110 minutes. After this incubation period, the trypsin was inactivated by the addition of a 10-fold molar excess of Tos-LysCH_2Cl. The supernate was recovered by centrifugation at 3,000g for 20 minutes. The red cells were washed one more time with phosphate-buffered saline, pH 7.2. Both supernatant and wash were combined and the same volume of 50% phenol was added. After stirring at ice-temperature for 20 minutes, the solution was centrifuged at 5,000 rpm for 1 hour in a Sorvall RC-3 centrifuge. The water phase was extensively dialyzed against distilled water at 4°C and finally lyophilized.

The lyophilized material was then subjected to gel filtration on Sephadex G150 superfine as described above, and the glycopeptides were finally purified by DEAE-cellulose chromatography as described.

Analytical data. Amino acid and carbohydrate analysis was performed as described previously [4, 18]. Norleucine and inositol, respectively, served as internal standards.

Two-dimensional immunoelectrophoresis. Total membrane proteins from freshly prepared human erythrocyte membranes and the isolated crude sialoglycoprotein mixture were analyzed by the technique described by Converse and Papermaster [19]. The protein loads for electrophoresis in the first dimension were 12.5 μg of membrane proteins and 0.5 μg of the isolated glycoprotein mixture. The concentration of the antiserum in the agarose layer was 6%. For a description of the antiserum see text and [9].

Labeling of SDS-polyacrylamide gels with specific antibodies [20]. Erythrocyte membrane proteins or the isolated sialoglycoprotein mixture were separated by SDS-polyacrylamide gel electrophoresis in tube gels, by using the gel and buffer system described by Laemmli [17]. The gels were fixed for 12 hours in 50% methanol, 8% acetic acid, and then rinsed extensively with several changes of buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.02% sodium azide, for a period of 12 hours. The gels were then transferred to small test tubes and covered with 3 ml of a 1:50 dilution of antiserum to a C-terminal determinant of glycophorin A [9] or rabbit normal serum, and after a 12-hour incubation nonbound protein was removed by several changes over a 12-hour period of the above buffer. The last step of the procedure consisted of a 12-hour incubation

with ^{125}I-labeled Protein A (Staph. aureus, Pharmacia), which had been diluted to $5 \times 10^5 - 10^6$ cpm/ml, and extensive washing of the gels with the buffer given above. The gels were sliced into 1-mm slices with a homemade gel slicer and counted in a Beckman Biogamma Counter.

Iodination of protein A [20]. To 50 μg of the protein in 10 μl 0.05 M sodium phosphate buffer, pH 7.5, were added 10 μl 0.2 M sodium phosphate, pH 7.5, 200 μCi in 10–20 μl of ^{125}I-Na (New England Nuclear, Carrier free) and 25 μg Chloramine T in 10 μl 0.05 M sodium phosphate, pH 7.5. After agitation for no longer than 1 minute at room temperature, 100 μl of 8 mM tyrosine was added to quench the reaction. Iodinated protein A was separated from free iodine and tyrosine by gel filtration on Sephadex G25 (1×20 cm) equilibrated in 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.02 NaN_3. The iodinated protein was stored at 4°C after 0.25% gelatin or 0.1% ovalbumin (Sigma) had been added.

Radioimmunoassay. The assays used have been described recently and employed the same antisera and reagents [9, 18].

RESULTS

Localization of Glycoprotein A Polypeptides on SDS-Polyacrylamide Gels

From previous work it has been concluded that glycophorin A polypeptides on SDS-polyacrylamide gels occupy two positions, the low-molecular-weight form being about half the size of the high-molecular-weight form [10, 21, 22]. Since these two electrophoretic forms were found to be interconvertible [10] and the high-molecular-weight form could be completely converted to the low-molecular-weight form by chemical modification [22] or by lowering the protein concentration [10], it has become clear that glycophorin, at least under the conditions of SDS-gel electrophoresis or gel filtration in SDS, is present as a complex. The data were consistent with the idea that a dimeric complex was dissociable into monomeric subunits. However, it has been impossible thus far to obtain reliable absolute molecular weights for these glycoconjugates by these techniques. Although no other PAS-stainable bands of molecular weight lower than the presumptive monomer have been observed, it is not clear how many subunits the glycophorin A complex contains.

Glycophorin A amounts to 1.6% of the total membrane protein as determined by radioimmunoassay (Fig. 1). We have used antibodies to a C-terminal protein determinant of glycophorin A in order to locate glycophorin A polypeptides on SDS-gels and with the hope to detect with this rather sensitive tool small amounts of glycophorin A subunits. In two-dimensional immuno-electrophoresis only two precipitin arcs are observed corresponding to the two known positions occupied by glycophorin A on the gel (Fig.2). The total amount of glycophorin A seen here is 200 ng. Since the formation of a visible antigen-antibody complex is dependent on optimal concentrations of both antigen and antibody, this technique may have missed additional products. Despite our attempts to cover a range of different concentrations, no additional precipitin arcs were observed.

The second technique we have used, namely direct staining of glycophorin A with antibodies on the gel, does circumvent this problem. In order to do the experiment, the gels have to be fixed to retain the protein during the somewhat lengthy procedure. The C-terminal antigenic determinant on glycophorin A is still reactive despite treatment with SDS and fixation with methanol-acetic acid and two radioactively labeled bands are seen

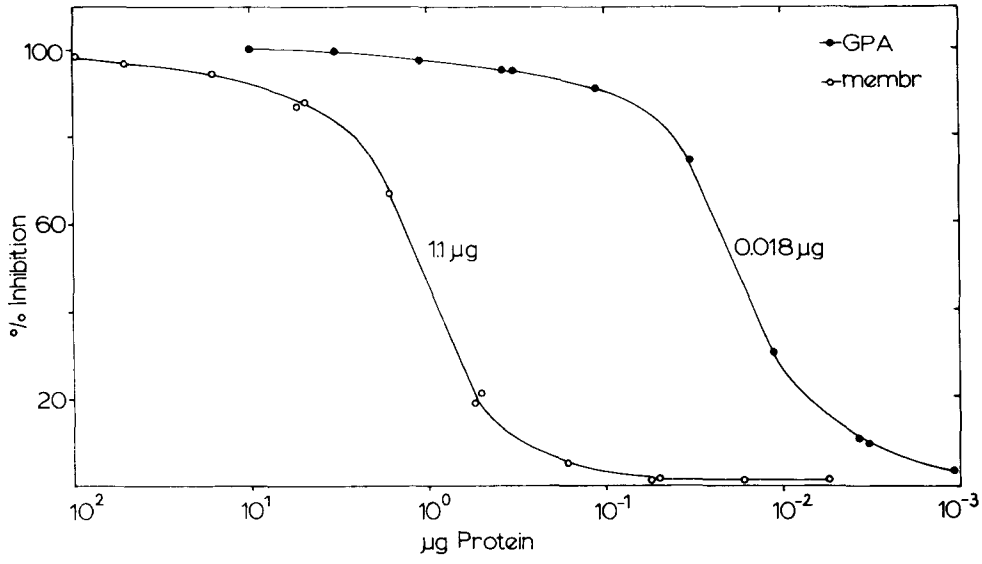

Fig. 1. Quantitation of glycophorin A in normal human erythrocytes by radioimmunoassay. Erythrocyte membranes (–○–) and isolated glycophorin A (–●–) were used as competitive inhibitors in the radioimmunoassay as described elsewhere [9, 18]. Inhibitor concentrations were determined by amino acid analysis and Lowry using norleucine and bovine serum albumin, respectively, as internal standards.

corresponding to the two positions known to contain glycophorin A (Fig. 3B). By varying the protein load on the gel, the amount of radioactive protein A bound to these regions was found to be nonlinear (data not shown) and starts to reach a plateau at a certain protein concentration. Since excess of antibody and ^{125}I-labeled protein A is used, the limitation seems to come from the fact that the protein has been fixed and that the rather large ligands do not penetrate sufficiently into the protein bands. The technique thus appears to be relatively more sensitive for smaller amounts of antigen in the gel. When membrane samples were heated at 100°C for 3 minutes at concentrations of 1 mg membrane protein per ml and loaded onto gels, 200 ng of glycophorin A was easily detectable. Heat treatment causes a considerable change in the amount of radioactivity found in the two positions on the gel, but apparently does not dissociate the low-molecular-weight form of glycophorin A further. If this form were still a dimer, it cannot be further reduced in size by simple manipulation. This conclusion is not invalidated by the fact that the technique of detection is not strictly quantitative.

Isolation of Glycophorin B and Glycophorin C

Several laboratories have shown that human red cell membrane proteins give a much more complex pattern in the SDS-gel system described by Laemmli [23, 24]. The sialoglycoprotein bands in particular are distinctly different from previously observed analysis. After isolation of the sialoglycoprotein fraction by the LIS-phenol method [14], the PAS-stainable pattern of this fraction on SDS-polyacrylamide gels was nearly identical to that of the original membrane protein mixture (Fig. 4T). The relative staining intensities of

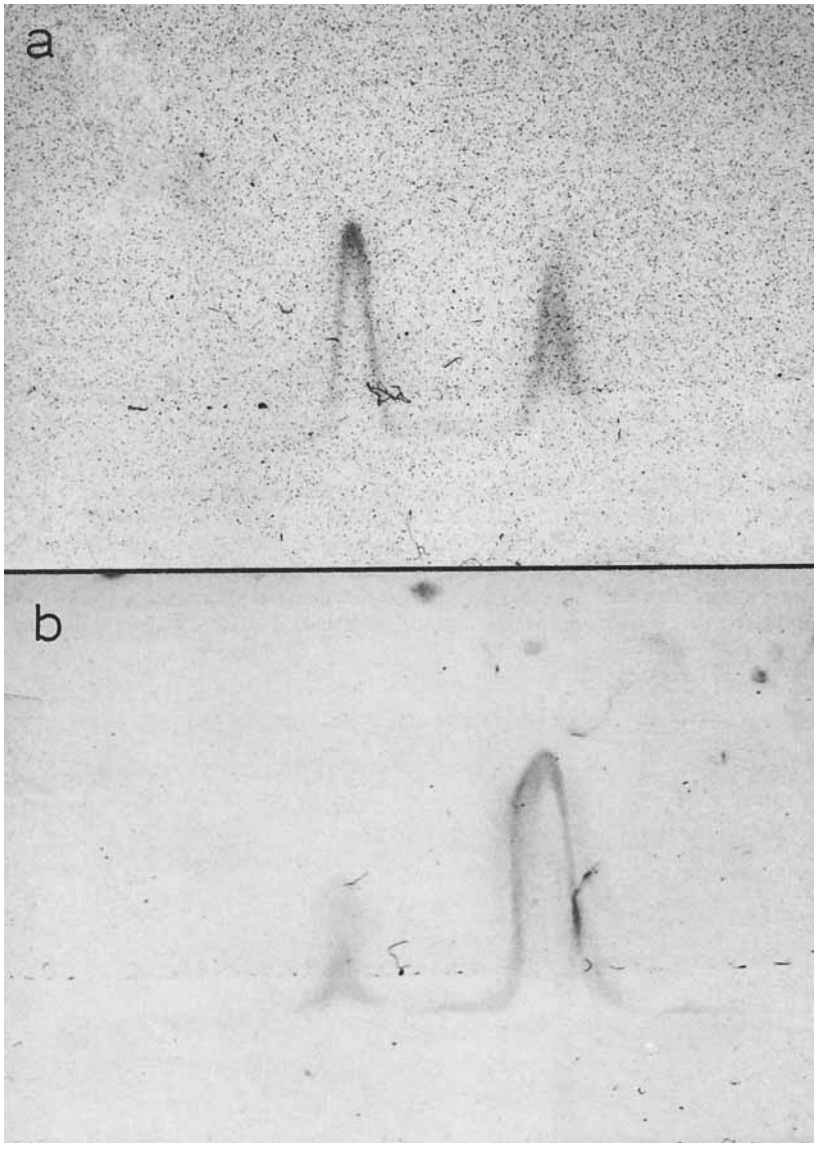

Fig. 2. Two-dimensional immunoelectrophoresis of human erythrocyte membranes. The antiserum was developed in rabbit against a fragment of glycophorin A and contains antibodies specific for a carboxy terminal protein determinant [9]. Red cell ghosts were dissolved in sample buffer and incubated for 30 minutes at 37°C (a) and for additional 3 minutes at 100°C (b) before loading on an SDS-polyacrylamide slab gel for separation in the first dimension (from left to right). Electrophoresis in the second dimension (from bottom to top) of the proteins into the antibody containing layer shows precipitin arcs formed at the two position where glycophorin A is found on gels in the first dimension.

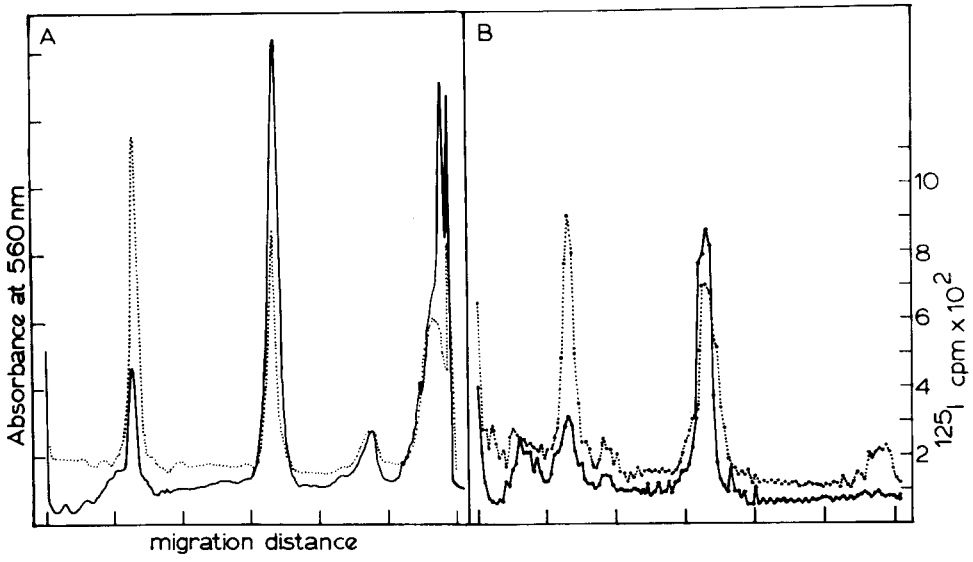

Fig. 3. Labeling of glycophorin A polypeptides on SDS-polyacrylamide gels with specific antibodies. 10-μl samples of erythrocyte membranes dissolved in SDS-loading buffer and containing approximately 25 μg protein were incubated at 37°C for 30 minutes (dotted line) and an additional 3 minutes at 100°C (solid line) before being subjected to electrophoretic separation on Laemmli tube gels. The gels in A were stained with PAS reagent and scanned at 560 nm. The absorbance is given in arbitrary units. In B the gels were treated with antiserum and ^{125}I-labeled protein A, as described in the Methods section, sliced and counted.

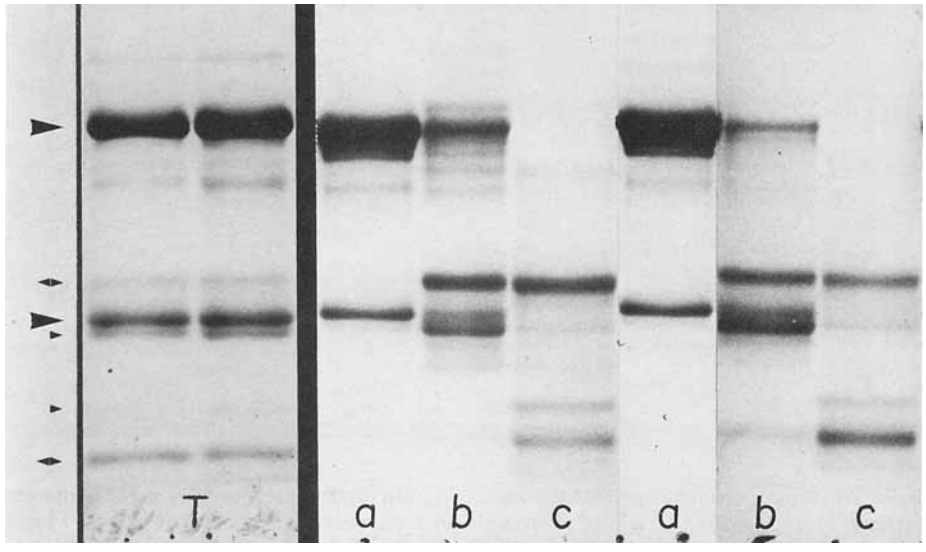

Fig. 4. SDS-polyacrylamide gel electrophoresis of sialoglycoprotein fractions obtained by gel filtration in the detergent Ammonyx-LO (cf [6, 15]). The gel system was as described by Laemmli [17] and about 50 μg of each sialoglycoprotein mixture was loaded. The gel was stained with PAS reagent. T designates two different sialoglycoprotein samples before fractionation; abc correspond to protein pools A, B, and C (in [6, 15]) from two separate gel filtration experiments.

TABLE I. Distribution of Glycophorins on SDS-Polyacrylamide Gels

Glycophorin	Band	%
A	1	51
	2, 2A	12
B	3	6
A	4	17
C	5	6
C	6	1
B	7	7

some of the bands changes to some extent in comparison to the membrane protein profile and a small amount of material of higher molecular weight is observed (Table I, cf [23].

It was shown previously that the major sialoglycoprotein species, glycophorin A, can be isolated in pure form and separated from other glycopeptide components by gel filtration in nonionic and other detergents [15]. When the glycopeptide pools A, B, and C, obtained by gel filtration on Biorad A 1.5 in the detergent Ammonyx-LO was analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli, the patterns shown in Figure 4a,b,c are obtained (for a more detailed description of the chromatogram see [6] and [15]). The first and major protein peak eluting from the column contains glycophorin A, seen as two bands on the gel indicated by large arrowheads. Small amounts of other bands are seen contaminating the preparation. This contamination can be avoided by pooling fractions of the major peak without including the fractions from the trailing shoulder which is frequently observed and presumably is caused by aggregation of the minor glycoproteins. The second peak eluting from the column which originally has been described to contain tryptophan as measured by its fluorescence [15], gives the peptide pattern shown in Figure 4b. Most of the peptide material indicated by the small arrowhead in the original sialoglycopeptide mixture is found in this fraction in addition to the high-molecular-weight form of peptides indicated by the double arrowhead. AMLO-pool C from the column contains mostly peptides indicated by double arrowheads in addition to small amounts of lower-molecular-weight peptides. As can be seen from the figure, the amount of individual peptides found in these positions varies from preparation to preparation.

The preparative step of gel filtration in detergents to remove glycophorin A and to enrich in a relatively efficient way for the minor components made it feasible to isolate adequate amounts of these polypeptides for chemical characterization. Conventional separation techniques like gel filtration in SDS or ion-exchange chromatography in nonionic detergents failed to further separate these glycoproteins and to produce proteins homogeneous as judged by SDS-electrophoresis. Since only the Laemmli gel system seems to distinguish these minor sialoglycoproteins we decided to isolate the proteins from polyacrylamide slab gels. In order to visualize the peptides, part of the AMLO-pool B and C material was chemically modified by dansylation; and after mixing with unlabeled protein and electrophoresis, fluorescent bands were cut from regions of slab gels as indicated in Figure 5. Although traces of fluorescent bands at other positions were observed for both fractions (cf Fig. 4 b,c), we made no attempt to recover these minute amounts. Re-electrophoresis of the isolated sialoglycopeptides demonstrated their remarkable tendency to aggregate and to form oligomers (Fig. 6). As judged from these gel patterns, peptides

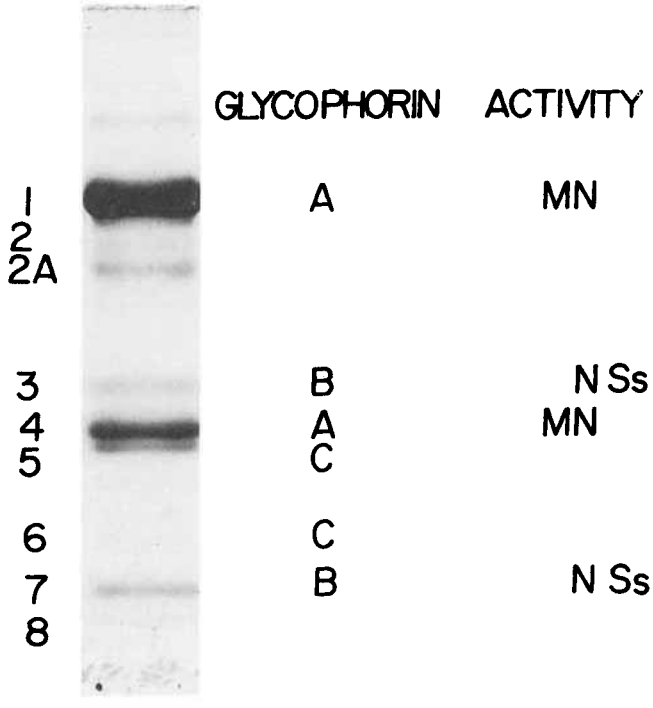

Fig. 5. Dansylated sialoglycoprotein mixture on SDS-polyacrylamide gels after staining with PAS reagent. The fluorescent profile (not shown) as identical to the stained gel shown here. The numbers on the left indicate positions 1–8 on the gel, from which fluorescent labeled proteins were isolated (cf Fig. 6). A, B, and C designate the glycophorin species, and on the far right, serological activities associated with glycophorin A and B are indicated.

isolated from regions 3–7 appear homogeneous by forming oligomers at distinct positions while those from regions 1 and 2 are mixtures of all the components from the original mixture. Although the data are shown only for AMLO-pool B material, identical findings were obtained for the pool C polypeptides. The original gel filtration in detergent thus appears to separate complexes of different size and does not distinguish between different molecules. Since the same polypeptides are found in both peaks, apparently these molecules can be incorporated into an aggregate of variable composition, which is either preformed in the membrane or is generated during the isolation technique. It is not clear if such sialoglycoprotein complexes contain only one species of protein.

Chemical Characterization of Glycophorin B and Glycophorin C

Amino acid analysis of the peptides isolated from the gels indicates that three proteins account for the complex electrophoresis pattern. We have named these proteins glycophorin A, B, and C (Table II). Glycophorin A is found, as expected, in positions 1 and 4, glycophorin B in 3 and 7, and glycophorin C was identified in positions 5 and 6 (cf Fig. 5). The three proteins lack cysteine, but are distinguished by considerable differences particularly in the values for aspartic acid, proline, glycine, alanine, valine, and isoleucine. Glycophorin C contains tryptophan, which is absent in the other two proteins, and glycophorin B lacks N-acetyl glucosamine and mannose (see below), markers for the com-

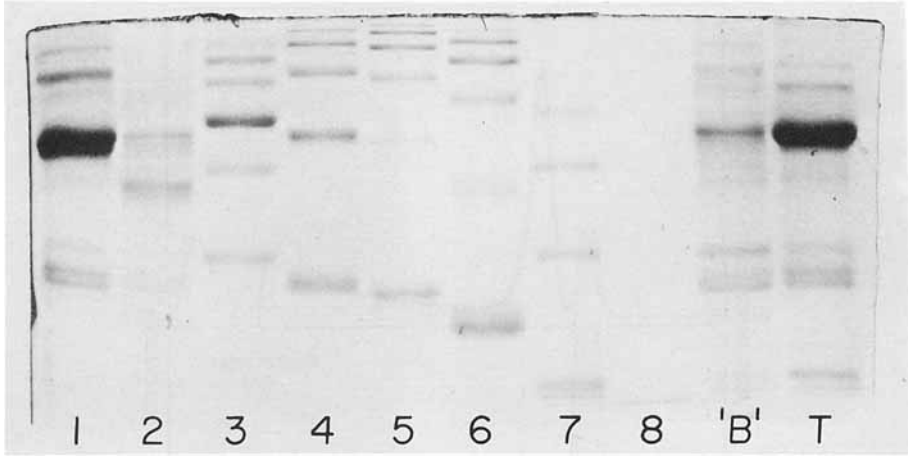

Fig. 6. Re-electrophoresis of sialoglycopeptides isolated from positions 1–8 (cf Fig. 5) of AMLO-pool B peptides ("B," of Fig. 4b), T indicates total sialoglycoprotein mixture and is used as an internal marker. The gel system was that of Laemmli and staining was done with the PAS reagent.

TABLE II. Amino Acid Composition of Human Erythrocyte Membrane Glycophorins*

	Glycophorin A			Glycophorin B	Glycophorin C
	mole/mole				
	Expected	Found	Mole %	Mole %	Mole %
Asp	8	8.9	6.8	3.9	8.9
Thr[a]	15	14.5	11.1	14.5	10.6
Ser	18.5	17	13.0	13.8	10.5
Glu	14.5	15.6	11.9	9.4	9.7
Pro	10	10	7.7	4.0	7.9
Gly	5.5	5.5	4.2	5.2	9.2
Ala	6	6.7	5.1	7.3	11.5
Cys	–[b]		–	–	–
Val	11	11.3	8.6	7.2	2.8
Met[c]	2	1.3	1.0	1.8	3
Ile	11	9.8	7.5	8.0	3.3
Leu	7.5	7.7	5.9	8.0	5.5
Tyr	4	3.8	2.9	3.1	3
Phe	2	2.1	1.6	2.1	2.3
His	5	4.6	3.5	3.3	3.2
Lys	5	5.1	3.9	3.3	2.9
Arg	6	6	4.6	5.1	4.6
Trp[d]		–		–	+
GalNAc[e]			+	+	+
GlcNAc[e]			+	–	+

*Amino acid analysis was done on a Durrum D500 analyzer. The values were calculated from duplicate analyses obtained for two preparations.

[a]Values for Thr and Ser are corrected for losses during hydrolysis by extrapolation to zero time after 24, 36, and 48 hours hydrolysis.

[b]Dash denotes 0.1 or less.

[c]Uncorrected values for Met.

[d]Determined spectrofluorometrically.

[e]Determined only semiquantitatively after acid hydrolysis on the amino acid analyzer.

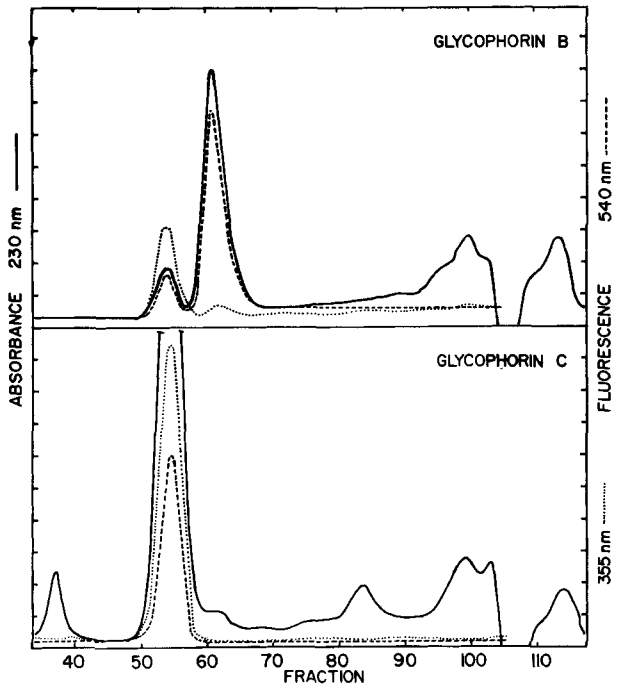

Fig. 7. Separation of soluble tryptic peptides of glycophorin B and glycophorin C by gel filtration on Aca 54. The column dimensions were 2.5 × 150 cm, flow rate 35 ml/hour, fraction volume 7 ml. ——) Absorbance at 230 nm in arbitrary units; ·····) tryptophan fluorescence (excitation 290 nm, emission 355 nm); - - - - -) dansyl chloride fluorescence (excitation 380 nm, emission 540 nm).

plex oligosaccharide linked N-glycosidically to an asparaginyl residue in glycophorin A and probably glycophorin C.

To confirm these findings the proteins isolated from the eight positions indicated in Figure 5 were treated with trypsin and after removal of insoluble peptide material, generated as a result of proteolysis, the soluble peptides were applied to a column of AcA 54. The elution of peptide material was monitored at 230 nm and by fluorescence measurements. In Figure 7 representative chromatograms for glycophorin B and C are given. Both proteins contain one major peptide, which carries the dansylchloride label and thus is derived from the amino terminal end of each of the polypeptide chains. The peptide in glycophorin C contains tryptophan as an additional marker, which is absent from the major peptide in glycophorin B. The small amount of peptide material in glycophorin B also containing tryptophan is present in nonstoichiometric amounts, elutes in the same position as the glycophorin C glycopeptide, and presumably is derived from contaminating material. These major peptides elute in exactly the same positions as the two amino terminal peptide products T1 and T2 from glycophorin A [5, 6, 15, 18], but they can be distinguished by ion-exchange chromatography on DEAE-cellulose [5]. Rechromatography of the amino terminal glycopeptides of glycophorin B and C on DEAE-cellulose gave uniform peaks (not shown), and the purity of the peptides was confirmed by amino acid analysis, by amino terminal analysis, and partial amino acid sequence studies [18 and unpublished].

TABLE III. Amino Acid and Carbohydrate Analysis of N-Terminal Peptides of Glycophorin A, B and Glycophorin C*

	A^{MN}-T1[a]	A^{M}-T1	A^{N}-T1	A^{MN}-T2	A^{M}-T2	A^{N}-T2	B^{MN}-T2	C^{MN}-T1[b]
Asp	3 (3)	2.9 (3)	3 (3)	2.2 (2)	2 (2)	2 (2)	1.3 (1)	3.1
Thr[c]	9 (9)	9 (9)	9 (9)	7.3 (7)	7 (7)	7.1 (7)	7.2 (7)	5.3
Ser	8.7 (8.5)	8.7 (9)	7.7 (8)	8.5 (8.5)	8.5 (9)	7.9 (8)	8.3 (8)	5
Glu	1.7 (1.5)	1.1 (1)	2.3 (2)	1.7 (1.5)	1.2 (1)	2.3 (2)	4 (4)	1.9
Pro	.9 (1)	.8 (1)	1 (1)	– (-)	–	–	.2 (-)	4.8
Gly	.6 (.5)	1.1 (1)	– (-)	.6 (.5)	1 (1)	–	2.2 (2)	2.7
Ala	2.9 (3)	2.7 (3)	2.9 (3)	1.2 (1)	1.1 (1)	1.1 (1)	1.2 (1)	3.2
Cys	–[d]	–	–	–	– (-)	–	– (-)	–
Val	2.2 (2)	2.1 (2)	2.1 (2)	2 (2)	2 (2)	2.1 (2)	2.7 (3)	–
Met[e]	.8 (1)	.9 (1)	.7 (1)	.4 (1)	.4 (1)	.4 (1)	.6 (1)	.6
Ile	1 (1)	1 (1)	1 (1)	1 (1)	.9 (1)	1 (1)	.9 (1)	1
Leu	.5 (.5)	– (-)	1.1 (1)	.6 (.5)	– (-)	1.1 (1)	2 (2)	1.4
Tyr	1.7 (2)	2 (2)	2 (2)	.8 (1)	.7 (1)	.8 (1)	.6 (1)	–
Phe	–	–	– (-)	–	– (-)	– (-)	– (-)	–
His	2.1 (2)	2 (2)	2.1 (2)	2 (2)	2 (2)	2 (2)	1.7 (2)	1
Lys	2.1 (2)	2 (2)	2.1 (2)	2 (2)	2 (2)	1.9 (2)	1.2 (1)	–
Arg	1.7 (2)	1.6 (2)	1.8 (2)	.5 (-/1)	.5 (-/1)	.6 (-/1)	.7 (1)	1.2
Total	39	39	39	30	30	30	35	
Carbohydrate composition[f]								
Fuc	.41	.5	.55	.4	.6	.2	–	.7
Man	2.7	2.8	3	3.2	2.8	2.6	–	2.4
Gal	13.5	12.3	12.4	11.1	11.9	10.5	10.7	10.6
GalNAc	10	9.8	10.1	7.9	8.3	7.6	13.8	8.1
GlcNAc	6.9	6.8	6.8	6.3	5.4	5.1	–	5.4
NANA	12.7	13	12	10.7	9.8	10	15.8	11.2

*Amino acid analysis was done on a Durrum D500 analyzer, using Nle as an internal standard. The values are given in moles/mole peptide with expected values in brackets.

[a]Superscripts M, N, and MN indicate the MN blood group type of the red cells, from which the glycophorin molecules were isolated.

[b]Contains tryptophan which was determined spectrofluorometrically only. The exact size of the peptide has not been determined.

[c]Values for Thr and Ser are corrected for losses during hydrolysis by extrapolation to zero time after 24, 36, and 48 hours hydrolysis.

[d]Dash denotes 0.1 or less

[e]Uncorrected values for Met.

[f]Moles/mole peptide. Carbohydrates were determined as trimethylsilyl derivatives of methyl glycosides by gas-liquid chromatography, on a Hewlett-Packard 5110 B gas chromatograph, using inositol as an internal standard [27].

Yields were obtained only for the purified peptides from glycophorin B and C and were in the order of 1 μmole from 20 units of blood. The amino acid and carbohydrate composition of the peptides derived from glycophorin B and glycophorin C (B^{MN}T2 and C^{MN}T1) is given in Table III in addition to the amino terminal peptides derived from glycophorin A^{M}, A^{N}, and A^{MN} [18]. Because of their chromatographic behavior, characteristic amino acid and carbohydrate composition these peptides thus provide reliable markers for the presence of the type of glycophorin in any given position on the SDS-gels. Isolation of these glycopeptides by the methods outlined above, confirm the scheme presented in Figure 5, namely that both, glycophorin B and C, occur in two positions on SDS-polyacryla-

mide gels. Incomplete cleavage was observed only for glycophorin C isolated from position 6 and may be due to modification of lysyl groups by dansylchloride.

Although the chromatograms of the tryptic digests of glycophorin B and C contain at least one other soluble peptide in stoichiometric amounts of low molecular weight which has not been further purified yet, the peptide patterns are strikingly different from that obtained for glycophorin A [5, 15, 18]. The abscence of peptides in the 3,000–4,000 dalton range may suggest that both glycophorin B and C are yet smaller than glycophorin A and do not contain an extended cytoplasmic segment.

Release of Sialoglycopeptides From Intact Human Erythrocytes With Trypsin

To determine which of the three glycophorins were accessible and susceptible to protease while in situ, intact red cells were treated with high concentrations of trypsin to release glycopeptides. Two different concentrations of enzyme were used at enzyme: substrate ratios of 1:20 and 1:3 when calculated on the basis of total membrane proteins of the red cells. The relative concentration of the enzyme is much higher, however, since only a fraction of the membrane proteins is accessible. The major sialoglycopeptides of glycophorin A partition into the water phase of a phenol-water mixture [Furthmayr, unpublished]. When the supernatant fluids after digestion of the intact cells and after phenol-partitioning were analyzed for glycopeptides by gel filtration, only two peptide peaks are obtained for both digests corresponding to the elution position of peptides T1 and T2 of glycophorin A [18]. Chromatography of the peptides from the first peak separated two peptides (T1A and T1B), as shown by Tomita et al [5]. The second peak was found homogeneous in this system. The amino acid composition of these three fragments was identical to the composition of peptides T1 and T2 of glycophorin A and peptide T1 of glycophorin C. These results indicate that even at high protease concentrations only glycophorin A and glycophorin C are sensitive and accessible to trypsin to release amino terminal peptides.

DISCUSSION

We have shown in this study that human erythrocyte membranes contain at least three different sialoglycoproteins, for which we propose the names glycophorin A, B, and C. Glycophorin A amounts to 1.6% of the total membrane protein as determined by radioimmunoassay. By using the figures on the relative abundance of red cell membrane proteins given by Fairbanks et al [16], the number of polypeptides per ghost is 420,000, corresponding to about 200,000 dimers of glycophorin A. Since glycophorin B and C contain similar amounts of sialic acid, the number of polypeptide copies per cell can be estimated as 70,000 and 35,000, respectively. There are possibly other glycoprotein species present in the crude sialoglycoprotein fraction, but the minute quantities of these peptides seen by PAS-stain or after dansylation made isolation unwarranted at this time. Admittedly, these minor components could be variants of the known proteins, which are separated in the gel system because of heterogeneity in carbohydrate structure. Small amounts of glycophorin C are found mainly in AMLO-pool C and migrate differently from the bulk of the glycophorin C polypeptides on SDS-gels for reasons which are unexplained at present. This component does not appear to be a subunit of a higher-molecular-weight complex of glycophorin C, since the electrophoretic mobilities of its oligomeric form differed from glycophorin C. On the other hand, glycophorin A and B apparently can readily undergo associations to form oligomers. These aggregates obviously are artifacts of the isolation

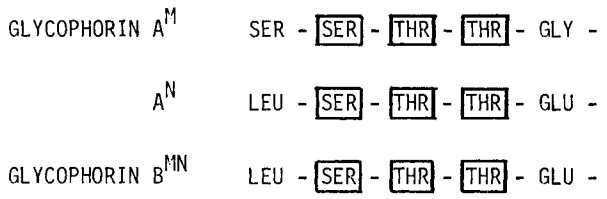

Fig. 8. Amino terminal sequences of glycophorin A^M, A^N and glycophorin B^{MN}. Superscripts M, N, and MN indicate the MN blood group type of the red cells, from which the glycophorin molecules were isolated. Residues in boxes indicate sites of glycosylation.

since they are not seen in membrane samples to the same extent. In fresh ghosts glycophorin A and B are always found in two electrophoretic forms which possibly correspond to monomers and dimers. With the exception of the high-molecular-weight form of glycophorin A, these complexes are stable in SDS and cannot be dissociated. Isolation of the sialoglycoprotein fraction by the method used causes some rearrangement, since higher amounts of glycophorin B dimers are found as compared to the ghost in addition to small amounts of aggregates of mixed composition.

Previous attempts to isolate the minor glycoproteins have dealt with impure preparations due to lack of separation on SDS-gels [15, 25]. Analytical data on amino acid and carbohydrate composition were significantly different from the results presented here. The compositional data on glycophorin A isolated from SDS-gels are comparable to those reported earlier on the column-purified protein [15]. Although the values for some amino acids deviate somewhat from the expected values, this seems to be a property of this highly glycosylated membrane protein rather than due to the fact that the proteins were isolated from SDS-gels. More accurate data can be obtained on smaller peptides, and in fact the isolation of sialoglycopeptides from the polypeptide mixtures has proven to yield reliable data, since the peptides obtained previously from AMLO-pool B [18] can now be shown to be derived from the amino terminal ends of glycophorin B and C.

Glycophorin B is related to glycophorin A in structure and antigenicity. It is, however, not a proteolytic degradation product of glycophorin A. The C-terminal peptide and antigenic determinant located in this region of glycophorin A [9] is apparently absent in glycophorin B, which could suggest shortening of the protein at the C-terminal end by proteases. However, peptide T3, located in the amino terminal half of glycophorin A (residues 40–61), is also not found in glycophorin B. These findings in addition to differences in amino acid sequence [18] make it rather unlikely that glycophorin B is derived from glycophorin A by post-translational modification or artificial degradation, but rather that it is the product of a different gene. The amino terminal sequence of B is identical to one form of glycophorin A, called A^N, in the first 22 amino acid residues (Fig. 8). In addition, the same sites are occupied by oligosaccharides in glycophorin B with the exception of N-glycosylation in position 26 of the sequence [18]. Correlated with this structural similarity or identity are serological characteristics. Glycophorin B carries the N antigen regardless of the MN type of the red cell which is determined predominantly by glycophorin A [18, 26]. Structural information on glycophorin C is limited and the relationship to the two other glycoproteins, if any, has to be defined yet.

The three proteins are integral membrane proteins [27] since portions of the polypeptide are interacting with the hydrophobic core of the lipid bilayer and hydrophobic peptides have been isolated which serve this function ([7] and Furthmayr, unpublished).

It is difficult at present to determine the molecular weight of glycophorin B and glycophorin C. The data presented here suggest however, that the polypeptide chains are considerably shorter than glycophorin A and may not exceed 100 amino acid residues. Further structural studies on the isolated peptides will be required to answer this question. The data available thus far also suggest that glycophorin B and glycophorin C lack the cytoplasmic segment of about 35 amino acid residues found in glycophorin A. The proteins may still span the entire thickness of the membrane with only rather short segments extending out of the inner face of the membrane. Alternatively, the hydrophobic segment is entirely buried within the membrane.

Analysis of membranes prepared from trypsinized intact cells by SDS-gel electrophoresis has indicated that glycophorin B is not digested in intact cells and that the cells retain their Ss activity [31]. Several groups have analyzed in some detail the glycopeptides released from cells by trypsin [32–34], but the fragments which have been described are only marginally related to the glycopeptides isolated here, since amino acid and carbohydrate compositions are rather different from those reported here. Presumably, these studies were dealing with insufficiently purified proteolytic fragments. It is interesting that in situ only glycophorin A and glycophorin C are sensitive to trypsin despite the high concentration of enzyme employed. The differential sensitivity of these proteins to proteases may be an indication for a definite arrangement of these molecules on the surface of the red cell.

In the red cells of certain individuals, some of the sialoglycoproteins are absent without apparent deficiencies for the individual, for red cell survival in the circulation, or red cell function. Such genetic variants have been described for glycophorin A and glycophorin B, either of which can be totally absent in homozygotes [18, 28]. This loss is associated with serologically recognizable changes of the red cell and lack of expression of MN and Ss antigens [29]. From this data it also has been concluded that glycophorin B (Fig. 5) carries the Ss determinants since both bands are absent in S-s-U-cells [28]. Combined absence of glycophorin A and B has been found only in heterozygotes with genetically determined loss of 50% of these proteins [18]. Variations with respect to glycophorin C have not been described and it is not known whether it carries determinants of any known antigenic system.

These latter findings put some strain on any hypothesis to explain the function of these complex glycoconjugates. Although they do not address the question, whether these proteins are synthesized at all at any stage during cell differentiation in these individuals, or at which stage of differentiation this loss occurs, these observations may indicate that a certain minimal amount of sialic acid conjugated to protein is required for normal function of the red cell. Reduction of the surface charge even lower than the "critical potential" [30] at which nonspecific aggregation of red cells occurs with specific ligands, may result in agglutination of cells in vivo in the presence of normal serum components. Alternatively, these proteins play a role much more important earlier during differentiation and genetic events which interfere with the expression of the proteins may result in nonviable conditions.

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