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TRYPTIC PEPTIDE MAP ANALYSIS OF THE MAJOR HUMAN BLOOD PLATELET MEMBRANE GLYCOPROTEINS SEPARATED BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

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Washed platelets were surface-labelled by lactoperoxidase catalyzed iodination and either the platelets or membranes were solubilized in detergent and applied to a wheat germ agglutinin-Sepharose column and a *Lens culinaris* lectin Sepharose column coupled sequentially. The glycoproteins eluted from the lectin columns were separated by two-dimensional gel electrophoresis. Alternatively, labelled whole platelets or membranes were solubilized and then directly separated by two-dimensional polyacrylamide gel electrophoresis. Spots corresponding to specific glycoproteins identified by apparent isoelectric point (p*I*), apparent molecular weight (M_r), staining and labelling characteristics were cut from the gels and analyzed by tryptic peptide mapping. The maps of the individual glycoproteins (GP) Ia, Ib, IIa, IIb, $GP^{4\text{-}4.5}_{132\text{-}135}$, IIIa, IIIb and IIIc were all different. Glycoproteins with the same M_r but different p*I* were distinct with the exception of regions of GP Ib. There were minor differences in the maps of glycoproteins separated in the reduced or non-reduced state. Tryptic peptide maps provide a valuable additional parameter for the identification and characterization of platelet glycoproteins.

1. Introduction

The combination of surface-labelling techniques or use of ^{125}I-labelled lectins with one or two-dimensional (non-reduced, reduced) high resolution sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis has shown that the platelet surface contains at least thirty membrane glycoproteins (GP) [1–7]. The use of two-dimensional gel electrophoresis (isoelectric focusing, SDS-polyacrylamide gel electrophoresis) has greatly added to the resolution of these platelet glycoproteins [7–10]. A number of newly identified glycoproteins have similar apparent molecular weights (M_r) but different isoelectric points (p*I*) to major, relatively well characterized platelet glycoproteins (GP Ib, IIb, IIIa, IIIb and V). It is not known if these newly identified glycoproteins are distinct entities or represent charge modifications of the major glycoproteins.

Little is known about the functions of the majority of the identified glycoproteins. Three platelet glycoproteins (GP Ib, IIb and IIIa), which are intensely radiolabelled by protein or glycopro-

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tein specific techniques or by ^{125}I-labelled lectins, have been implicated in aspects of the platelet haemostatic function. A substantial part of the evidence for the role of GP Ib, IIb and IIIa in platelet adhesion and aggregation comes from studies on platelets from patients with congenital blood disorders [11,12]. Less is known about GP V which is cleaved from the platelet surface by thrombin. The cleavage of this glycoprotein is probably related to the thrombin generated signal for platelet activation [1,12].

A necessary preliminary step in studying the functions of the different glycoproteins and in comparison of different separation and analytical techniques is the characterization of the large number of glycoproteins by more sophisticated parameters than those currently in use. In addition it is necessary to investigate possible relationships between different glycoproteins.

In this paper a number of major platelet glycoproteins isolated by lectin affinity chromatography and/or separated by two-dimensional electrophoresis have been characterized by their tryptic peptide maps and their interrelationships investigated.

This study is part of an investigation aimed at establishing the tryptic peptide maps of platelet membrane glycoproteins as a parameter for their identification in addition to other known properties (M_r, pI, staining with Coomassie blue and periodic acid-Schiff's reagent, labelling by various techniques and the effect of removal of sialic acid by neuraminidase on pI and M_r).

Material and Methods

Platelet washing and labelling

Platelets separated from 400 ml of fresh blood obtained from informed, consenting donors were immediately washed and surface-labelled with ^{125}I as previously described [3]. Labelled platelets were either used for lectin affinity chromatography or membranes were prepared by the method of George et al. [13].

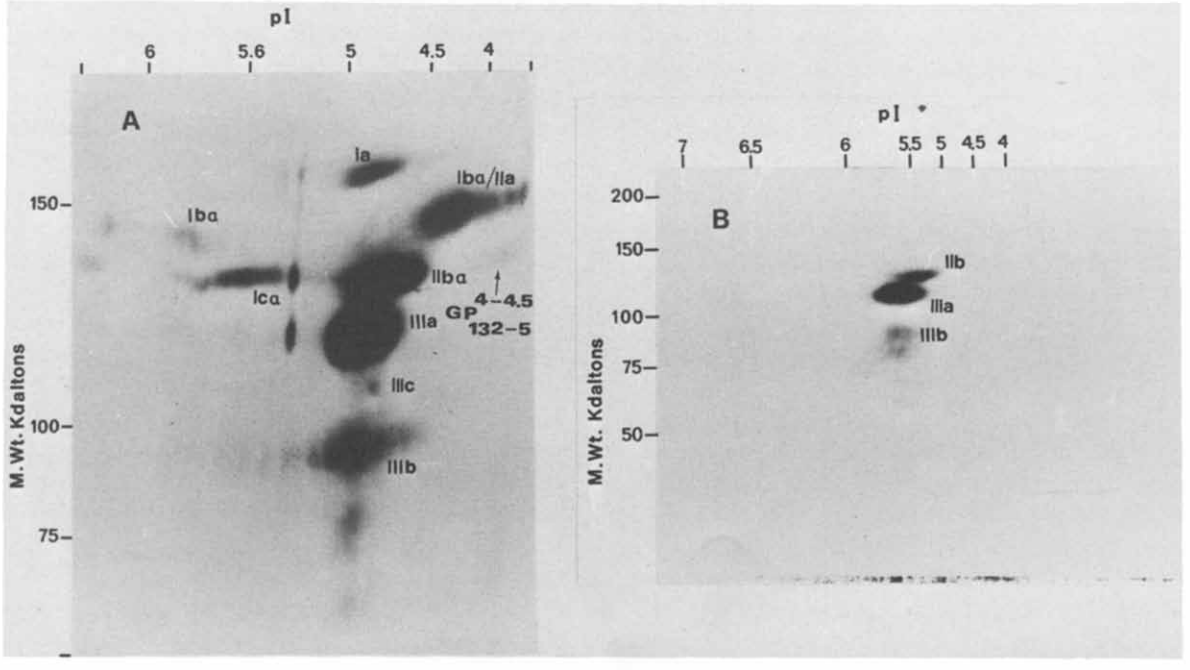

Fig. 1. (A) Autoradiograph of two-dimensional separation of ^{125}I-surface labelled platelet samples (reduced). (B) Autoradiograph of two-dimensional separation of ^{125}I-labelled glycoproteins (reduced) eluted from a *Lens culinaris* Lectin-Sepharose column after affinity chromatography of ^{125}I-surface labelled platelets, solubilized in 1% sodium deoxycholate on sequentially coupled wheat germ agglutinin-Sepharose and *Lens culinaris* lectin columns.

Lectin affinity column chromatography

Whole platelet or platelet membrane suspensions were solubilized by adding solid sodium deoxycholate to a final concentration of 1% (w/v), in the presence of 2 mM phenylmethylsulphonyl fluoride and vortexing until the detergent had dissolved. The samples were then centrifuged at $8000 \times g$ for 10 min and the supernatant centrifuged at $100000 \times g$ for 60 min in an SW 50.1 rotor using an L5 65 Beckman ultracentrifuge. Lectin affinity chromatography on *Lens culinaris* lectin-Sepharose or sequentially on wheat germ agglutinin-Sepharose and *Lens culinaris* lectin-Sepharose was performed as described by Clemetson et al. [14]. The chromatography was followed by measuring the A_{250} and the radioactivity of the column effluent. Fractions containing the glycoproteins eluted with the appropriate sugars (2% methyl α-mannopyranoside or 2.5% *N*-acetyl glycosamine) were pooled, extensively dialysed against $(NH_4)\ HCO_3$, then lyophilized.

Two-dimensional electrophoresis

Lyophilized glycoprotein samples were resuspended in 0.05 M Tris-HCl (pH 6.8) plus *N*-ethylmaleimide (2 mM) and SDS was added to a 2% (w/v) final concentration (for unreduced samples). Samples were then heated at 100°C for 10 min. Two aliquots were taken to measure the protein concentration by the method of Lowry et al. [15] and for determination of radioactivity (^{125}I). Reduced samples were prepared by adding SDS to 2% final concentration and heating to 100°C then removing the aliquots for protein determination. The samples were then made 30 mM in dithiothreitol and heated for a further 5 min at 100°C.

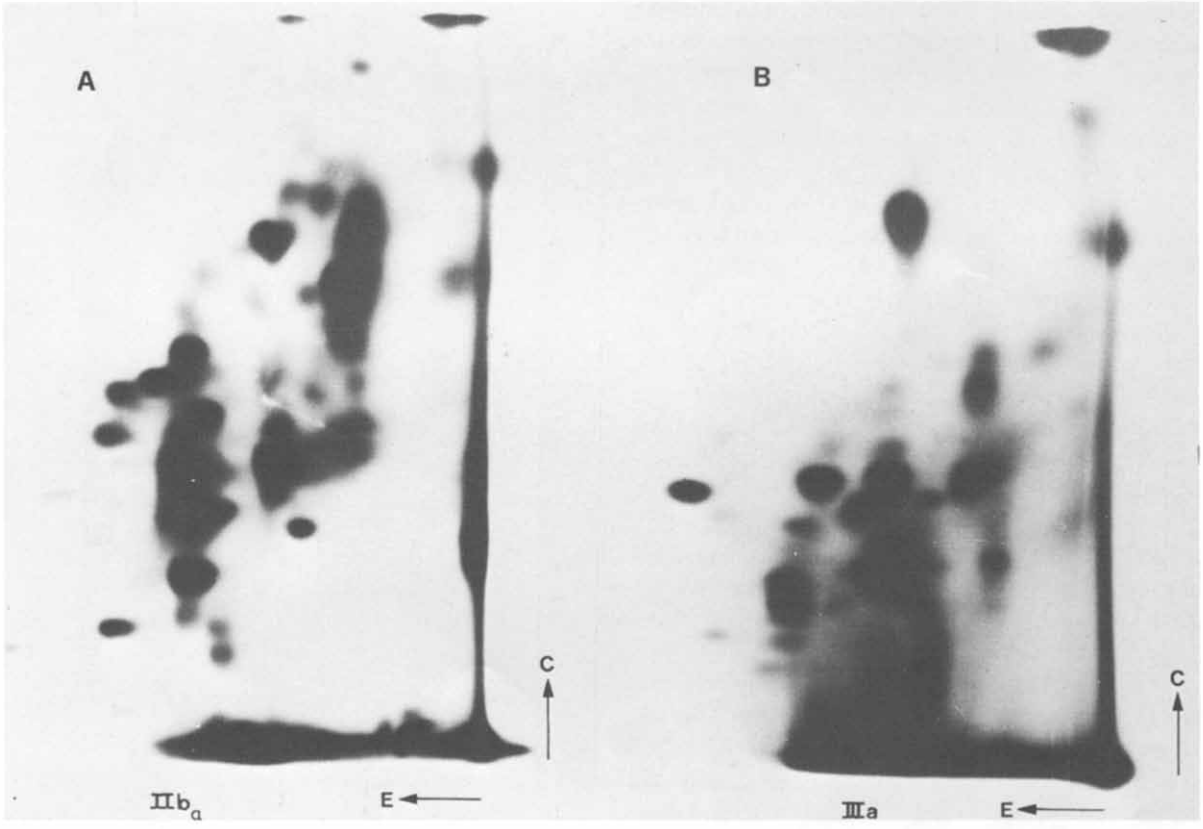

Fig. 2. Autoradiographs of ^{125}I-labelled tryptic peptide maps of glycoproteins cut from two-dimensional gels such as shown in Fig. 1. The origin (application point of tryptic digest) is in the bottom right corner. E denotes the direction of high-voltage electrophoresis and C the direction of thin-layer chromatography. (A) Glycoprotein IIbα. (B) Glycoprotein IIIa.

Samples were either used directly for electrophoresis or were stored at −70°C.

Labelled glycoproteins isolated by lectin affinity chromatography or labelled whole platelet samples were separated either by two-dimensional non-reduced/reduced SDS-polyacrylamide gel electrophoresis [5,16] or by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis [8,17]. After electrophoresis, the gels were fixed, briefly stained with Coomassie blue, destained and either dried or frozen before indirect autoradiography was carried out using preflashed Kodak X-Omat S film and Cronex Lightning Plus (Du Pont) intensifying screens as described by Laskey and Mills [18].

Two-dimensional tryptic peptide map analysis

Glycoproteins identified by molecular weight, p*I*, staining and ^{125}I-labelling properties were cut out from dried or freshly run gels and tryptic peptide mapping was performed as described by Elder et al. [19]. The pieces of gel were extensively washed with 25% isopropanol and then with 10% methanol in a siliconized tube and were then dried under an infrared lamp. To each piece of gel was added 20 μl of sodium phosphate buffer (0.5 M, pH 7.5), 300 μCi of ^{125}I in 5 μl (Amersham International, specific activity 16 Ci/mg) and 5 μl of chloramine T (1 mg/ml). After 30 min, 1 ml of sodium bisulphite (1 mg/ml) was added to stop the reaction. After 15 min the bisulphite solution was removed and the pieces of gel extensively washed with 10% methanol. The gel pieces were then placed in fresh siliconized tubes, dried under an infrared lamp and treated with 0.5 ml of trypsin (50 μg/ml, Sigma) in 0.05 M NH_4HCO_3 buffer, pH 8.0, at 37°C overnight. The supernatants were lyophilized and then analysed by two-dimensional chromatography. The first dimension high voltage electrophoresis was performed on a Desaga Desaphor apparatus cooled to 4°C or 10°C using either (4 or 8) 10 × 10 cm or (1 or 2) 20 × 20 cm cellulose thin-layer chromatography plates (0.1 mm, Merck, Darmstadt, F.R.G) at 1 kV for 50 min. After the second dimension thin-layer chromatography the plates were dried, wrapped in Saran wrap (Dow Chemical Co.) and indirect autoradiography carried out using a pre-flashed X-Omat S film and a barium tungstate intensifier screen as described by Laskey and Mills [18]. Duplicate runs were carried out with each sample. Using surface-labelled platelets and two-dimensional electrophoretic separations [16 or 17] glycoproteins Ia, Ib, IIa, IIb, $Gp^{4\text{-}4.5}_{132\text{-}135}$, IIIa, IIIb and IIIc from six different donors (male and female) were examined. Each glycoprotein sample separated by lectin affinity chromatography was analyzed in quadruplicate by the O'Farrell technique and tryptic peptide mapping was performed on the glycoproteins.

Results

The tryptic peptide maps of glycoproteins IIb and IIIa separated by *Lens culinaris* lectin affinity chromatography followed by two-dimensional polyacrylamide gel electrophoresis [17] of the

Fig. 3. Autoradiograph of ^{125}I-labelled tryptic peptide map performed as described in Fig. 2. Clycoprotein IIb (unreduced), the arrows mark peptides absent compared to the tryptic peptide map of GP IIbα, shown in Fig. 2A.

lectin-bound fractions, were distinctly different. Fig. 1A shows the two-dimensional separation of ^{125}I-surface-labelled reduced whole platelets and Fig. 1B that of the lectin bound fractions in the reduced state. Fig. 2 shows the tryptic peptide maps of glycoproteins IIbα and IIIa isolated from gels like these. When equal concentrations of trypsin digests of glycoproteins IIbα and IIIa were analyzed together as a mixture on the same plate, no peptides with identical mobilities were found (results not shown). Tryptic peptide maps of glycoproteins IIbα and IIIa isolated from SDS solubilized ^{125}I-labelled, whole platelets separated directly by two-dimensional gel electrophoresis (not subjected to lectin affinity chromatography) gave identical results to those obtained using a combination of lectin affinity chromatography and two-dimensional gel electrophoresis [17]. Glycoprotein IIIa separated by two-dimensional unreduced-reduced gel electrophoresis [16] gave a peptide map similar to those obtained for glycoprotein IIIa by the two other techniques (results not shown).

The tryptic peptide maps of GP IIb excised from two-dimensional polyacrylamide O'Farrell gels [17] run under non-reducing or reducing conditions showed minor differences. Under reducing conditions two spots were present in the maps of GP IIbα (Fig. 2A) which were absent or very much reduced in intensity in the maps of non-reduced GP IIb (Fig. 3). A glycoprotein with the same M_r as GP IIb but with a much more acidic pI ($GP^{4\text{-}4.5}_{132\text{-}135}$) on two-dimensional O'Farrell gels gave a different tryptic peptide map to GP IIb (Fig. 4).

Two glycoprotein bands with different pI (4 and 5.5) but similar M_r, previously designated as regions of GP Ib [9] were cut from two-dimensional gels and their tryptic peptide maps prepared. The maps differed slightly with the absence

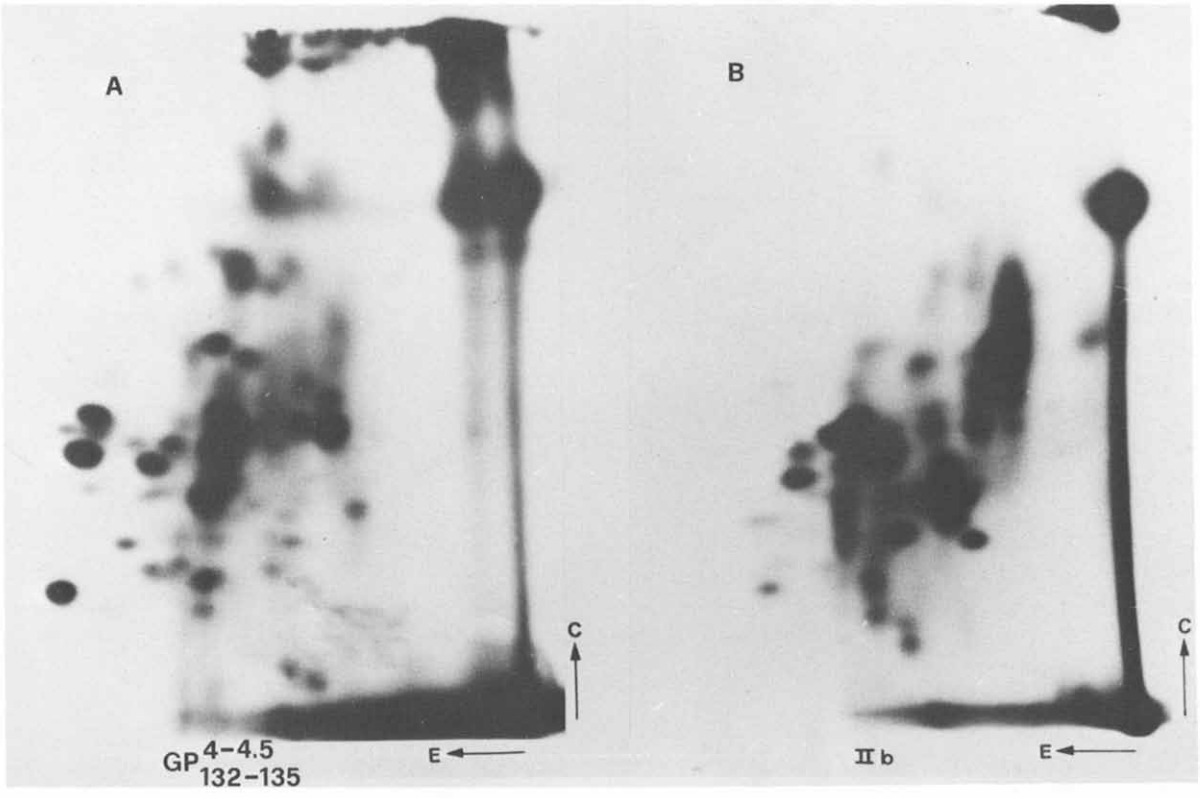

Fig. 4. Autoradiographs of ^{125}I-labelled tryptic peptide maps performed as described in Fig. 2. (A) $GP^{4\text{-}4.5}_{132\text{-}135}$. (B) Glycoprotein IIb (unreduced).

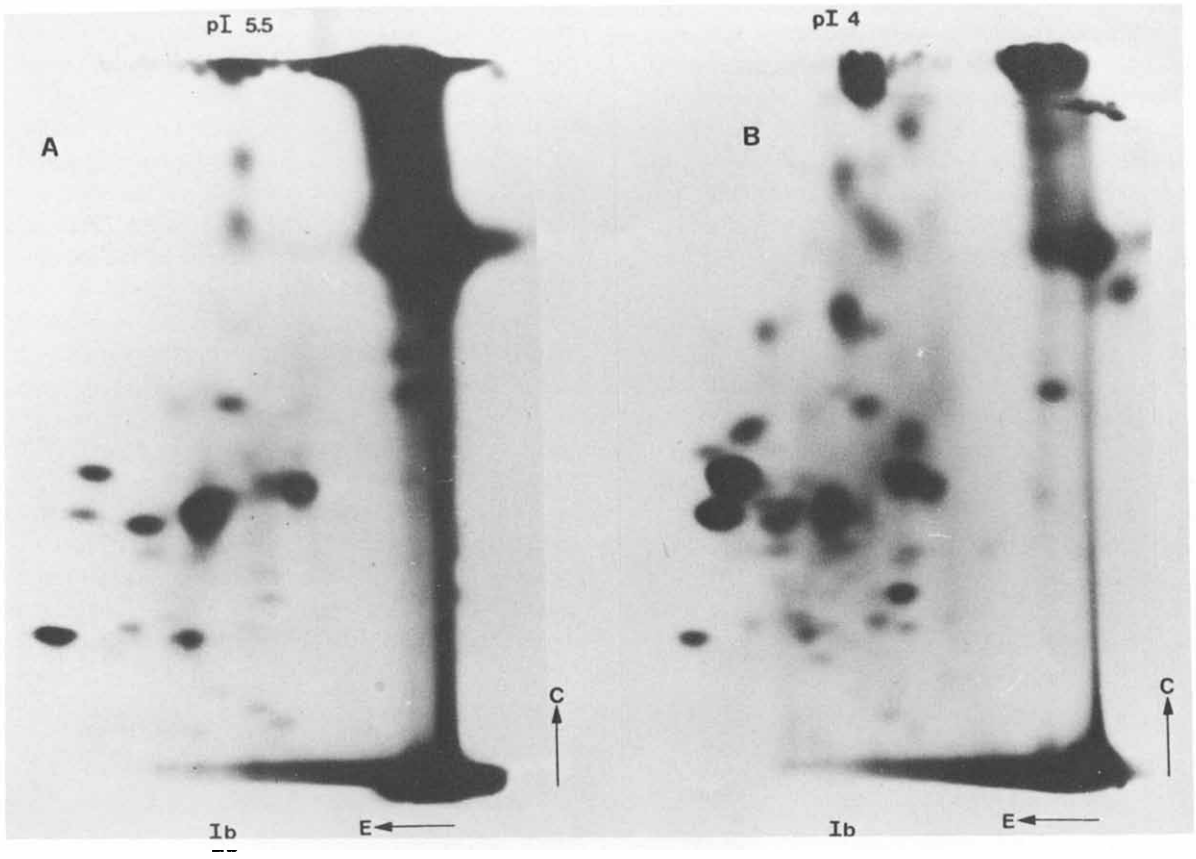

Fig. 5. Autoradiographs of ^{125}I-labelled tryptic peptide maps performed as described in Fig. 2. (A) Glycoprotein Ib (sample cut from the two-dimensional gel in the p*I* 5.5 region. (B) Glycoprotein Ib (Sample cut from the two-dimensional gel in the p*I* 4.0 region).

of a number of peptides and a reduction in the labelling intensities of the peptides of the less acidic region of GP Ib compared to the more acidic region (Fig. 5).

Tryptic peptide maps of a glycoprotein designated GP IIa were carried out on unreduced and reduced material. The map of the reduced form had more peptides than that of the unreduced form (Fig. 6).

Minor differences were also observed between the tryptic peptide maps of non-reduced and reduced GP Ia (Fig. 7). Tryptic peptide maps of GP IIIb and GP IIIc (Fig. 8) also differed from each other and from other platelet glycoproteins.

Discussion

Platelet membrane glycoproteins IIb and IIIa have different tryptic peptide maps indicating that there is little if any sequence homology and implying that both glycoproteins are not derived from a single structural gene. It is unlikely that the different electrophoretic mobilities of the peptides can be ascribed solely to differences in carbohydrate moieties. These results suggest that glycoproteins IIb and IIIa are distinct entities which may form complexes on the platelet surface via non-covalent bonds. Glycoproteins IIb and IIIa may be derived from different structural genes under coordinated expression or the synthesis of both proteins may be necessary for their insertion into the membrane. A further possibility is that both glycoproteins are derived from a single gene and the product is then cleaved after insertion in the membrane. Studies on the amino acid sequence and the glycosylation of these glycoproteins will be necessary to completely resolve this problem while investigations on the precursors synthesized in the megakaryocyte will be necessary in order to completely exclude

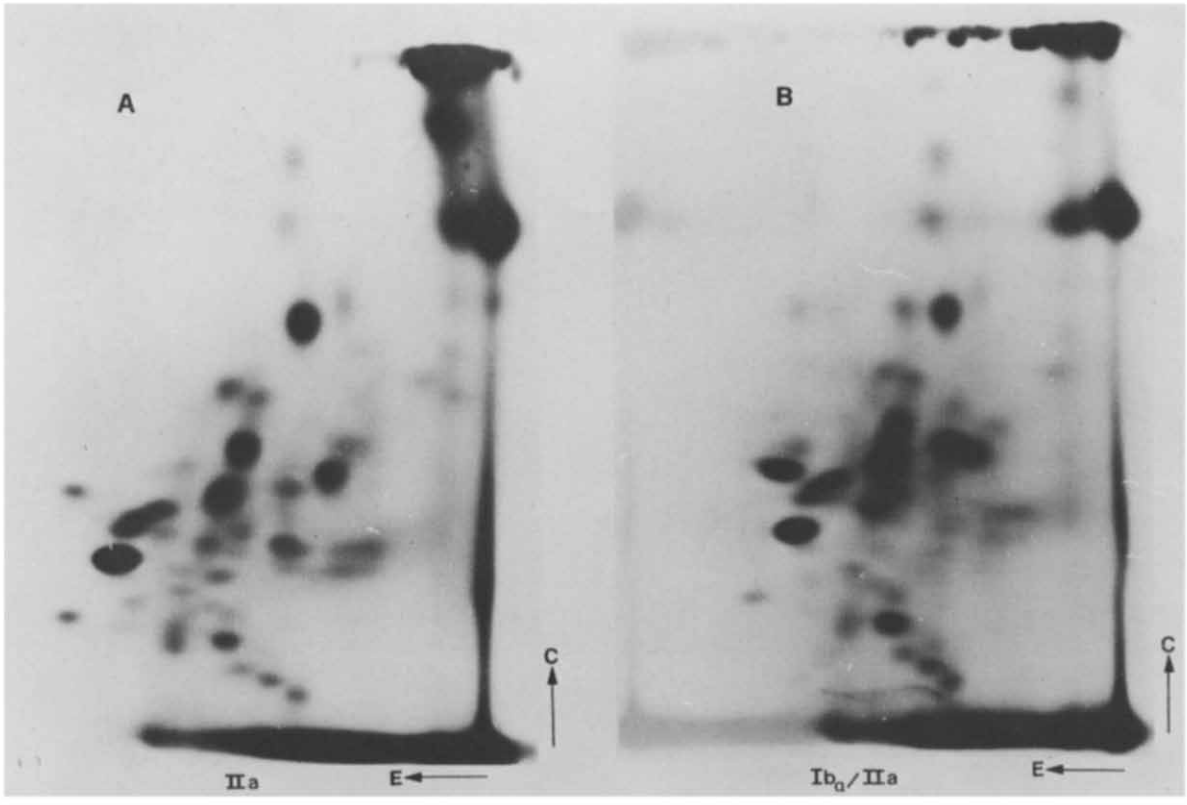

Fig. 6. Autoradiographs of ^{125}I-labelled tryptic peptide maps performed as described in Fig. 2. (A) Glycoprotein IIa (unreduced). (B) Glycoprotein IIa (reduced) cut from the glycoprotein Ibα region of the two-dimensional gel.

the single gene hypothesis.

These results confirm those recently obtained by Nachman et al. [20] using *Lens culinaris* lectin chromatography together with one-dimensional gel electrophoresis. However, in view of the fact that several major platelet membrane glycoproteins have similar M_r but different p*I* (e.g. Ib, Ic, IIa and IIb on the one hand and IIIa and IIIc on the other), to be sure of having an optimal purity it is safer to separate glycoproteins purified on lectin affinity columns by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis. In fact, the tryptic peptide maps obtained by the separation of whole platelets directly on two-dimensional gels shows that this technique alone is adequate to give pure samples. Glycoprotein IIIa could also be isolated in a pure state by two-dimensional unreduced-reduced SDS-polyacrylamide gel electrophoresis, however this was not possible with glycoprotein IIbα due to its spoor resolution from a neighbouring glycoprotein probably glycoprotein Ic.

Glycoprotein IIb was shown to be composed of two nonidentical subunits, an α and a β, joined together by intermolecular disulphide bridges, with the larger subunit also containing intramolecular disulphide bridges [5]. A combination of carbohydrate-specific surface labelling and two-dimensional gel electrophoresis has shown that GP IIbβ is more acidic than GP IIbα (McGregor, J.L., unpublished data). It had been expected that the tryptic peptide map of GP IIb (non-reduced) would show more peptides than IIbα, since it contains both subunits, however, this was not the case. It is possible that GP IIbβ is highly sialylated and thus less easily iodinated or digested by trypsin. Any peptides derived from GP IIbβ would only be visible after longer exposure times which would

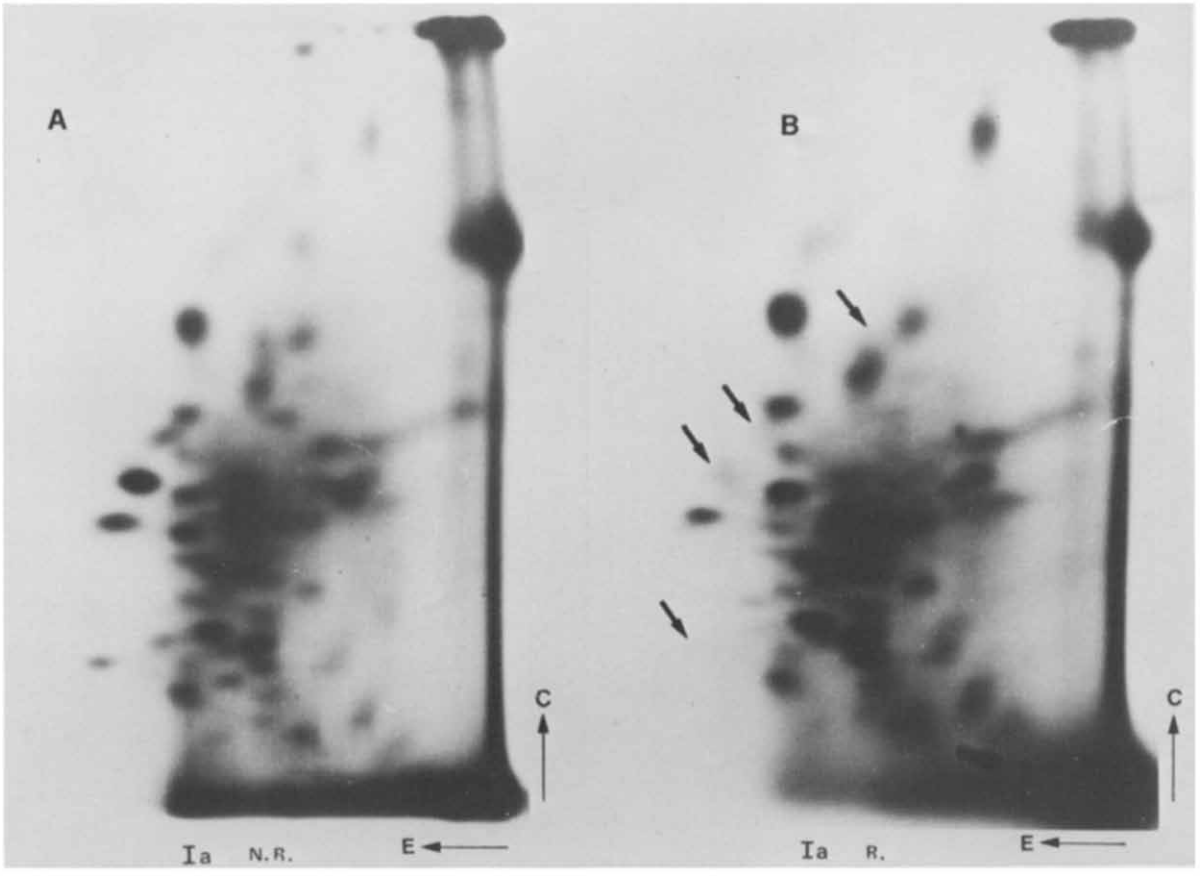

Fig. 7. Autoradiographs of ^{125}I-labelled tryptic peptide maps performed as described in Fig. 2. (A) Glycoprotein Ia (unreduced). (B) Glycoprotein Ia (reduced); the arrows indicate peptides absent in the tryptic digest of the reduced form compared to the unreduced.

overexpose the peptides from GP IIbα. Alternatively the disulphide bridges in non-reduced GP IIb may sterically hinder access by either lactoperoxidase or trypsin. The presence of two additional peptides in the map from GP IIbα may be due to cleavage of the intramolecular disulphide bonds existing between tryptic fragments.

The glycoprotein with the same M_r as GP IIb but a more acidic pI ($GP^{4\text{-}4.5}_{132\text{-}135}$) seems to be a separate entity as it gives a different tryptic peptide map. In addition when platelet samples were first isoelectrofocused under non-reducing conditions and then reduced before second-dimension SDS-gel electrophoresis no equivalent to the GP IIbβ subunit found under GP IIbα was found under $GP^{4\text{-}4.5}_{132\text{-}135}$.

The tryptic peptide maps of GP Ib are similar to those obtained by Nachman et al. [20] and by Clemetson et al. [21] using intact GP Ib and asialo GP Ib, respectively. Qualitative and quantitative differences between the tryptic peptide maps of the two pI regions of GP Ib may reflect differences in glycosylation of GP Ib which has a highly heterogeneous pI on two-dimensional O'Farrell gels [7–9]. An alternative explanation for the heterogeneity is that normal platelet samples contain a mixture of younger and older platelets and that the latter may have lost terminal sialic acid from GP Ib which would eventually lead to their removal from the circulation by the liver or the spleen as is the case for desialylated erythrocytes [22].

GP IIa contains intramolecular disulphide bridges and when these are reduced the molecule

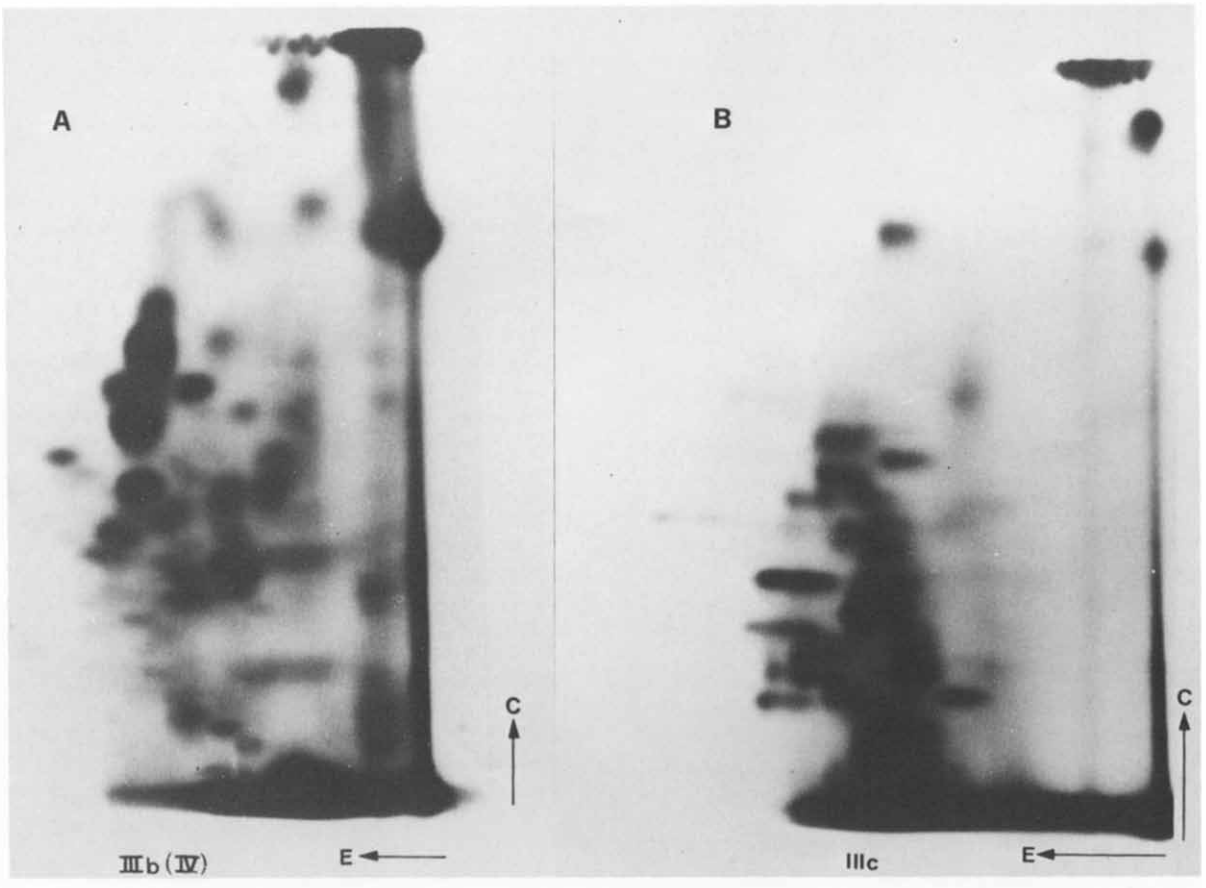

Fig. 8. Autoradiographs of ^{125}I-labelled tryptic peptide maps performed as described in Fig. 2. (A) Glycoprotein IIIb (also known as GP IV). B. Glycoprotein IIIc.

can unfold so that it runs at a higher M_r on SDS-gel electrophoresis [5] and partially blends into GP Ibα on two-dimensional O'Farrell gels. GP IIa could be identified in the GP Ibα area by its staining and labelling properties and by the very similar tryptic peptide map of the glycoprotein cut from this region to that of unreduced GP IIa.

Minor differences between the tryptic peptide maps of the non-reduced and reduced forms of GP Ia are probably due to intramolecular disulphide bridges thought to be present in this molecule [5].

A glycoprotein with an M_r slightly lower than GP IIa in the reduced state and with a more acidic pI which gives a round spot by Coomassie blue staining gave a tryptic peptide map different from that of GP IIIa. The M_r of GP IIIc was not affected by reduction. GP IIIc was labelled by iodination techniques and could be clearly detected on two-dimensional gels using either ^{125}I-labelled *Lens culinaris* lectin or ^{125}I-labelled concanavalin A (McGregor, J.L., unpublished data).

Thus, tryptic peptide map analysis of a number of major platelet glycoproteins shows that they have unrelated structures and give characteristic individual maps. However more sophisticated techniques will be necessary to resolve completely the problem as to whether certain platelet glycoproteins are structurally related.

A number of techniques are currently used to characterize platelet membrane glycoproteins (pI, M_r, staining, labelling and effect of neuraminidase). The use of two-dimensional tryptic peptide mapping provides a valuable additional parameter

for the identification of platelet glycoproteins separated by different techniques.

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