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CHARACTERIZATION OF HUMAN BLOOD PLATELET MEMBRANE PROTEINS AND GLYCOPROTEINS BY THEIR ISOELECTRIC POINT (p*I*) AND APPARENT MOLECULAR WEIGHT USING TWO-DIMENSIONAL ELECTROPHORESIS AND SURFACE-LABELLING TECHNIQUES

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Summary

Intact human blood platelets were radioactively labelled at the surface by techniques specific for proteins or glycoproteins. Labelled platelet samples were analyzed by a high-resolution two-dimensional separation system involving isoelectric focusing in the first dimension and discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis in the second. The major platelet membrane glycoprotein (GP) bands (Ib, IIb, IIIa and IIIb) were found to be highly heterogeneous even after removal of terminal sialic acid residues. Lactoperoxidase-catalyzed iodination of platelets showed that the major labelled proteins (Ib, IIb, IIIa and IIIb) had altered isoelectric points (p*I*) and molecular weights after neuraminidase treatment. A number of membrane glycoproteins previously undetected by one-dimensional gel electrophoresis were demonstrated and good evidence provided that the major platelet surface proteins are glycosylated.

Introduction

Membrane glycoproteins have been implicated in vital platelet activities such as adhesion and aggregation by studies on platelets with genetically transmitted membrane defects [1—3].

Four major glycoproteins (Ib, IIb, IIIa and IIIb) and a number of minor glycoproteins have been identified depending on the electrophoretic separation,

staining and labelling techniques used [4—8]. It was shown that in Bernard-Soulier syndrome where GP Ib is absent the platelets do not agglutinate to factor VIIIR:WF and ristocetin [5] and in Glanzmann's thrombasthenia where GP IIb and IIIa are greatly reduced or absent no platelet aggregation to ADP, collagen or adrenalin occurs [9,10]. However, very little is yet known about the chemical composition, the structure-functional relationship and the mode of action of these membrane glycoproteins. Platelets are very rich in membrane sialic acid, having approx. 11 times the concentration found in red blood cells [11]. The question therefore arises of the role which this high concentration of sialic acid plays in specialized platelet function.

We have demonstrated previously that several major platelet glycoproteins show charge heterogeneity on isoelectric focusing [12]. In this paper we have examined the distribution of surface-exposed platelet proteins and glycoproteins, radioactively labelled by various techniques, after separation by two-dimensional gel electrophoresis and have studied the effect of neuraminidase treatment on the heterogeneity of the glycoproteins.

Materials and Methods

Ampholines (3.5—10, 5—7) were from LKB Produkter, Bromma, Sweden. Urea, NaOH, H_3PO_4 and glycerol were from Merck, Darmstadt, FRG. Agarose (Indubiose A37) was from Industrie Biologique Française, Paris. Nonidet P-40 (NP-40) was from Fluka, Switzerland. All other chemicals, enzymes, radioactive-labelling reagents and photographic films used were obtained as previously indicated [8]. Platelets isolated from blood (50—60 ml) from a number of consenting, healthy, adult donors were washed as previously shown [8]. Washed platelets were surface-labelled by galactose oxidase and NaB^3H_4; neuraminidase, galactose oxidase and NaB^3H_4; sodium metaperiodate and NaB^3H_4; and lactoperoxidase and ^{125}I as previously described [8]. Platelets treated by neuraminidase, lactoperoxidase and ^{125}I were first incubated with neuraminidase prior to labelling with ^{125}I. Labelled platelets were then immediately prepared for two-dimensional separation. The two-dimensional electrophoretic separation was similar to that of Clemetson et al. [12], separation of isolated platelet membrane proteins or whole platelet proteins using the technique adapted from that of O'Farrell [13] except for the following changes.

Isoelectric focusing. A mixture of ampholytes was used which gave a better resolution in the pH 6 region of the gel. The isoelectric focusing gels were pre-run for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. Platelet samples solubilized and reduced, containing 80 μg of protein were loaded on the gels. The isoelectric focusing was carried out for 18 h at 300 V and 1 h at 800 V.

SDS-polyacrylamide gel electrophoresis. A 1.5 mm thick, 7.5% polyacrylamide Laemmli slab gel with a 3% polyacrylamide stacking gel was used as the second dimension. A slot was made in the stacking gel to allow standard proteins or labelled platelet samples to be electrophoresed in parallel. The gels were fixed, stained and prepared for fluorography or indirect autoradiography as previously described [8].

TABLE I

CHARACTERIZATION OF PLATELET MEMBRANE PROTEINS AND GLYCOPROTEINS BY THEIR p*I* AND APPARENT MOLECULAR WEIGHT AFTER SURFACE LABELLING AND SEPARATION BY POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING FOLLOWED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

P + ^{3}H = periodate and NaB^{3}H$_4$; G + ^{3}H = galactose oxidase and NaB^{3}H$_4$; N + G + ^{3}H = neuraminidase, galactose oxidase and NaB^{3}H$_4$; (these labelling techniques specifically label glycoconjugates found on the cell surface). L + ^{125}I = lactoperoxidase and ^{125}I; N + L + ^{125}I = neuraminidase, lactoperoxidase and ^{125}I (these labelling techniques label proteins including glycoproteins found on the cell surface). p*I* = Isoelectric point. The glycoprotein (GP) nomenclature used for one-dimensional electrophoresis [8] (see left-hand column) was modified in order to differentiate between the acidic and basic components of a particular glycoprotein (see right-hand column) (see Nomenclature).

GP	Nomenclature	Labelling technique									
		P + ^{3}H		G + ^{3}H		N + G + ^{3}H		L + ^{125}I		N + L + ^{125}I	
Old	New	Ranges of p*I*	Apparent molecular weight (X10^3)	Ranges of p*I*	Apparent molecular weight (X10^3)	Ranges of p*I*	Apparent molecular weight (X10^3)	Ranges of p*I*	Apparent molecular weight (X10^3)	Ranges of p*I*	Apparent molecular weight (X10^3)
Ia	Ia	5.4—6.2	162	5.3—6.4	164	5.4—5.6	164	4.8—5.4	168	4.5—5.4	166
	IbA2	4.6—5.4	150	4.7—5.6	156	4.4—6.4	151	4.1—5.4	152	4.5—5.7	156
Ib	IbA1	5.5—6.3	139	5.3—6.2	146	4.4—6.4	151	5.0—6.0	137	4.5—5.7	156
	IbB1	7.4	143	6.4—7.6	143	7.5	145				
	Ic/IIa(?)			5.9—6.5	136	6.1	140			5.1—5.7	137
	IIbA2	4.3	133			4.4	136			4.5—5.2	130
IIb	IIbA1	5.3—5.8	132	5.4—6.1	129	5.5—6.1	136	4.6—5.2	131	5.2—5.8	130
	IIIaA2	4.3	117			4.4	119			4.5—5.0	113
IIIa	IIIaA1	5.3—5.6	113	5.6—6.0	111	5.4—5.8	118	4.8—6.0	112	5.0—5.7	113
	IIIc					5.6—5.8	107				
	IIIbA2	4.3	93	4.4	86						
IIIb	IIIbA1	5.3—6.3	95	5.5—6.9	91	6.9—7.6	96.0	3.4—5.0	95	6.6—8.1	89
IV	IV	7.6—7.9	83	6.6—7.4	82	7.9	86.0				
V	V	4.3	62			4.4	66				

Nomenclature. The platelet glycoprotein nomenclature is based on the separation of glycoproteins on SDS-polyacrylamide gels according to their molecular weight. It is inadequate in the bi-dimensional system presented in this paper since it does not take into consideration glycoprotein components which have a similar molecular weight but different p*I* values. We have adopted the following modification of the present nomenclature: where several spots or bands are observed at a given molecular weight but with different p*I* values we have added an extra letter to represent the acidic (A) or basic (B) p*I*. Where more than one acidic or basic component is observed an extra number is added working outwards from pH 7.0 towards the acidic or basic extremes (e.g., IbA1 which is less acidic than IbA2 which is the most acidic spot and IbB1 which is in the basic region of the pH gradient). However, when only one component was observed for a glycoprotein the established glycoprotein-nomenclature was used (see Table I).

Results

Sodium metaperiodate and NaB^3H_4 labelling

Fig. 1 shows the fluorogram of the two-dimensional separation after 4 weeks of exposure at −70° C. The most intensively labelled glycoproteins (GP) are, respectively: Ib, IIb, IIIa, IIIb, IV and V. With the exception of GP Ia (5.4–6.2, 162 000)(within the brackets are shown the apparent p*I*, and the apparent molecular weight, respectively), GP IV (7.6–7.9, 83 000) and GP V (4.3,

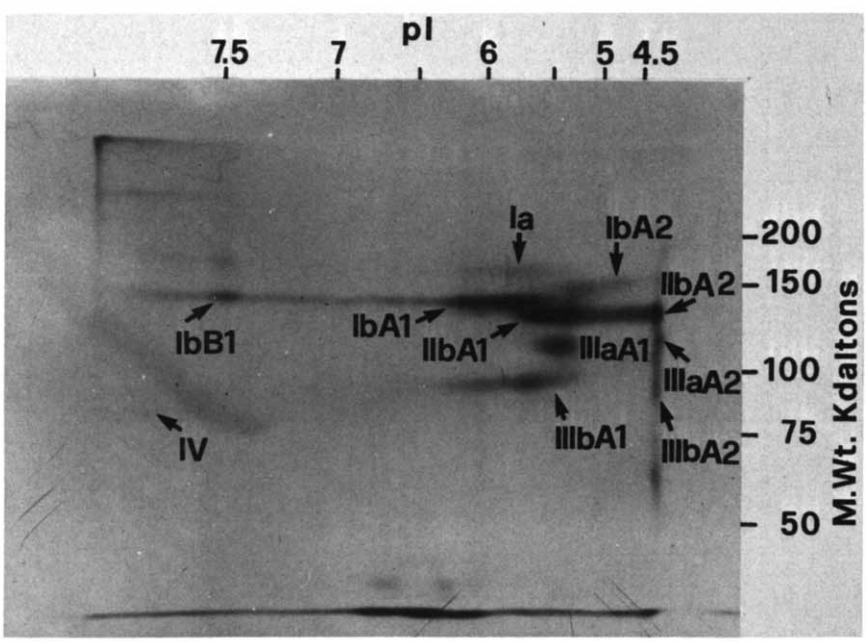

Fig. 1. Fluorogram of two-dimensional separation of intact platelets labelled by sodium metaperiodate and NaB^3H_4 after 4 weeks exposure.

62 000), all the major glycoproteins (Ib, IIb, IIIa and IIIb) have minor bands migrating at the same molecular weight but with different p*I* values. In this respect the most interesting glycoprotein is GP Ib which displays three bands (IbA1, IbA2, IbB1) with different p*I* values, slight differences in apparent molecular weights and with one of them (GP IbA1) intensely labelled. GP

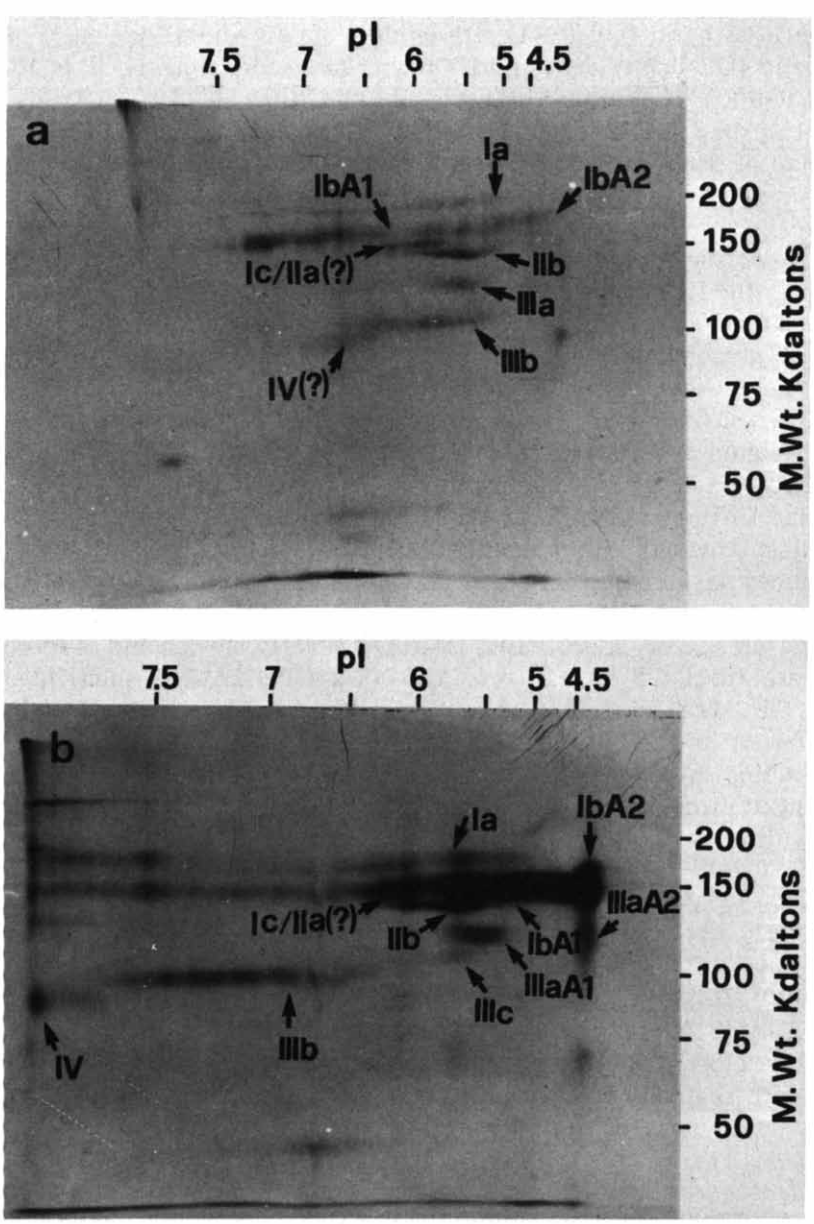

Fig. 2. Fluorograms of two-dimensional separations of intact platelets labelled by: (a) galactose oxidase and NaB^3H_4 after 8 weeks exposure; (b) neuraminidase, galactose oxidase and NaB^3H_4 after 4 weeks exposure.

IbA1 migrated close to GP IIbA2, masking another glycoprotein (GP Ic/IIa?) which was clearly visible after treatment of platelets with neuraminidase.

Galactose oxidase and NaB^3H_4 labelling

Fig. 2a shows the fluorogram of the two-dimensional separation after 8 weeks exposure at −70° C. Two intensely labelled bands (GP Ib and IIIb) and five other faintly labelled bands (GP Ia, Ic/IIa, IIb, IIIa and IV) can be seen. GP Ia (5.3—6.4, 164 000) is slightly above GP Ib which displays two bands, one elongated band (GP IbA1) stretching from an acidic to a basic p*I* and an acidic band (GP IbA2) slanting to a higher molecular weight. GP Ic/IIa (?) (5.9—6.5, 136 000) is located between Ib and IIb. GP IIb (5.4—6.1, 129 000), IIIb (5.5—6.9, 91 000) and IV (6.6—7.4, 82 000) are elongated and made up of a line of discrete spots whereas GP IIIa (5.6—6.0, 111 000) is compact.

Neuraminidase, galactose oxidase and NaB^3H_4 labelling

Fig. 2b shows the fluorogram of the two-dimensional separation after 4 weeks exposure at −70° C. There is a drastic alteration in the p*I* and molecular weight of all glycoproteins compared to the results obtained by labelling terminal sialic acid (see above). The least affected are GP Ia (5.4—5.6, 164 000), IV (7.9, 85 600) and V (4.4, 66 000) which have altered p*I* values and molecular weights and one of them (GP IV) changed from an elongated to a circular spot. A large intensely labelled glycoprotein band (4.4—6.4, 151 000) is found in the normal positions of GP IbA1 and GP IbA2, with its more basic end subdivided into several minor components, GP IbB1 (7.5, 145 000) is reduced in labelling intensity and GP Ic/IIa (?) (6.1, 140 000) is found between the major GP Ib band and GP IIA2. GP IIbA1 (5.5—6.1, 135 000) became elongated, more basic, migrated slightly slower and is more distinctly separate from GP IIbA1 (4.4, 136 000). GP IIIaA1 is subdivided into two bands, GP IIIaA1 (5.4—5.8, 118 000) and GP IIIc (5.6—5.8, 107 000), of slightly different p*I* and molecular weight. GP IIIaA2 (4.4, 119 000) increased in labelling intensity whereas GP IIIbA2 is not visible. GP IIIbA1 completely changed from an acidic to a basic position (6.9—7.6, 96 000) and in place of many discrete spots now shows three to four major spots.

Lactoperoxidase and ^{125}I-labelling

As shown in Fig. 3a, GP IIIa (4.8—6.0, 112 000) is the most intensely labelled band followed, respectively, by GP IIb (4.6—5.2, 131 000), IbA1 (5.0—6.0, 137 000), IbA2 (4.1—5.1, 152 000), Ia (4.8—5.4, 168 000) and IIIb (3.4—5.0, 95 000). GP IIIa and IIb are elliptical with GP Ic/IIa (?) and part of GP Ib migrating close to GP IIb. GP Ib is composed of two bands (GP IbA1 and GP IbA2) with slight differences in their molecular weights, one of them (GP IbA1) with quite an extensive p*I* range.

Neuraminidase, lactoperoxidase and ^{125}I-labelling

As with neuraminidase, galactose oxidase and NaB^3H_4, extensive changes in the shape, p*I* and molecular weight of the glycoproteins can be seen (Fig. 3b). GP IIIa consists of two bands one which moved to a more basic p*I* (GP IIIaA1,

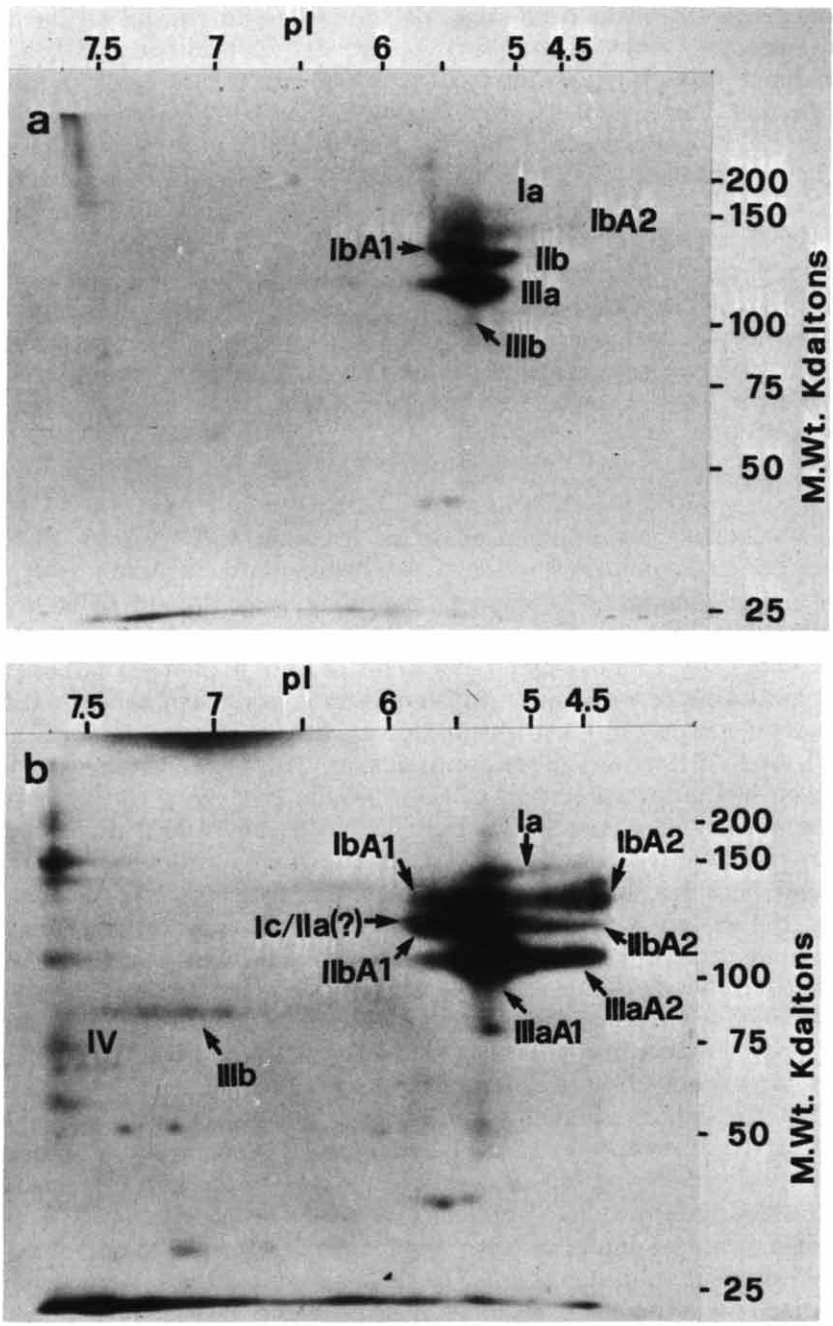

Fig. 3. Indirect autoradiograms of two-dimensional separations of intact platelets labelled by: (a) lactoperoxidase and ^{125}I after 2 days exposure; (b) neuraminidase, lactoperoxidase and ^{125}I after 2 days exposure.

5.0—5.7, 113 000) and the other (GP IIIaA2, 4.5—5.0, 113 000) was an elongated band stretching to the acidic edge of the gel. GP IIb also moved to a more basic p*I* (GP IIbA1, 5.2—5.8, 130 000) but it has a small, intensely labelled

band stretching from the main band towards the basic end of the gel (as in neuraminidase, galactose oxidase and NaB^3H_4, see Fig. 2b) and it also has a faint labelled band (GP IIbA2, 4.5—5.2, 130 000) stretching towards the acidic end of the gel. Part of GP Ib which migrated close to GP IIb (see lactoperoxidase and ^{125}I, or periodate and NaB^3H_4, Figs. 1 and 3) moved to a higher molecular weight so that GP Ib (5.7—4.5, 156 000) is made up of one band except for its acidic end which was split with a minor component migrating at a slightly higher molecular weight (4.5, 161 000). GP Ic/IIa (?) (5.1—5.7, 137 000) is clearly present between IIb and Ib as in neuraminidase, galactose oxidase and NaB^3H_4. GP Ia (4.5—5.4, 166 000) migrated with slight differences in its p*I* and molecular weight. GP IIIb (6.6—8.1, 89 000) has a drastically altered p*I*, moving towards the basic end of the gel. All five major glycoproteins (Ia, Ib, IIb, IIIa and IIIb) labelled by lactoperoxidase are affected by neuraminidase pretreatment.

Discussion

The two-dimensional separation (isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis) of surface-labelled platelets shows heterogeneity of the major membrane glycoproteins which were thought to be homogeneous by one-dimensional SDS-polyacrylamide gel electrophoresis [7,8]. The charge heterogeneity found may be due to differences in charged carbohydrates present in the glycoprotein, to differences in peptide sequence, to the altered positions of amide groups or disulphide bridges in otherwise identical molecules [16], or to differences in phosphorylation [15]. However, great care has to been taken in the interpretation of these results in view of the possible effects that surface-labelling techniques may have on the p*I* and molecular weight of the various glycoproteins (e.g., effects of neuraminidase, galactose oxidase and NaB^3H_4, Fig. 2 and Table I). Artifacts may also be present in the first dimension which alter the p*I* of separated components. However, the two-dimensional patterns obtained were consistently reproduced a great number of times and the individual components showed the same p*I* and/or heterogeneity when longer isoelectric focusing times were used despite some gradient drift. Furthermore, results obtained by staining two-dimensional gels of labelled or unlabelled platelet samples with periodic acid-Schiff reagent (which has a bias for sialic acid residues of glycoproteins) and Coomassie blue R250 were similar to those obtained with labelling and fluorography or autoradiography. Different concentrations of urea (8 M, 9.5 M) in the first dimension gels and the initial solubilization of platelets in 1% NP-40 instead of SDS in the presence of a protease inhibitor gave similar results for the major glycoproteins and proteins (unpublished results) to those presented here.

Results obtained by Clemetson et al. [12] using a two-dimensional separation with staining techniques (periodic acid-Schiff reagent, Coomassie blue R250) on isolated membranes and whole platelets were largely similar to those shown here for the major glycoproteins. Differences (e.g., the absence of certain minor bands, or different band shapes) may be due to the different type and pH range of ampholytes used and to the poor sensitivity of the periodic acid-Schiff reagent for sugars other than sialic acid.

GP Ib (or GP I) is thought to be a receptor for thrombin and for ristocetin:

factor VIIIR:WF induced agglutination [17—19]. Furthermore, with a severe reduction or absence of GP Ib platelet adhesion to subendothelium does not occur [20]. Two-dimensional separation of GP Ib shows three components: IbA1, IbB1 having similar molecular weights but different p*I* values and IbA2 having a slightly higher molecular weight and a very acidic p*I*. It is possible that variation of sialic acid content of this glycoprotein could account for part of the observed heterogeneity. Platelets homogenized in the absence of EDTA or treated with 3 M KCl release a soluble glycoprotein [14,21] known as glycocalicin which has been reported to inhibit thrombin-induced aggregation and ristocetin-induced agglutination in normal platelets [22]. However, detergent-solubilized GP Ib was shown by Cooper et al. [23] to inhibit bovine vWF whereas glycocalicin under similar conditions did not. In addition, glycocalicin has been found to have a different tryptic peptide map from 'GP Ib' [24]. The two-dimensional results may therefore represent GP Ib (GP IbA1), glycocalicin (GP IbA2) and another component having less sialic acid and a more basic p*I* (GP IbB1). The most acidic of the GP Ib components was situated at about pH 4.3 (see Figs. 2 and 3) or 3.5 (unpublished results) depending on the pH gradient used. Pepper and Jamieson [25] using proteolytically cleaved platelet sialoglycopeptides and column electrofocusing found a p*I* of 1.8—2.0. Since the p*I* value of the carboxyl group of free sialic acid is about 2.8 [26] components of GP Ib and probably other acidic glycoproteins migrate to a p*I* lower than that obtained on the pH gradient and therefore may migrate off the gel into the anode electrolyte. Treatment with neuraminidase made the p*I* of the glycoprotein more basic and increased the size of the GP Ib spot, presumably due to the acidic glycoprotein part which formerly was lost.

Intact GP Ib and glycocalicin are not well labelled with lactoperoxidase and ^{125}I [4]. The use of neuraminidase greatly helps in labelling the different GP Ib components probably by removing the sialic acid and allowing lactoperoxidase to penetrate more easily to the peptide backbone. All the major iodinated platelet membrane proteins are greatly affected by neuraminidase treatment. This would indicate that all the major proteins on the platelet surface are glycosylated in agreement with the results of lectin binding studies [5] and with studies on red blood cells and other types of cell [27].

GP Ic/IIa (?) which migrates very close to IIb is clearly visible after neuraminidase treatment. This glycoprotein may be the same as that of Phillips and Poh Agin [6], GP Ic. Desialylation of platelet membrane glycoproteins clearly shows that in addition to the variation of sialic acid, heterogeneity may be due to other charge differences present in the glycoproteins. GP IIIa cross-reacts with an antiserum to muscle α-actinin [28] and is associated with the PL^{A1} alloantigen [29]. After neuraminidase treatment the GP IIIa spot splits into two components having slightly different p*I* values and molecular weights. This subdivision of IIIa may represent either two different glycoproteins which have similar p*I* values and molecular weights or two differently glycosylated versions of the same polypeptide possibly with different functions.

GP IIIb appeared characteristically as a band composed of a number of spots. Neuraminidase treatment produced a dramatic shift from an acidic

to a basic p*I*. Although, after rigorous treatment with neuraminidase, the pattern observed for GP IIIb is less heterogeneous, nevertheless some heterogeneity remains. This glycoprotein has been found to be resistant to trypsin treatment [4]. In a family with the platelet disorder 'Storage pool disease' GP IIIb stained more strongly with periodic acid-Schiff reagent than in normals [30].

GP IV may be the glycoprotein that is released from the platelet membrane by thrombin treatment [31,32] (GP V or thrombin-cleavable glycoprotein). This glycoprotein which was observed as an elongated spot in the basic part of the gel changed to a circular spot after neuraminidase treatment.

The combination of surface-labelling techniques together with two-dimensional electrophoresis showed a much greater number of glycoproteins than were previously found by other electrophoretic separation systems. Rigorous treatment of intact platelets with neuraminidase does not remove completely the heterogeneity of glycoprotein bands suggesting that neuraminidase-resistant or inaccessible sialic acid or differences in peptide sequence or in phosphorylation may be sources of this heterogeneity. Human erythrocyte glycoproteins have been shown not to contain any O-acetylated sialic acid residues, which would not be cleaved by bacterial neuraminidases [33]. It is not yet known if this is the case for human platelet membrane glycoproteins also. Treatment of intact platelets with neuraminidase removes about 60% of the sialic acid present in human platelets [11], however, it is known that internal components such as the granule glycoprotein, thrombospondin [34], contain sialic acid and this could account for the difference. Further work to resolve this problem is in progress.

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