ISOLATION OF A SURFACE GLYCOPROTEIN OF HUMAN PLATELETS*

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1. Introduction

A *macroglycopeptide* has been isolated by proteolytic digestion of intact platelets [1] or isolated platelet plasma membranes [2]. This macroglycopeptide has a mol. wt. of 120 000 and may be related to the inhibitor of viral hemagglutination present on the platelet membrane [3] and to the thrombocyte-specific antigen [4].

In order to obtain further information on the structural and immunochemical relationships of this macroglycopeptide we have now isolated a precursor glycoprotein in pure form and have obtained preliminary information on its size, chemical analysis and proteolytic conversion.

2. Materials and experimental procedures

Platelet concentrates were freed of erythrocytes by differential centrifugation and washed 3 times in 0.01 M Tris–HCl buffer (pH 7.4) containing 0.001 M EDTA and 0.15 M NaCl.

The platelets were lysed and the soluble fraction obtained from two different sources:

(1) From the soluble material obtained during the isolation of platelet membranes by the glycerol-lysis technique [5].

(2) From the soluble material released by the sonication of washed platelets. The washed platelets were subjected to 4 × 15 sec bursts in a Bronson sonifier in an ice bath and insoluble material was removed by centrifugation at 105 000 *g* for 1 hr.

The following method was routinely applied to the soluble material derived from platelets isolated from 40 units of whole blood:

(i) The supernatant solution obtained after centrifugation, or from glycerol-lysis, was dialyzed against 100 vol of distilled water in the cold for 72 hr with 2–3 changes per day. A precipitate formed gradually and was separated from the soluble fraction by centrifugation at 10 000 rpm (18 000 *g*) for 20 min.

(ii) The pellet was dissolved in 50 ml of buffer (0.05 M Tris–HCl; 0.001 M EDTA, pH 8.4); this step was facilitated by the use of the sonicator.

(iii) The pH was lowered to 7.4 with dilute hydrochloric acid and the mixture was centrifuged in the S.W. 30 rotor of the Beckman Model L ultracentrifuge at 30 000 rpm (105 000 *g*) for 4 hr.

(iv) The clear supernatant solution was allowed to come to room temperature and saturated ammonium sulfate solution, adjusted to pH 7.4, was added to give a final concentration of 30% saturation.

(v) The mixture was immediately centrifuged at 10 000 rpm (18 000 *g*) for 20 min and the resulting precipitate was discarded.

(vi) The supernatant solution was desalted by gel filtration on a column of Sephadex G-25 (5 × 34 cm) which had been equilibrated with 0.05 M Tris–HCl buffer (pH 7.4), and was concentrated by pressure dialysis to a volume of 10 ml.

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(vii) The solution was cooled to 4°C, adjusted to pH 5 with 0.1 N HCl and immediately centrifuged at 10 000 rpm (18 000 *g*) for 20 min.

(viii) The precipitate was discarded and the supernatant solution dialyzed against Tris–HCl (0.05 M, pH 7.4) and concentrated to 5 ml by pressure dialysis.

(ix) The dialysate was subjected to gel filtration on a column (2.5 X 90 mm) of Sephadex G-200 in 0.05 M Tris–HCl (pH 7.4). Two main peaks were obtained (fig. 1); the first peak, which contained the soluble glycoprotein, was eluted at close to the V_0 value of the column and was well resolved from the second peak, which was devoid of sialic acid.

(x) In the last step of purification, the pooled fractions of Peak 1 were reduced in volume to 5 ml by pressure dialysis and saturated ammonium sulfate solution was added in the cold to a final concentration of 60% of saturation. The pH was carefully adjusted to 3 with 0.2 N sulfuric acid using a pH meter and the mixture was centrifuged at 10 000 rpm for 20 min at 4°C. The supernatant solution was dialyzed exhaustively against distilled water at 4°C and then freeze-dried.

3. Results

Analysis of isolated platelet membranes in SDS-acrylamide gel showed a complex pattern of protein banding (fig. 2, tube 1) together with three main glycoprotein components of relatively low anodal mobility and, in addition, a faint, discrete glyco-

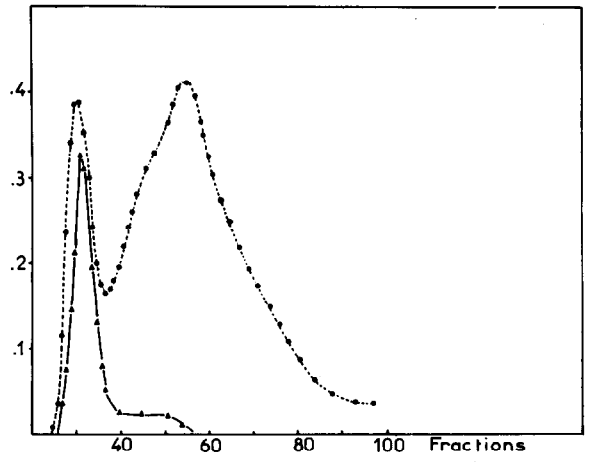

Fig. 1. Gel filtration of partially purified glycoprotein on Sephadex G-200. Column size, 2.5 X 90 cm; fraction volume, 5 ml; buffer, 0.05 M Tris–HCl, pH 7.4; flow rate, 9 ml/hr ordinate; OD_{280} ···●···; OD_{549} ——▲——.

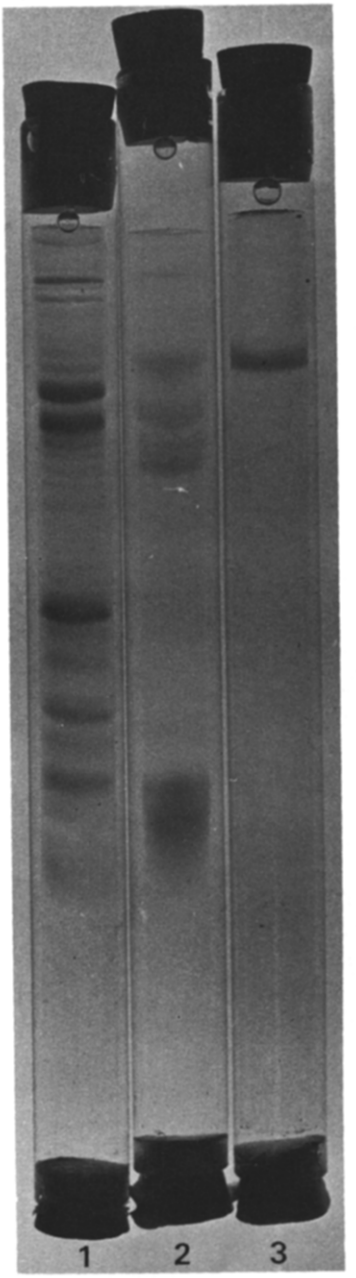

Fig. 2. SDS-acrylamide gel electrophoresis: (1) platelet membranes, Coomassie Blue stain; (2) platelet membranes, PAS stain; (3) soluble cytoplasm, PAS stain.

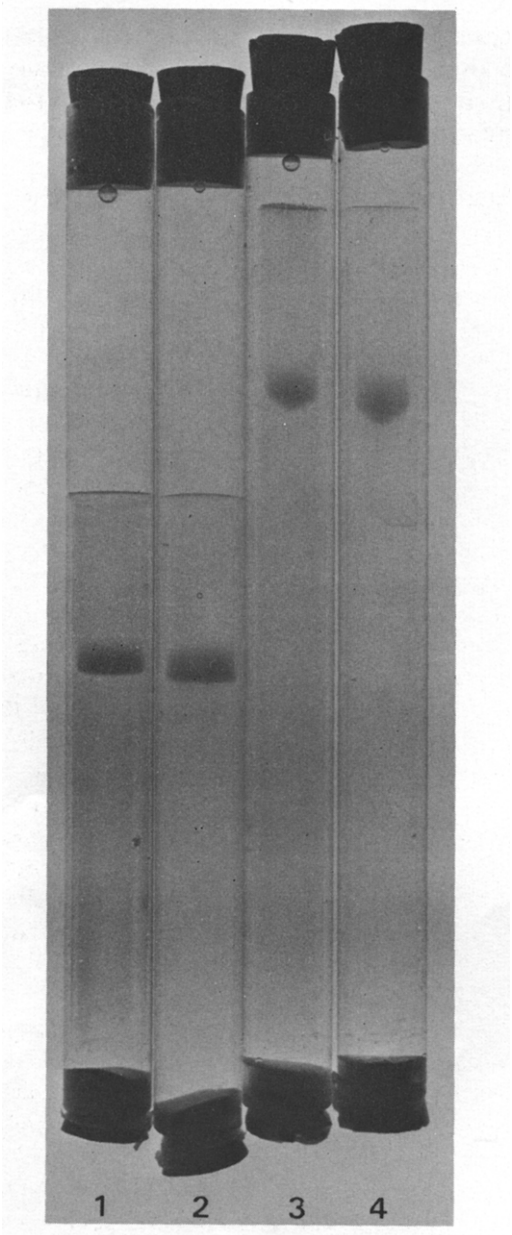

Fig. 3. Acrylamide gel electrophoresis of purified platelet glycoprotein: (1) standard gel, Coomassie Blue stain; (2) standard gel, PAS stain; (3) SDS gel, Coomassie stain; (4) SDS gel, PAS stain.

protein band close to the origin and a pronounced glycolipid band of high mobility (fig. 2, tube 2). The slowest of the three glycoprotein bands was also found to be present in the soluble supernatant fraction resulting from the glycerol-lysis technique, or from platelet sonication (fig. 2, tube 3).

The procedure described here permits the isolation of the soluble glycoprotein in pure form. The product gave a single band on polyacrylamide gel electrophoresis, both in the presence and absence of SDS, using either the Coomassie Blue or periodic acid–Schiff stains (fig. 3).

Treatment with trypsin gave rise to the macroglycopeptide as determined by its behavior on gel filtration and gel electrophoresis, both in the presence and absence of SDS. The molecular weight of the glycoprotein was 148 000, as determined by SDS-acrylamide gel electrophoresis using standard markers (fig. 4). In the absence of SDS the molecular weight was 138 000, as determined on the basis of

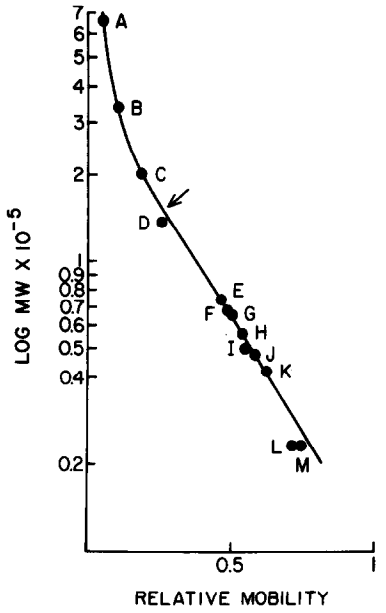

Fig. 4. Determination of molecular weight of glycoprotein by electrophoresis in SDS-polyacrylamide gel. Standard markers A, thyroglobulin (670 000); B, fibrinogen (330 000); C, myosin (200 000); D, bis albumin (135 000); E, transferrin (74 000); F, fibrinogen, α-chain (68 000); G, albumin (67 000); H, fibrinogen, β-chain (56 000); I, γ-globulin H-chain (50 000); J, fibrinogen γ-chain (47 500); K, ovalbumin (45 000); L, γ-globulin L-chain (23 500); M, trypsin (23 000). Relative mobility was calculated as the ratio of the distance travelled by the protein compared with that travelled by the marker dye. The arrow shows the relative mobility of the purified glycoprotein.

the ratio of the logarithms of the electrophoretic mobilities of the glycoprotein and its tryptic macroglycopeptide, which is known to have a molecular weight of 120 000 [1].

The principal amino acids present in the glycoprotein are threonine and serine but with significant amounts of leucine and aspartic acid, and, to a lesser extent, of lysine, proline and glycine; neither cysteine nor methionine were detected (table 1). Carbohydrate comprises nearly two-thirds of the glycoprotein, on a molar basis. Galactose is the principal monosaccharide, with sialic acid being present in about half the amount, and with glucosamine and galactosamine being present in equimolar quantities. Fucose, mannose and glucose are present in trace amounts only.

Table 1
Amino acid composition of platelet glycoprotein (moles %)

Carbohydrate		Amino acids			
Man	1.4	Lys	3.0	Gly	2.9
Fuc	2.4	His	1.2	Ala	1.4
Gal	25	Arg	1.6	Cys	Not detected
Glc	2.6	Val	1.4	Met	Not detected
NANA	13	Asx	3.4	Ileu	0.9
$GlcHN_2$	9.2	Thr	3.9	Leu	3.3
$GalNH_2$	8.1	Ser	3.7	Tyr	0.9
	61.7	Glx	2.5	Phe	2.1
		Pro	3.1		
					35.3

4. Discussion

Three well defined glycoprotein bands can be seen on SDS-acrylamide gel electrophoresis of platelet membranes. Attempts to purify these glycoproteins following solubilization of the membranes with lithium iodosalicylate, with detergents such as SDS or Triton X-100, or with organic solvents such as pyridine, methyl cellosolve or chloroethanol, were unsatisfactory because of the tendency of these platelet components to aggregate and become insoluble in aqueous buffers.

An alternative method for the purification of one of the glycoproteins was isolation from the soluble fraction obtained from whole platelets after sonication or following glycerol-lysis. The slowest of the three bands is present in the supernatant solution following centrifugation of either of these preparations at 105 000 *g*.

Since aggregation phenomena were still encountered with this fraction an attempt was made to put these to use for further purification. These aggregates precipitated at very low ionic strength, as in distilled water, and the soluble glycoprotein co-precipitated with them. On the other hand, the aggregates dissociated at high ionic strength near their isoelectric point [6], as in the case of the ammonium sulfate precipitation step, and the dissociated soluble glycoprotein remained in the supernatant solution. This step was essential for the succes of the gel filtration step on Sephadex G-200 which resolved the components into two distinct peaks only after salt fractionation.

Two other points appeared to be of importance in the purification procedure. First, in order to avoid the action of proteases present in the platelet [7] the isolation procedure should be carried out as rapidly as possible and, except for the ammonium sulfate step, the temperature should not be allowed to rise above +4°C. The dialysis step following homogenization should also be accelerated by changing the water several times in the first few hours. Second, the time of centrifugation is critical at the ammonium sulfate step, too brief a period resulting in incomplete separation and losses due to precipitation.

The glycoprotein produced by this procedure appears not to be aggregated, for the following reasons; first, it runs as a single band in both regular and SDS gels; second, all the carbohydrate material enters the gels; third, it elutes slightly behind the V_0 on Sephadex G-200. It corresponds to the membrane glycoprotein of lowest electrophoretic mobility. The similarity of the molecular weights calculated from electrophoresis in the presence and absence of SDS strongly suggests that this glycoprotein occurs as a single polypeptide chain.

The isolation of this glycoprotein in soluble form permits studies on its structural and immunochemical relationships. Its isolation in a form which is apparently identical with that present on the surface of the platelet, or of the isolated membrane, also raises questions of possible biosynthetic relationships.

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