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ISOLATION AND PARTIAL CHARACTERIZATION OF TWO MINOR GLYCOPROTEINS FROM HUMAN ERYTHROCYTE MEMBRANES*

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SUMMARY

Two minor glycoproteins GP-II and GP-III, were isolated from human erythrocyte membranes and characterized chemically and immunologically. The chemical composition of GP-II and GP-III was similar: GP-II consisted of 81 % protein and 19 % carbohydrate of which 4.9 % was hexose, 5.4 % hexosamine and 7.8 % sialic acid. GP-III consisted of 76 % protein and 24 % carbohydrate of which 7.6 % was hexose, 7.2 % hexosamine and 8.1 % sialic acid. The amino acid composition of GP-II and GP-III was also similar. GP-II and GP-III, however, differed in chemical composition from the MN glycoprotein. GP-II and GP-III were associated with the blood group activities Ss, I and A, but not with the MN antigens. GP-III had higher blood group activities per μg of protein than did GP-II. The specific activities for the Ss blood group antigens were increased 3–10-fold by purification of GP-III from the aqueous phase of chloroform methanol extracts.

INTRODUCTION

Human erythrocyte membranes contain several glycoproteins as constituents [1–3]. Three glycoproteins have been shown to extend through the entire membrane from the inner surface to the outer surface [4–6]. The major glycoprotein, the so-called MN-glycoprotein, has been the subject of extensive studies [7–15]. Only limited information is available on the other minor glycoproteins [3]. In a previous report it was shown that three glycoproteins can be recovered in the aqueous phase after extraction of red cell membranes with a mixture of chloroform and methanol [16]. The two minor glycoproteins were associated with blood group antigens which were not found in the major MN glycoprotein [16].

In this paper the isolation of these two minor erythrocyte membrane glyco-

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proteins is described and data on their biochemical and immunological characteristics are presented.

METHODS

Protein concentrations were determined by the method of Lowry et al. [17] using three times crystalized bovine pancreatic ribonuclease as standard. Phosphorous was measured by the method of Bartlett [18]. Cholesterol was measured according to Zlatkis et al. [19]. Sialic acid was determined chemically by the method of Warren [20].

Analytical disc gel electrophoresis was carried out with 0.5 % sodium dodecylsulfate on 7.5 % polyacrylamide gels in 0.5 % sodium dodecylsulfate/0.1 M phosphate buffer at pH 7.1 [2]. Proteins were separated at 3 V/cm for 8 h in tubes of 5 mm diameter and 12 cm length. Gels were stained with Coomassie Brilliant Blue or with the periodic acid-Schiff reagent [2].

Amino acid analysis was carried out on a Beckman model 120-B automatic analyzer (Beckman Instruments, Inc., Calif.) using the system of Moore and Stein [21]. Carbohydrates were analyzed by gas-liquid chromatography using 3 % OV17 on 80–100 mesh chromosorb W. Nitrogen gas was used as carrier gas. Methanolysis of glycoprotein in 0.5 M HCl in dry methanol and trimethylsilyl-derivatization of methylated carbohydrate were carried out according to the method of Reinhold [22]. Gas-liquid chromatogram was temperature-programmed from 125 °C to 230 °C at 2 °C/min and 230 °C was kept until the peak for sialic acid appeared.

Blood group activities of glycoproteins were measured by hemagglutination inhibition tests. The test was carried out on serial two-fold dilution of glycoproteins. To glycoprotein dissolved in 0.15 M NaCl solution specific antiserum was added to a concentration of four hemagglutinating doses in the final reaction mixture. The glycoproteins and antisera were incubated for 2 h at 37 °C for A, S and s, at room temperature for M and N and 4 °C for I. After the incubation erythrocytes were added to the reaction mixture and incubated for 1 h at the same temperature. The agglutination of erythrocytes was read after centrifugation with a serological centrifuge for 30 s. Activity was expressed as minimum amount of protein which completely inhibited the agglutination of erythrocytes by four hemagglutinating doses of antiserum. Human anti-A serum, rabbit anti-M and anti-N sera were purchased from Behring Diagnostics, Inc. Human anti-S serum was kindly provided by Dr F. H. Allen, Jr., of the New York Blood Center. Anti-s serum and anti-human globulin serum were purchased from Pfizer Inc. Serum from a patient with cold agglutinin disease served as anti-I reagent.

RESULTS

Fresh human blood in acid citrate dextrose from individual donors was provided by the New York Blood Bank. Preparation of membranes and extraction of glycoproteins from erythrocyte membranes with a mixture of chloroform and methanol was carried out according to the method described by Hamaguchi and Cleve [16].

Extracted glycoproteins were fractionated further by preparative electro-

phoresis on polyacrylamide gels in the presence of 0.5 % sodium dodecylsulfate. To 2 parts of chloroform/methanol extracts was added one part of a solution consisting of 6 % sodium dodecylsulfate, 3 % β-mercaptoethanol, 30 % glycerol, 0.003 % Bromphenol Blue and 30 mM phosphate buffer (pH 7.1). The mixture was incubated at 70 °C for 20 min. For electrophoresis, gels of 0.5 % sodium dodecylsulfate and 7.5 % polyacrylamide were prepared; electrophoresis was carried out in 0.5 sodium dodecylsulfate-0.1 M phosphate buffer at pH 7.1. A current of 5 mA per gel was used for the first 30 min, subsequently 10 mA per gel was employed until the tracking dye, Bromphenol Blue, reached a mark 8 cm from the origin. After completion of the electrophoresis, gels were cut into segments according to the migration rate of each glycoprotein in relation to the tracking dye. The segments were homogenized and immersed in 0.5 % sodium dodecylsulfate solution to elute the glycoproteins. Small fragments of gel were removed by filtration on a sintered glass filter. Urea was added to the filtrate to a final concentration of 6 M. Subsequently sodium dodecylsulfate was removed on Dowex (AG) 1-X8 columns (Bio-Rad Lab., Calif.) [23, 24]. Urea was removed by extensive dialysis against distilled water. Glycoprotein preparations were lyophilized afterwards.

Usually 150 ml of blood were processed. On the average 2390 μg of GP-I, 816 μg of GP-II and 914 μg of GP-III were obtained during one preparative procedure. Considerable variations in the yield were observed from preparation to preparation. After lyophilization the preparations of GP-I, GP-II and GP-III appeared as white, fluffy powder that could be dissolved completely in distilled water.

Fig. 1. Banding pattern of human erythrocyte membrane glycoproteins on 0.5 % sodium dodecylsulfate/7.5 % polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue. Gels 2–4 represent the isolated glycoproteins.

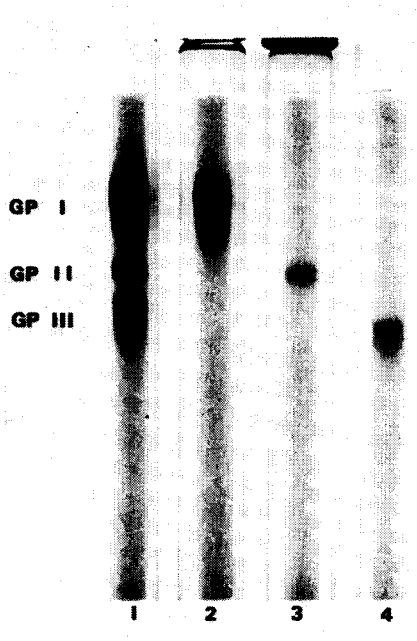

Fig. 2. Banding pattern of human erythrocyte membrane glycoproteins on 0.5 % sodium dodecylsulfate/ 7.5 % polyacrylamide gels. Gels were stained with Periodic acid-Schiff reagent. Gels 2–4 represent the isolated glycoproteins.

The purity of preparations was examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Figs 1 and 2 show the electrophoretic patterns of chloroform-methanol extracts, isolated GP-I GP-II and GP-III. GP-I is the so-called MN-glycoprotein [13]. GP-II appeared as a sharp band on the gels stained for protein and for carbohydrate. GP-III of chloroform-methanol extracts and the isolated material appeared as a blurred broad band. Although no additional bands can be seen on gels in Figs 1 and 2, a band with slightly slower migration rate than GP-I was sometimes observed on gels of purified GP-II and of purified GP-III. This additional band stained with Coomassie Blue but not with the periodic acid-Schiff reagent. As seen in the pattern of chloroform-methanol extracts, the GP-III fraction in some cases appeared two-banded, especially when large amounts of protein were applied to the gels. The two band pattern could not be observed in preparations of isolated and purified GP-III. The isolated preparations of GP-I, GP-II and GP-III had the same migration rate as the fractions in the aqueous phase of chloroform/methanol extracts.

Chemical composition of minor glycoproteins

The chemical composition of the two minor glycoproteins is shown in Table I. The protein and carbohydrate concentrations given in Table I were calculated from the sums of protein and carbohydrate content in the lyophilized material. Protein was found to represent approximately 81 % in GP-II and 76 % in GP-III. The carbohydrate composition of GP-II and GP-III was very similar. Sialic acid was determined by the chemical method; the contents were 7.75 % in GP-II and 8.12 %

TABLE I

CHEMICAL COMPOSITION OF MINOR GLYCOPROTEINS OF HUMAN ERYTHROCYTE MEMBRANE, GP-II AND GP-III

Values for sialic acid were mean values of 3 preparations for GP-II and of 5 preparations for GP-III. Sialic acid was determined by the method of Warren [20]. Values for the other carbohydrates were mean values of two preparations from 0 type erythrocytes, determined by gas-liquid chromatography (see Methods).

Constituent	GP-II (g/100 g)	GP-III (g/100 g)
Protein	80.95	76.26
Carbohydrate	19.05	23.74
Fucose	1.04	0.85
Mannose	0.59	0.40
Galactose	3.02	5.46
Glucose	1.31	1.73
N-Acetyl-glucosamine	5.42	7.15
Sialic acid	7.75	8.12

in GP-III. By gas-liquid chromatography, however, no sialic acid was detected in two preparations of GP-II, while sialic acid content in GP-III varied from 0 to 4 %. The discrepancy between the results of the determination by the method of Warren [20] and by gas-liquid chromatography may be due to degradation of sialic acid during methanolysis of materials for gas-liquid chromatography and to the lower sensitivity of gas-liquid chromatography for sialic acid compared to other carbohydrates, especially when small amounts of material are used. *N*-Acetylgalactosamine could not be found in GP-II or GP-III prepared from O-type erythrocytes, however, 1.3 % and 0.3 % *N*-acetylgalactosamine were found in GP-II and GP-III, respectively, prepared from A-type erythrocytes. In several preparations, peaks with similar retention time to L-arabinose and L-xylose were observed. To assess their significance, material was obtained by the following procedure: The purification protocol was carried through all steps without application of any proteins. Preparative electrophoresis on polyacrylamide gels was followed by elution of glycoproteins from gel segments, removal of sodium dodecylsulfate and urea, and lyophilization. This blank control resulted in a white-grey powder. On gas-liquid chromatography this material revealed peaks from L-arabinose, L-xylose and D-glucose. Comparative analysis of gas-liquid chromatography chromatograms indicated that the peaks for L-arabinose and L-xylose observed occasionally could be ascribed completely to contamination which occurred during the purification procedure. D-Glucose, however, appeared to be, in part, a genuine constituent of GP-II and GP-III, since D-glucose peaks were never observed in analyses of GP-I preparations.

Cholesterol and phosphorous could not be detected in preparations of GP-I, GP-II and GP-III. There is, thus, no evidence for the presence of cholesterol and phospholipids in purified samples of GP-II and GP-III. Protein determinations of the lyophilized materials revealed, however, a protein content of only 12.7 % and 14.4 % of the dry weight of GP-II and GP-III, respectively. We presume that the balance is largely due to contamination with material which is inert in the analytical procedures employed and which is to be ascribed to contamination during the preparation.

TABLE II

AMINO ACID COMPOSITION OF MINOR GLYCOPROTEINS OF HUMAN ERYTHROCYTE MEMBRANE, GP-II AND GP-III

Values were means of 5 preparations for GP-II and 7 preparations for GP-III analyzed in duplicate. Values for MN glycoprotein were means of 9 preparations from 9 different individuals analyzed in duplicate [13]. Composition of MN glycoprotein [13] is given for comparison.

Amino acid	GP-II (mol %)	GP-III (mol %)	MN glycoprotein (mol %)
Lys	5.58	5.48	4.31
His	2.12	2.13	3.72
Arg	4.37	4.30	4.23
Asp	9.04	8.73	6.25
Thr	6.14	6.58	10.40
Ser	6.27	6.87	12.26
Glu	10.55	10.32	10.85
Pro	4.93	4.49	7.13
Gly	11.12	10.15	5.24
Ala	11.14	11.08	5.07
Cys/2	0.70	0.77	–
Val	6.93	6.87	7.87
Met	2.48	2.43	1.75
Ile	4.44	4.53	7.17
Leu	8.29	8.05	6.57
Tyr	2.08	3.21	5.52
Phe	3.83	4.01	1.72

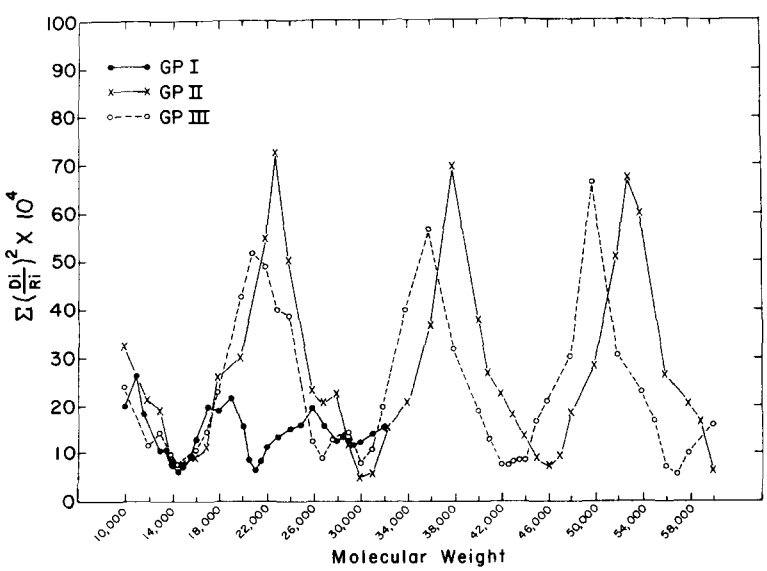

Fig. 3. Calculation of minimal molecular weight by the method of Katz [25]. i. represents the ith kind of amino acid residue. Di is the difference between analytical composition of an amino acid and its nearest integer for a trial molecular weight, and Ri is mol of i in 10^5 g protein.

The amino acid analysis of the human erythrocyte membrane glycoproteins is shown in Table II. 5 preparations of GP-II and 7 preparations of GP-III were analyzed in duplicate. Amino acid compositions of GP-II and GP-III were quite similar. Glycine and alanine were found in high concentrations. Serine and threonine were relatively low compared to MN glycoprotein. Half-cystine residues which were absent from the MN glycoprotein were found in both minor glycoproteins. There were no differences in amino acid and carbohydrate compositions of GP-III when the SS phenotype and ss phenotype were compared.

From the amino acid composition, the minimal molecular weights of the peptide parts of GP-II and GP-III were calculated [25]. The amino acid compositions were compatible with molecular weights of approx. 14 500, 30 000 and 45 000 for GP-II and GP-III (Fig. 3). The calculated minimal molecular weights for GP-II and GP-III were similar. Since the peptide portions were 81 % in GP-II and 76 % for GP-III, minimal molecular weights were calculated as approx. 18 000, 37 000 or 56 000 for GP-II and 19 000, 39 500 or 59 000 for GP-III. The migration rates on 0.5 % sodium dodecylsulfate/7.5 %-polyacrylamide gels corresponded to molecular weights of 37 000 for GP-II and 24 000 for GP-III.

Immunological properties

The blood group activities associated with the purified glycoproteins are

TABLE III

BLOOD GROUP SPECIFICITIES OF GLYCOPROTEINS OF HUMAN ERYTHROCYTE MEMBRANE

Hemagglutination inhibition activities were expressed by the minimum protein concentration (μg/ml) which inhibited completely the agglutination of erythrocytes at four hemagglutinating doses. The figures for activities were mean values of preparations tested, numbers of which are given in parenthesis.

Blood group	S		s		I	A	M
	SS	Ss	ss	Ss			
Chloroform/methanol extracts	55.8 (1)	105.5 (2)	40.1 (3)	71.4 (2)	30.2 (4)	56.9 (4)	72.7 (2)
GP-I	N.A.[a] (1)	N.T.	N.A.[c] (2)	N.T.	W.A.[e] (4)	335.0 (3)	17.5 (3)
GP-II	N.A.[b] (1)	N.T.	36.7[d] (2)	N.T.	26.6 (4)	41.7 (3)	N.A.[f] (3)
GP-III	10.0 (1)	10.0 (2)	12.7 (3)	20.7 (2)	13.5 (4)	38.1 (4)	N.A.[g] (3)

N.A., no inhibitory activity.
N.T., not tested.
W.A., very weak inhibition.
[a] No inhibitory activity at 320 μg/ml.
[b] No inhibitory activity at 60 μg/ml.
[c] No inhibitory activity at 466.7 μg/ml.
[d] One preparation had no inhibitory activity at 53.3 μg/ml.
[e] Very weak inhibition at 350 μg/ml.
[f] No inhibitory activity at 73.3 μg/ml.
[g] No inhibitory activity at 106.7 μg/ml.

shown in Table III. The data for the MN-glycoprotein, GP-I, were also included for comparison.

Blood group activities for Ss, I, A and M were found in the aqueous phase of chloroform/methanol extracts. GP-III had high activities for Ss, I and A blood groups, but not M activity. Specific activities for Ss and I were increased substantially in GP-III compared to chloroform/methanol extracts. A dosage effect was observed for s, but not clearly for S in GP-III, while there was a clear dosage effect for S and s in chloroform/methanol extracts. In GP-II, one preparation from a ss phenotype had specific activity for s in similar magnitude to that of chloroform/methanol extracts. The other two preparations from SS and ss phenotypes failed to inhibit hemagglutination at protein concentrations of 60 μg/ml and 53.3 μg/ml. GP-II had I and A activities in similar magnitude to those of chloroform/methanol extracts. No M activity was observed in GP-II. GP-I had M blood group activity and very weak activities for antigens I and A. No Ss blood group activity was observed in GP-I. While M activity was comparable to that of the MN glycoprotein prepared by another method [13], N activity was very weak even in GP-I. For complete inhibition of hemagglutination approx. 200 μg/ml and 400 μg/ml of protein were required in chloroform/methanol extracts and GP-I respectively. The specific activity for N was decreased in GP-I as compared to chloroform/methanol extracts. This implies that N activity was specifically affected by the isolation procedure while M activity was preserved intact.

DISCUSSION

In a previous study [16] the separation of three human erythrocyte membrane glycoproteins by gel filtration in the presence of sodium dodecylsulfate was demonstrated. In the present report an improved method for the isolation of the three glycoproteins is described in which preparative gel electrophoresis is employed in the presence of sodium dodecylsulfate. Three glycoproteins are obtained in highly purified state and in quantities sufficient for further analysis. The electrophoretic pattern on polyacrylamide gels revealed migration rates identical with those observed in the unfractionated material. Thus, there was no indication of an alteration of the structural integrity of the glycoproteins.

The two minor glycoproteins, GP-II and GP-III, are quite similar in their chemical composition and closely resemble the proteins E and F described by Tanner and Boxer [3]. When compared to the MN glycoprotein several differences are observed: The ratio of protein to carbohydrate is approx. 4 : 1 instead of 1 : 1. The sialic acid content is lower; the fucose content is higher. Concentrations of threonine and serine are lower, glycine and alanine are higher compared to the MN glycoprotein. Particularly important is the presence of half-cystine residues, which are absent from the MN glycoprotein. This finding excludes the possibility that the two minor glycoproteins are in vivo or in vitro degradation products of the MN glycoprotein. The minor glycoproteins are associated with Ss, I and A blood group activities but lack MN blood group activity. The MN glycoprotein, on the other hand, did not have Ss blood group activity. In purified GP-III the specific inhibitory activities for the Ss blood group antigens were increased 3–10-fold when compared with the aqueous phase of chloroform/methanol extracts.

Several recent reports have been concerned with the formation of aggregation products between the major membrane glycoprotein and the minor glycoproteins and with the conditions favoring disaggregation to forms of lower molecular weight [26–28]. In particular the interactions between GP-I and GP-II have been examined [28]. Our results indicate that the MN glycoprotein and the two minor glycoproteins are chemically and immunologically distinct entities. Whether they share a common subunit cannot be decided on the basis of present information.

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