Studies on the Major Sialoglycoprotein of the Human Red Cell Membrane. Isolation and Characterization of Tryptic Glycopeptides†

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ABSTRACT: Tryptic digestion of the major sialoglycoprotein of the human red cell membrane has yielded four unique glycopeptides. These four peptides, designated α-1, α-2, α-3, and β, have been isolated by a combination of ion-exchange chromatography and gel filtration. Each glycopeptide has been characterized with respect to amino acid and carbohydrate compositions and receptor activities for phytohemagglutinin (PHA), wheat germ agglutinin (WGA), and influenza virus (Flu). The α-1 glycopeptide contained 34 amino acids, had receptors for PHA, WGA, and Flu, had substantial amounts of galactosamine and glucosamine, and had a calculated molecular weight of approximately 16,000. The α-2 peptide had 22 amino acids and had a calculated molecular weight of approximately 6000. The α-3 peptide contained only 8 amino acids and had an apparent molecular weight of approximately 2000. The β-glycopeptide had 27 amino acids, 8 residues of galactosamine, and receptors for PHA and Flu. The calculated molecular weight of β was approximately 11,000. The linear arrangement of these glycopeptides in the intact molecule has been determined by sequential tryptic digestion of the glycoprotein while *in situ*. When intact red cells were incubated with trypsin, α-1, α-2, and probably α-3 were released. The β-glycopeptide was generated only after the partially digested glycoprotein had been isolated from the membrane with lithium diiodosalyate and treated a second time with trypsin. The results of these studies add further support to the concept that the glycoprotein is oriented at the cell surface with its oligosaccharide-rich N-terminal half exposed to the external environment of the cell.

On the basis of previous studies from this laboratory (Marchesi *et al.*, 1972; Segrest *et al.*, 1971, 1973) and elsewhere (Winzler, 1970), it has been established that most of the carbohydrate residues associated with the human red cell membrane are bound to a sialoglycoprotein. The carbohydrate residues of this species represent approximately 60% of the total mass of this molecule and are localized along the N-terminal half of the polypeptide chain (Segrest *et al.*, 1973). On the basis of these data we have proposed a tentative model for the orientation of the glycoprotein in the membrane (Marchesi *et al.*, 1972) which represents an extension of previous ideas (Winzler, 1969; Morawiecki, 1964), and is based on the distribution of the carbohydrate in the glycoprotein (Segrest *et al.*, 1973) and its accessibility to enzymatic iodination (Phillips and Morrison, 1971) and proteolytic degradation (Wallach, 1972).

In the present study we have described the isolation and partial characterization of glycopeptides prepared by both tryptic digestion of the isolated glycoprotein and by tryptic digestion of intact red cells.

Methods

Preparation of Glycoprotein. Human red cell membrane glycoprotein was isolated as described previously (Marchesi and Andrews, 1971). [^{125}I]Glycoprotein was prepared by iodination with purified lactoperoxidase as described by Phillips and Morrison (1971) and Segrest *et al.* (1973).

Electrophoresis. Sodium dodecyl sulfate–acrylamide gel electrophoresis was performed in 0.1% sodium dodecyl sulfate–0.1 M sodium phosphate buffer (pH 7.0) and stained with either Coomassie Blue or periodic acid–Schiff reagent as described previously (Segrest *et al.*, 1971).

Radioactivity Estimation. LIQUID SCINTILLATION SPECTROSCOPY. Radioactivity of samples in aqueous media (2–500 μl) was estimated by using 10 ml of a scintillation mixture (400 ml of Fluoralloy TLX-500 ml of Bio-Solv BBS-3 in 3100 ml of toluene, Beckman reagents) and a Beckman liquid scintillation system Model LS-250.

Carbohydrate Analysis. Hexose was determined by the phenol–sulfuric acid method (Plummer and Hirs, 1964). Glucosamine and galactosamine were determined following hydrolysis in 3 N HCl at 100° for 4 hr on the basic column of the amino acid analyzer. Sialic acid was determined by the thiobarbituric acid method (Warren, 1959).

Amino Acid Analysis. Samples were subjected to hydrolysis in 1.0 ml of 6 N constant-boiling HCl in an evacuated sealed tube at 110° for 22 hr. After hydrolysis the acid was removed by evaporating under reduced pressure in a rotary evaporator. The amino acids were determined with a Hitachi Model KLA-3B, amino acid analyzer.

Receptor Activities. The receptor activities of the glycoprotein and of the isolated glycopeptides were determined by their ability to inhibit the agglutination of red cells in the presence of phytohemagglutinin, influenza virus, or wheat germ agglutinin. The assays were carried out using a 4% suspension of saline-washed erythrocytes and serial dilutions of the glycoproteins or glycopeptides.

Alkaline–Borohydride Reduction. To determine the number of *O*-glycosidic linkages, the glycopeptides were treated with

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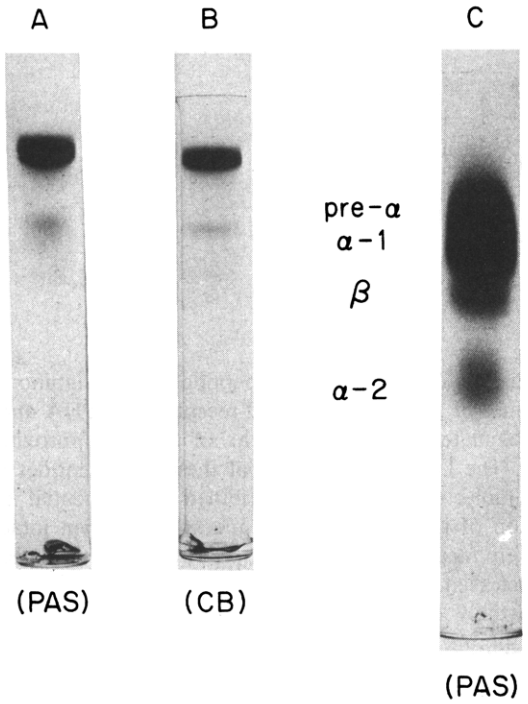

FIGURE 1: Sodium dodecyl sulfate–acrylamide gel electrophoresis of native glycoprotein and glycopeptides produced from trypsin hydrolysis. Electrophoresis was performed with 12.5% acrylamide, containing approximately 50 μg of digest.The gels were stained with periodic acid–Schiff reagent (PAS) for carbohydrate or with Coomassie Blue (CB): (A, B) native glycoprotein; (C) tryptic glycopeptides.

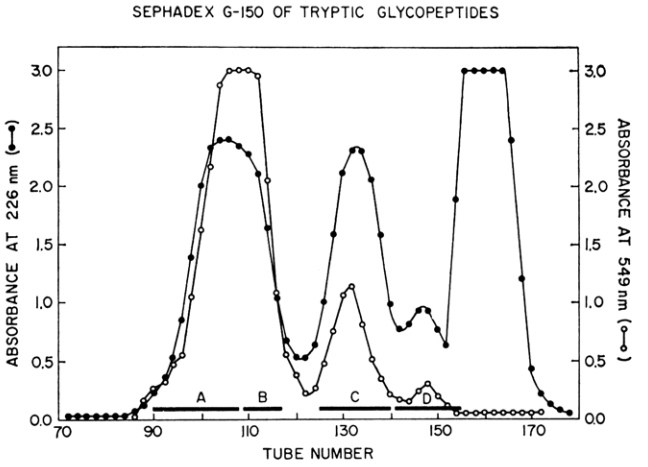

FIGURE 2: Gel filtration of tryptic glycopeptides. Fractions from Aminex A-5 column were pooled, concentrated by a rotary evaporator and applied to a Sephadex G-150 column (2.5 × 200 cm), which was previously equilibrated with 0.10 M ammonium acetate (pH 7.0) at 25°. The column was eluted with the same buffer at a flow rate of 45 ml/hr, and 6-ml fractions were collected. Peptides were detected by measurement of their absorbance at 226 μm. Glycopeptides were detected by the thiobarbituric acid assay for sialic acid (Warren, 1959).

0.5 ml of 0.1 M sodium borohydride in 0.2 M NaOH at room temperature. After 50 hr, the samples were neutralized with HCl and amino acid analysis was performed.

Hydrolysis with Carboxypeptidase B. In a typical experiment, 0.10 μmol of peptide was added to a 5-ml conical tube and taken to dryness with a stream of nitrogen. The sample was dissolved to 0.2 ml of 0.1 M Tris-HCl buffer (pH 8.2), containing 0.5 M NaCl. The reaction was initiated by the addition of 100 μg of carboxypeptidase B (Worthington). After 5 hr at 37°, the reaction was stopped by the addition of 0.025 ml of glacial acetic acid. The solution was evaporated to dryness with a stream of nitrogen and the residue was analyzed directly on the amino acid analyzer.

Results

Tryptic Hydrolysis of Glycoprotein. In a typical experiment, 350 mg of delipidated glycoprotein was dissolved in 35 ml of 0.05 M Tris-HCl buffer and the solution was adjusted to pH 8.2 with NaOH. To the solution was added 9.4 mg of Tos-PheCH_2Cl[1]-treated trypsin (Worthington) in 1 ml of 0.05 M Tris-HCl buffer (pH 8.2). The solution was shaken gently at 37° for 24 hr, after which time the trypsin was inactivated by the addition of a 10 molar excess of Tos-Lys-CH_2Cl. The solution was adjusted to pH 3.5 with 1 N HCl; a precipitate developed that was removed by centrifugation. The precipitate contained negligible amounts of carbohydrate, but has as one of its major components a portion of the C-terminal end of the glycoprotein (Segrest *et al.*, 1973). The soluble peptides were lyophilized and dissolved in 5.0 ml of water; the solution was adjusted to pH 3.5 with 1 N HCl. Sodium dodecyl sulfate–acrylamide gel electrophoresis of the intact glycoproteins and of the soluble tryptic peptides is presented in Figure 1. With periodic acid–Schiff reagent, five glycopeptides could be detected and have been designated pre-α, α-1, α-2, α-3, and β. Using standard urea gels (Ornstein, 1964) with Tris–glycine buffer (pH 8.6), all the peptides moved with the front and could not be identified. The differences observed in the color intensity of each peptide with the periodic acid–Schiff reagent is probably due to differences in their carbohydrate composition as described below. The pre-α-glycopeptide was not a unique peptide since it could be degraded by a second tryspin treatment to yield α-1 and α-2. The α-3-glycopeptide was a small peptide and diffused so rapidly that it was difficult to demonstrate by electrophoresis.

Fractionation of Glycopeptides. Cation-exchange chromatography on Aminex A-5 was used to separate the sialic acid containing peptides from the neutral and basic peptides. The column (0.9 × 60 cm) was equlibrated with 0.2 M pyridine–acetate buffer (pH 3.1). After application of the sample, the column was eluted with the same equilibrating buffer. Based on the recovery of carbohydrate, 92% of the sialoglycopeptide applied to the column was present in the unretarded fraction. Sodium dodecyl sulfate–acrylamide gel electrophoresis of this fraction was identical with that of the total tryptic digest and indicated that no glycopeptide fraction had been lost.

Further fractionation of the glycopeptide mixture presented many problems, and it is important to emphasize that not all of the final products were completely homogeneous with regard to their amino acid composition. Three different factors were responsible for the difficulties involved in the isolation of each glycopeptide fraction. (1). The general lack of satisfactory procedures for the resolution of acidic sialoglycopeptide mixtures; (2) the inhibitory influence of the carbohydrate side chains on proteolytic cleavage with trypsin; (3) the probable microheterogeneity of the polysaccharide side chains.

[1] Abbreviations used are: PAS, periodic acid–Schiff reagent; tlg, thin-layer gel filtration; PHA, phytohemagglutinin; Flu, influenza virus; WGA, wheat germ agglutinin; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl 1-ketone; Tos-LysCH_2Cl, *N-p-α*-tosyllysyl chloromethyl ketone; RBC, red blood cell.

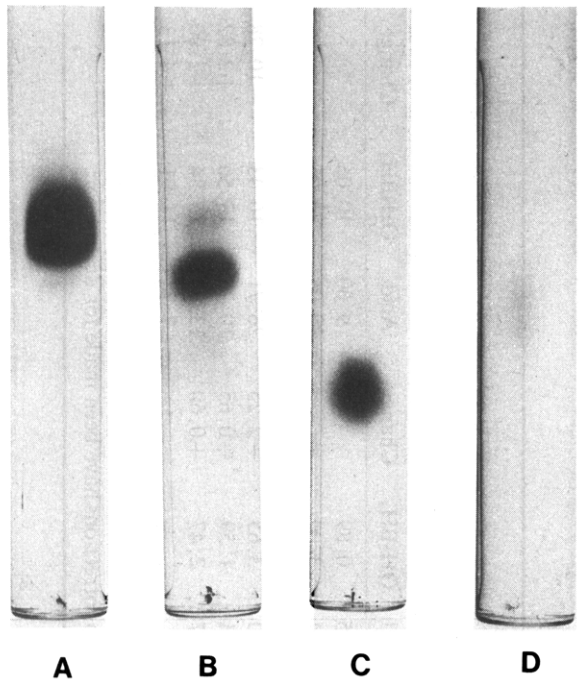

FIGURE 3: Sodium dodecyl sulfate–acrylamide gel electrophoresis of tryptic glycopeptides purified as described in the text. Gels A, B, C, and D correspond to α-1, β, α-2, and α-3, respectively. Approximately 100 μg of each glycopeptide was electrophoresed on 12.5% acrylamide gels and stained with periodic acid–Schiff reagent. Comparable gels stained with Coomassie Blue showed no detectable bands. The α-3-glycopeptide appears as a faint diffuse band probably because it is too small to fix and stain permanently. However, α-3 can be detected using ^{125}I as described in Figure 8A,B.

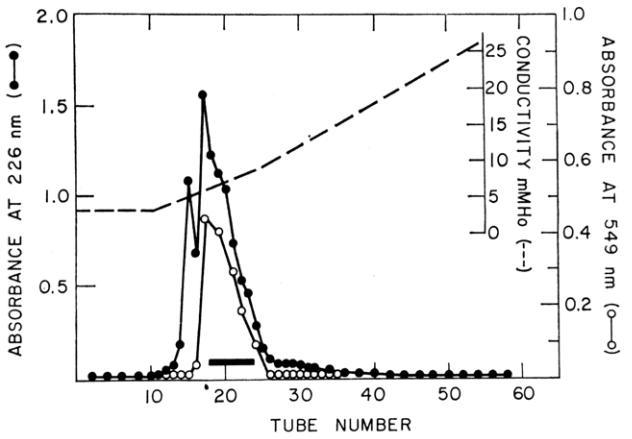

FIGURE 4: Purification of α-2-glycopeptide by chromatography on DEAE-cellulose. The peptides eluting at zone C of the G-150 gel filtration column (Figure 2) were applied to a column(0.9 × 30 cm) previously equilibrated with 0.05 M sodium formate buffer (pH 6.3) at room temperature. The peptides were eluted with the equilibrating buffer at a flow rate of 100 ml/hr. At tube 10, a linear gradient was started consisting of 400 ml of 0.5 M NaCl in the same buffer and mixed in a two-chambered apparatus; 15-ml fractions were collected. The fractions were monitored by the methods indicated in Figure 2; absorbance at 226 μm (●) and sialic acid (○). The fraction indicated by the solid bar represents α-2 and is shown in Figure 3C.

The initial fractionation of the glycopeptide mixture was performed on Sephadex G-150 and is shown in Figure 2. Three zones containing sialic acid were detected. The complexity of the major zone (zone A and B) became apparent when appropriate fractions from the elution profile were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis; three components were evident. From the results of the sodium dodecyl sulfate gels, two zones of glycopeptides were pooled such that zone A contained all three components, pre-α, α-1, and β, and zone B a single glycopeptide, designated β.

After rechromatography of zone A over the same Sephadex G-150 column and sodium dodecyl sulfate–gel electrophoresis of appropriate fractions, pre-α and α-1 were isolated. Incubation of the pre-α component with trypsin yielded α-1 and α-2.

For the purpose of isolating α-1, the entire zone A of Figure 2 was lyophilized and treated a second time with trypsin as was described for the glycoprotein. The digestion mixture was rechromatographed on Sephadex G-150 as described in Figure 2. This procedure yielded the α-1 glycopeptide essentially free of pre-α and of α-2. Gel electrophoresis of the purified α-1 is presented in Figure 3A.

Zone B was subjected to rechromatography on Sephadex G-150. The tubes containing the β-glycopeptide were pooled and lyophilized, and gel electrophoresis of the purified β-glycopeptide is illustrated in Figure 3B.

Zone C of Figure 2 was a mixture of two peptides: one a glycopeptide and the other a peptide with no detectable carbohydrate. The two peptides were fractionated by chromatography on DEAE-cellulose as shown in Figure 4. The non-glycopeptide emerged as a shoulder of the glycopeptide fraction. The glycopeptide in tubes 18–23 were pooled and lyophilized and gel electrophoresis shows that it is α-2 (Figure 3C).

Zone D of Figure 2 contained an octaglycopeptide. Disc gel electrophoresis of the fraction, α-3, is shown in Figure 3D. Owing to the small size, the peptide diffused rapidly from the gels and did not show a distinct band with the periodic acid–Schiff reagent. However, this glycopeptide was observed when [^{125}I]glycoprotein was digested as will be described below.

Properties of Glycopeptides. The individual fractions were subjected to analysis for amino acid and carbohydrate as described in the Methods section, and the results are presented in Tables I and II. The most striking aspect of the amino acid composition of the trypsin fragments is the high content of the hydroxylamino acids, serine and threonine, in α-1 and β; the sum of both residues accounts for approximately 50% of the amino acids in each peptide. From the specificity of trypsin, each peptide should contain only one lysine or arginine, but, as seen in Table I, all of the peptides except α-3 possess more than one basic amino acid. However, carboxypeptidase B digestions of α-1, α-2, α-3, and β released only arginine from each of the glycopeptides in amounts equivalent to 0.51, 0.95, 0.05, 1.02 mol of arginine per mol of peptide, respectively. For some unexplained reason, the single arginine residue in the α-3 peptide was not released with carboxypeptidase B. The lack of cleavage with trypsin is not completely clear but it might possibly be due to the negative charge of the sialic acids and/or by the influence of a bulky heterosaccharide side chain close to the site of cleavage.

Each of the glycopeptides possessed at least one residue of tyrosine and is consistent with the results of experiments using lactoperoxidase to specifically iodinate tyrosines as described below.

The most striking feature of the carbohydrate compositions (Table II) was the high content of sialic acid, which explains the very acidic properties of the peptides. From the amino acid and carbohydrate compositions α-1, α-2, α-3, and β had calculated molecular weights of 16,000, 6000, 2000 and 11,000, respectively.

TABLE I: Amino Acid Composition of Purified Glycopeptides.[a]

Amino Acid	Glycopeptide			
	α-1	α-2	α-3	β
Aspartic acid	2.14 (2)	0.18	0.93 (1)	0.75 (1)
Glutamic acid	1.96 (2)	5.84 (6)	0.18	3.04 (3)
Threonine	7.59 (8)	2.10 (2)	2.03 (2)	4.49 (5)
Serine	8.02 (9)	2.19 (2)	0.10	5.90 (7)
Proline	0.56 (1)	2.68 (3)	1.18 (1)	
Alanine	1.61 (2)	0.97 (1)	2.13 (2)	0.94 (1)
Glycine	0.36	0.98 (1)	0.06	1.87 (2)
Valine	1.99 (2)	3.10 (3)		2.11 (2)
Methionine	0.98 (1)			1.10 (1)
Isoleucine	0.98 (1)	1.04 (1)		0.88 (1)
Leucine	1.07 (1)	0.09		1.23 (1)
Tyrosine	1.34 (2)	0.79 (1)	0.65 (1)	0.69 (1)
Phenylalanine				
Lysine	1.92 (2)	0.11		1.11 (1)
Histidine	ND[b]	ND	ND	ND
Arginine	1.00 (1)	2.01 (2)	0.91 (1)	0.89 (1)
Total	34	22	8	27

[a] Amino acid compositions are expressed as molar amino acids per mole of peptide based on molecular weights of 16,000, 6000, 2000, and 11,000 for α-1, α-2, α-3, and β, respectively. The results are from duplicate analyses of 22-hr hydrolysates; no corrections have been made for destruction or incomplete hydrolysis. [b] ND, not done.

One aspect of the carbohydrate composition that deserves mentioning was the apparent lack of glucosamine in α-2, α-3, and β (Table II). In contrast, 40% of the amino sugar content of α-1 was glucosamine. To show that the glucosamine was attached through an alkali-stable linkage to asparagine in a β-asparginylglucosylamine bond, the peptides were treated with alkaline borohydride (Marshall and Neuberger, 1964). Since the glucosamine was not destroyed (Table III), it was suggested that *N*-acetylglucosamine was linked to one or both of the aspartic acid residues in α-1.

As shown in Table III, 50% of the serine and threonine residues are involved in *O*-glycosidic linkages with carbohydrate. Since there was loss of an equivalent amount of galactosamine, the data indicated that galactosamine was

TABLE II: Carbohydrate Composition of Purified Glycopeptides.[a]

Sugar	α-1	α-2	α-3	β
Sialic acid[b]	19.64	7.75	1.90	17.70
Hexose[c]	8.40	2.18	1.06	4.60
Glucosamine[d]	8.33			0.10
Galactosamine[d]	12.71	4.33	1.38	7.82

[a] The compositions are expressed as moles of carbohydrate per mole of peptide. [b] Determined by the procedure of Warren (1959). [c] Determined by the phenol–sulfuric acid method (Plummer and Hirs, 1964). [d] Determined, after mild acid hydrolysis, on the basic column of the amino acid analyzer.

TABLE III: Effect of Alkaline Borohydride on Amino Acid Composition of Tryptic Glycopeptides.[a]

Amino Acid or Sugar	α-1 Glycopeptide			α-2 Glycopeptide			α-3 Glycopeptide			β-Glycopeptide			Native Glycopeptide		
	Acid	$OH\text{-}BH_4$	Change	Acid	$OH\text{-}BH_4$	Change	Acid	$OH\text{-}BH_4$	Change	Acid	$OH\text{-}BH_4$	Change	Acid	$OH\text{-}BH_4$	Change
Aspartic acid	2.14	2.24								0.75	0.89		9.60	10.06	
Glutamic acid	1.96	2.28		5.84	5.28		0.93	1.05		3.04	3.00		20.05	20.05	
Threonine	7.59	2.69	−4.90	2.10	0.62	−1.48	2.03	1.18	−0.85	4.49	1.80	−2.69	20.00	11.74	−8.26
Serine	8.02	4.33	−3.69	2.18	0.42	−1.76				5.90	3.65	−2.25	26.71	16.43	−10.28
Alanine	1.61	2.62	+1.01	0.97	1.64	+0.67	2.13	1.94		0.94	1.59	+0.65	10.74	13.56	+2.82
Glycine	0.36	1.21	+0.85	0.98	1.17					1.87	2.47	+0.60	13.34	15.34	+2.00
Glucosamine	4.32	6.79	+2.47										7.78	11.91	+4.13
Galactosamine	9.94	3.71	−6.23	2.10	0.14	−1.96	0.88	0.68	−0.20	6.62	2.39	−4.23	17.28	10.00	−7.28

[a] Compositions are expressed as moles of carbohydrate per mole of peptide. The results are from 22-hr hydrolysates and no corrections have been made for destruction.

TABLE IV: Receptor Activity.[a]

Fraction	PHA[b]	Influenza[c]	WGA[d]
Native glycoprotein	<10	<10	<10
α-1-Glycopeptide	40	40	40
α-2-Glycopeptide	>100	>100	>100
α-3-Glycopeptide	>100	>100	>100
β-Glycopeptide	40	40	>100

[a] Activities are expressed as the micrograms of peptide required to inhibit completely red cell agglutination in the standard system. [b] Phytohemagglutinin. [c] Influenza virus. [d] Wheat germ agglutinin. The peptides were dissolved in water and preincubated with agglutinin or influenza virus adequate to give 4+ agglutination of washed red cells in 15 min. A 50-μl aliquot was added and the degree of agglutination measured after 15 min at 37°.

probably linked to serine or threonines in the peptide backbone and is consistent with data presented previously (Winzler *et al.*, 1967).

Since the receptor sites for both phytohemagglutinin and wheat germ agglutinin are reported to be dependent on glucosamine, it was of interest to assay each of the glycopeptide fractions for receptor activities. When measured by hemagglutination–inhibition (Table IV) both the α-1 and β-glycopeptides had PHA activity, but only α-1 had significant WGA activity.

Trypsin Digestion of Intact Red Cells. Six units of freshly drawn type A blood were centrifuged and the supernatant discarded. The red cells were washed with 0.01 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl, and centrifuged; the washing procedure was repeated three times. To 1500 ml of washed packed cells was added 1500 ml of 0.2 M sodium phosphate buffer (pH 8.0), containing 0.2 M NaCl. Trypsin hydrolysis was initiated by the addition of 250 mg of Tos-PheCH$_2$Cl-treated trypsin (Worthington) and the solution was shaken gently at 37°. After 90 min the trypsin was inactivated by the addition of a 10 molar excess of Tos-LysCH$_2$Cl. The solution was centrifuged at 3000*g* for 20 min, and the supernatant fraction containing the tryptic fragments was decanted. The red cells were washed one more time with 0.01 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl and centrifuged, and membranes were prepared as described below. Sodium dodecyl sulfate–gel electrophoresis of the supernatant fraction is illustrated in Figure 5. Many peptides were detected when the gel was stained with Coomassie Blue (gel A). However, only one major carbohydrate staining band was observed when the gel was treated with periodic acid–Schiff reagent (gel B). A second glycopeptide (α-2) was detected, but its resolution was hindered because of the large quantities of heme-containing peptides present in the supernatant solution.

Fractionation of Trypsin Fragments Derived from Intact Cells. The supernatant (1500 ml) was lyophilized to dryness. The powder was dissolved in 100 ml of distilled water and the solution was adjusted to pH 4.1 with 0.2 M citric acid, and then dialyzed for 12 hr against 20 l. of 0.02 M sodium citrate buffer (pH 4.1). Cation-exchange chromatography on phosphocellulose was utilized to separate the carbohydrate-containing peptides from other peptides. The dialyzed sample was applied to a column (2.5 × 30 cm) containing phosphocellulose (Cellex P Bio-Rad Laboratories) that had been previously

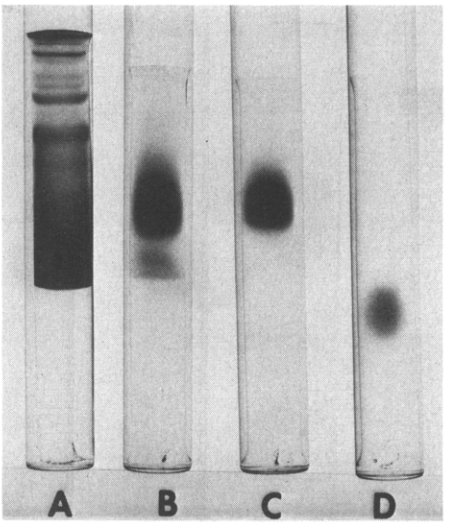

FIGURE 5: Sodium dodecyl sulfate–acrylamide gel electrophoresis of peptides released from intact red cells by tryptic digestion. Washed red cells were incubated with trypsin as described in the text and a portion of the total digest treated with 0.5% sodium dodecyl sulfate and electrophoresed directly on 12.5% acrylamide gels in 0.1% sodium dodecyl sulfate. Duplicate samples were stained with Coomassie Blue (A) or periodic acid–Schiff reagent (B). The approximate position of the tracking dye is indicated by the arrow. Gels C and D show the α-1- and α-2-glycopeptides, respectively, after their purification as described in the text.

equilibrated with 0.02 M sodium citrate buffer (pH 4.1). The glycopeptides were eluted with the equilibrating buffer, and, as expected, they emerged unretarded by the column. The glycopeptide fractions were pooled, the pH was adjusted to 7.0 with NaOH, and the solution was lyophilized to dryness. Further fractionation of the glycopeptides was achieved by chromatography on Sephadex G-150 and is illustrated in Figure 6. Two sialic acid containing peaks were detected which corresponded to the glycopeptides α-1 and α-2. The peptides in the zones α-1 and α-2 were pooled and lyophilized to dryness. Gel electrophoresis of these fractions is shown in Figure 5-C and 5-D. Neither the β-glycopeptide nor α-3 was isolated from the Sephadex column. In an attempt to show that the β-glycopeptide remained on the red cells after trypsin treat-

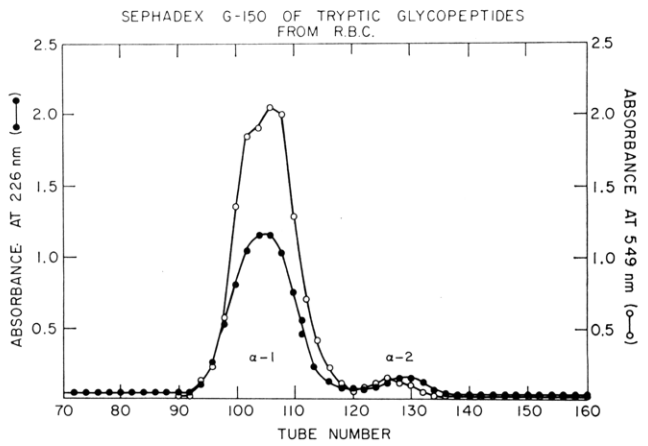

FIGURE 6: Purification of the α-peptides released from intact red cells by trypsin. The peptides were purified on phosphocellulose and DEAE-cellulose columns as described in the text and then chromatographed on Sephadex G-150. The column and the elution conditions were the same as those described in Figure 2. Both peaks were pooled and samples were electrophoresed as shown in Figure 5C, D.

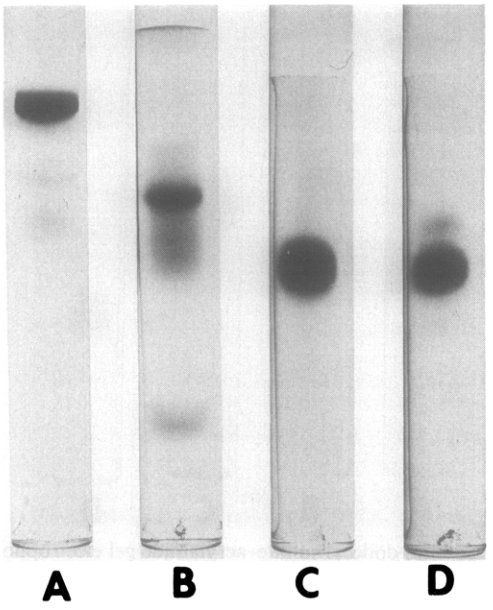

FIGURE 7: Sodium dodecyl sulfate–acrylamide gel electrophoresis of the glycoprotein fragment extracted from trypsin-treated red cells and the isolated β-glycopeptide. A partially degraded glycoprotein fragment (B) was extracted from the red cell membranes after the cells have been treated with trypsin. Compare the mobility of this fragment to that of the native glycoprotein (A). The β-glycopeptide (C) is produced by a second tryptic digestion of the isolated fragment, and is identical with the β-glycopeptide (D) produced by tryptic hydrolysis of the intact glycoprotein. Gel A was 10% acrylamide and gels B and C were 12.5%. All were stained with PAS reagents.

ment, the partially digested glycoprotein (minus α-glycopeptides) was isolated from trypsin-treated cells as described in detail below.

Purification of "Fragmented Glycoprotein" from Trypsinized Red Cells. The washed red cells, that had been treated with trypsin as described above, were lyzed in dilute phosphate buffer and the membranes were prepared as described previously (Marchesi and Andrews, 1971). The isolated membranes were suspended in 100 ml of 0.3 M lithium diiodosalicylate and the "fragmented glycoprotein" was isolated as described for the intact glycoprotein (Marchesi and Andrews, 1971). Sodium dodecyl sulfate–acrylamide gel electrophoresis of the isolated fragmented glycoprotein indicated a small amount of the native glycoprotein was also present, but attempts to purify the fragmented glycoprotein from the native glycoprotein by chromatography on Sephadex G-150 in 8 M urea were not successful. Therefore, the fragmented glycoprotein was purified by sodium dodecyl sulfate–acrylamide gel electrophoresis. Sodium dodecyl sulfate gels were run in phosphate buffer as described in Methods; 500 μg was applied to each gel, and after electrophoresis one of the gels was removed and stained with Coomassie Brilliant Blue to locate the protein. The other gels were sliced at the appropriate positions and the protein was eluted by suspending the gels in 20 ml of 1% sodium dodecyl sulfate and homogenizing with a glass motor-driven homogenizer. The suspension was centrifuged and the supernatant solution was retained. The sodium dodecyl sulfate was removed by the method of Lenard (1971).

To show that the fragmented glycoprotein possessed the β-glycopeptide, 23 mg of the protein was treated with 2% trypsin at 37°. After 20 hr the trypsin was inactivated with Tos-LysCH$_2$Cl, and the peptide mixture was chromatographed on Aminex A-5 as described previously for the native glycoprotein. The glycopeptide fraction was lyophilized and sodium dodecyl sulfate–gel electrophoresis of this fraction is shown in Figure 7C and is comparable to the β peptide from the isolated native glycoprotein (Figure 7D). No pre-α, α-1, α-2, or α-3 were present.

Since both β-peptide fractions have comparable mobility on sodium dodecyl sulfate gels and essentially identical amino acid compositions, we conclude that the β-glycopeptide isolated by tryptic hydrolysis of the fragmented glycoprotein is identical with the β-glycopeptide isolated from the native glycoprotein.

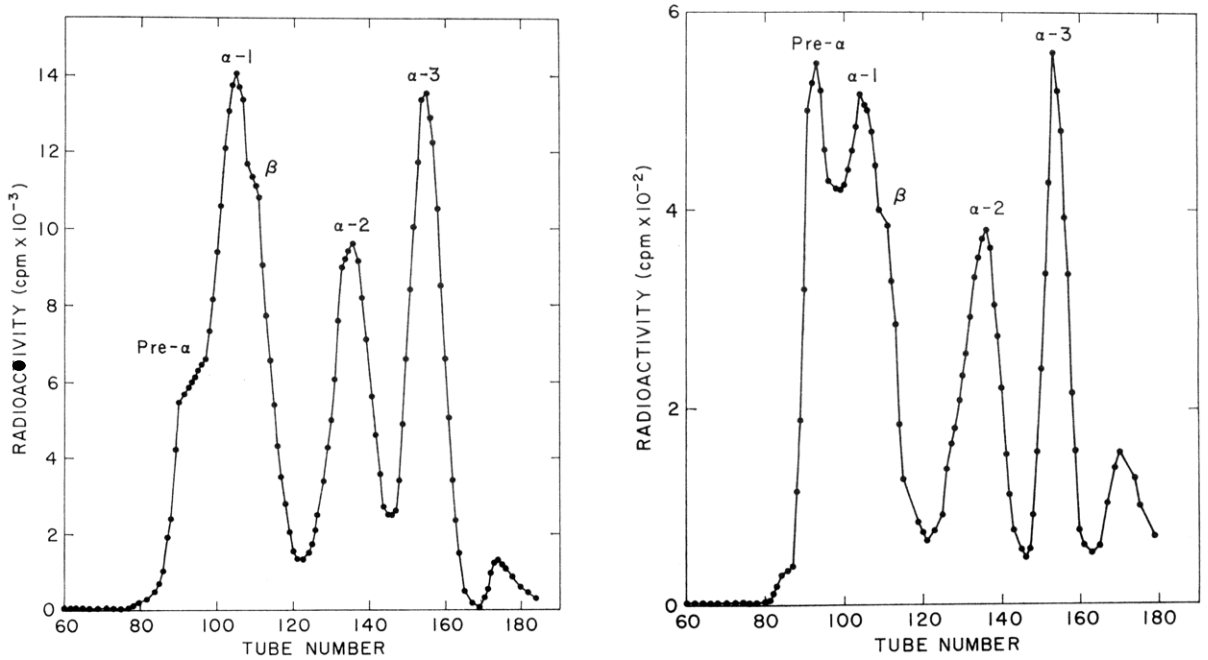

FIGURE 8: Gel filtration of tryptic-cleaved [^{125}I]glycopeptides. A 2.5 × 150 cm Sephadex G-150 column was used and the elution conditions were the same as for Figure 2: (A) 7 mg of [^{125}I]glycopeptides which were derived from glycophorin that was iodinated in solution (after extraction); (B) 3 mg of [^{125}I]glycopeptides derived from glycophorin which was extracted from iodinated ghosts; 0.5-ml fractions were sampled for radioactivity.

Orientation of the Sialoglycoprotein in the Membrane. Using lactoperoxidase and ^{125}I for the specific iodination of tyrosines on intact red cells, Phillips and Morrison (1971) have shown that only two proteins are exposed to the exterior of the cell. One of these proteins had an apparent molecular weight of approximately 90,000 and was a glycoprotein and probably corresponded to the glycoprotein in the present study. It was of interest, therefore, to determine if the number of tyrosines iodinated was different when the glycoprotein was labeled while *in situ* as opposed to its being labeled after isolation from the membrane.

[^{125}I]Glycophorin was prepared as described in Methods. After tryptic hydrolysis of [^{125}I]glycophorin (7 mg), the glycopeptides were purified through Aminex A-5 as described above and then over G-150 Sephadex as shown in Figure 8A. The elution profile was very similar to that of Figure 2. By electrophoresis and autoradiography of fractions 94, 104, 109, 135, 153 of Figure 8A, it was confirmed that these fractions corresponded respectively to pre-α, α-1, β, α-2, and α-3 peptides. These results suggested that the tyrosine residues in each of the glycopeptides was available for iodination by lactoperoxidase. The nature of the small ^{125}I peak at fractions 165–175 is not clear, but since it eluted later than phenol red, it would have a very small molecular weight.

To determine whether the same peptides were accessible while *in situ*, red cell ghosts were iodinated with lactoperoxidase and the labeled glycoprotein was isolated and treated with trypsin in the usual manner. Gel filtration of these iodinated peptides produced an elution pattern (Figure 8B) which was indistinguishable from that described above. This result suggested that the tyrosine residues of all the glycopeptides are exposed to the exterior of the cell.

There does appear to be a difference in the amount of the pre-α peptide between the elution pattern in Figure 8A,B. This difference was probably the result of a less effective digestion causing an incomplete cleavage of pre-α to its corresponding derivatives α-1 and α-2.

Although the glycoprotein is composed of a major electrophoretic unit when analysed by sodium dodecyl sulfate gels, most preparations have two to three minor bands which also stain with the periodic acid–Schiff reagent. To eliminate the possibility that some of the tryptic glycopeptides might be derived from the minor bands, the major band was isolated from sodium dodecyl sulfate gels, and, after removal of sodium dodecyl sulfate, was trypsinized and the products analyzed by thin-layer gel filtration. All four tryptic glycopeptides were produced by digestion of the major component.

From these data a model is proposed which is similar to that suggested by Winzler (1969) and Morawiecki (1964), in which the major sialoglycoprotein of the red cell is organized in the membrane with its oligosaccharide-rich portions exposed to the exterior environment of the cell. This is illustrated in Figure 9.

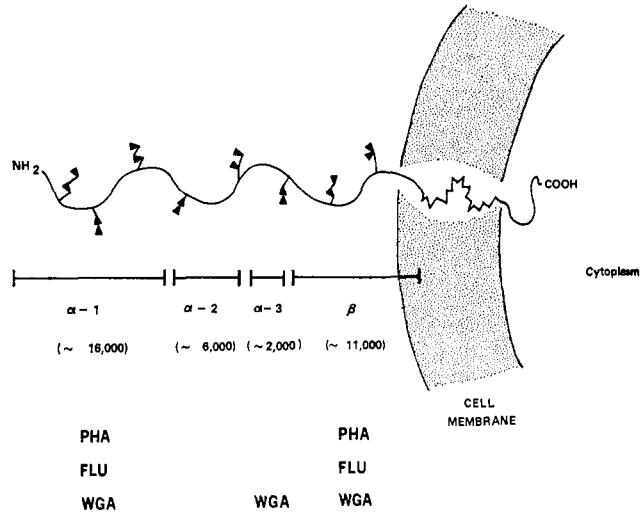

FIGURE 9: Schematic representation of the order of glycopeptides in red cell membrane glycophorin.

Discussion

Four unique carbohydrate-containing peptides (α-1, α-2, α-3, and β) were produced by tryptic digestion of the major sialoglycoprotein of the human red cell membrane. These glycopeptides represented over 90% of the total protein-bound carbohydrates in the glycoprotein. Although previous investigators have shown that glycopeptides could be released from intact red cells by tryptic digestion (Wallach, 1972), the results presented here demonstrated that not all the trypsin-sensitive sites are susceptible to cleavage when the molecule is digested while *in situ*.

Winzler *et al.*, (1967), have analysed in some detail glycopeptides released from red cells by trypsin. One major glycopeptide was isolated from DEAE-cellulose and it was found to have a molecular weight of 11,000. On the basis of this value and the reported amino acid compositions, it is likely that this glycopeptide corresponded to the α-1-glycopeptide described here. The purification procedure used by these investigators did not separate the other α-glycopeptides.

More recently, Jackson and Seaman (1972) have isolated and characterized three glycopeptides that were released from intact red cells with trypsin. Although one of their fractions has a similar amino acid composition to α-2, there is considerable difference with respect to carbohydrate composition of their glycopeptides and those reported here. It is not clear why these results differ from the present study.

Neither Winzler *et al.* (1967) nor Jackson and Seaman (1972) have isolated the β-glycopeptide from the trypsin-treated red cells nor have they demonstrated that the glycopeptides released from the intact cell were identical with those of the completed glycoprotein extracted from the membrane.

The presence of substantial amounts of sialic acid on the β-glycopeptide explains the well-documented observation that only 60% of the total protein-bound sialic acid can be released from human red cells by trypsin (Cook and Eylar, 1965). Since the linkage of the β-peptide to the rest of the polypeptide chain is susceptible to trypsin when either the complete molecules or the partially digested molecules are exposed to trypsin after their isolation from the membrane, we have tentatively aligned the peptides along the polypeptide chain as shown in Figure 9. The α-3-glycopeptide was not identified from the tryptic digestion of intact red cells. However, since the peptide was not present in the fragmented glycoprotein, it was assumed that α-3 was penultimate to β.

Each of the four glycopeptides described here have alkali-labile carbohydrate–protein linkages, and this is consistent with their high content of serine and threonine. Thomas and Winzler (1969) have reported that alkali treatment of tryptic glycopeptides obtained from intact red cells caused the release of a major portion of the carbohydrate in the form of a tetrasaccharide which contains one residue each of galactosamine and galactose and two residues of sialic acid. From the carbohydrate compositions presented in Table II, it is clear that this tetrasaccharide is a constituent of each of the peptides. From Table II it appears that the complete molecule may have

at least 18 alkali-labile carbohydrate linkages and two or three asparagine–carbohydrate linkages.

Finally, Kornfeld and Kornfeld (1970) have isolated a tryptic glycopeptide from intact red cells and have shown that it contains a receptor site for red kidney bean phytohemagglutinin (*Phaseolus vulgaris*) in the form of an oligosaccharide containing *N*-acetylglucosamine, mannose, galactose, and sialic acid. This is probably the PHA binding site on the α-1-glycopeptide. The β-glycopeptide also reacts with PHA, but it has a carbohydrate composition which differs from that of the PHA receptor described above. It is possible that there may be some variation in the makeup of receptors depending upon their position along the polypeptide chain.

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