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TANDEM SEPARATION OF LABELLED HUMAN BLOOD PLATELET MEMBRANE GLYCOPROTEINS BY ANION-EXCHANGE AND GEL FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

Studies were made of the separation of surface-labelled platelet glycoproteins, solubilized in a non-ionic detergent (Berol 185), on a Mono Q anion-exchange column, coupled to a Superose 12 gel fast protein liquid chromatographic column. Peaks eluted from the anion-exchange and gel columns were subjected to electrophoresis on sodium dodecyl sulphate-polyacrylamide gels in the presence (non-reducing conditions) or absence (reducing conditions) of disulphide bridges. Labelled electrophoresed glycoprotein bands were rendered visible by fluorography or indirect autoradiography. Platelet membrane glycoproteins Ib, IIb, IIIa, IIIb, V and IX were identified by their apparent molecular weights and their surface labelling characteristics. It was concluded that tandem chromatography can be used to separate platelet membrane proteins and glycoproteins rapidly and with good resolution.

INTRODUCTION

Membrane glycoproteins (GP) are thought to play an important role in human blood platelet haemostatic functions. Two-dimensional polyacrylamide gel electrophoresis combined with different surface labelling techniques has shown the presence of a large number (*ca.* 40) of glycoproteins on the surface of human blood platelets[1,2]. A number of these platelet membrane glycoproteins (Ib, IIb and IIIa) have been

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shown to take part in platelet aggregation or adhesion[1,3]. The functions and structures of most separated platelet membrane glycoproteins remain to be elucidated.

Although it allows tryptic peptide map analysis of membrane glycoproteins[4], two-dimensional polyacrylamide gel electrophoresis provides very little material for immunological studies. In view of the recent advances in protein separation by liquid chromatography (LC)[5], it was interesting to separate platelet membrane glycoproteins by a tandem LC system. However, no work has been reported on the rapid separation of platelet glycoproteins by tandem LC. Newman and Kahn[6] applied the detergent phase of a Triton X-114 extraction of platelet membranes to a DEAE-cellulose column and then chromatographed the eluate from the column on a high-performance liquid chromatographic (HPLC) TSK-4000SW gel column. The technique used by Newman and Kahn, although providing relatively pure preparations of GPIIb and GPIIIa, has not dealt with the large number of glycoproteins present on the platelet surface.

The aim of this study was to separate surface-labelled platelet glycoproteins, solubilized in a non-ionic detergent (Berol 185), by Mono Q anion-exchange followed by Superose 12 gel fast protein liquid chromatography (FPLC). Platelet membrane glycoproteins were specifically labelled in their terminal sialic acid residues or protein moieties. Peaks eluted from the anion-exchange and gel columns were subjected to electrophoresis on sodium dodecyl sulphate (SDS)-polyacrylamide gels (PAGE) in the presence (non-reducing conditions) or absence (reducing conditions) of disulphide bridges. Labelled, electrophoresed glycoprotein bands were rendered visible by fluorography or indirect autoradiography. Staining with Coomassie G250 of SDS-PAGE showed the protein bands present in the eluted peaks. Platelet membrane glycoproteins Ib, IIb, IIIa, IIIb, V and IX were identified by their apparent molecular weights (M_r) and their surface-labelling characteristics.

The results show that tandem chromatography can be used to separate platelet proteins and glycoproteins rapidly with good resolution.

EXPERIMENTAL

The Mono Q HR 5/5 prepacked anion-exchange column (50 × 5 mm I.D.) and the Superose 12 HR 10/30 pre-packed gel column (300 × 10 mm I.D.) (Pharmacia, Uppsala, Sweden) were installed in a fast protein liquid chromatographic (FPLC) system. Materials, chemicals, enzyme and radioisotopes used for washing and labelling platelets and for separation in the FPLC system were as previously indicated[7,8]. Berol 185 (an oligooxyethylene–fatty alcohol adduct) was obtained from Berol (Stenungsund, Sweden).

Isolation, washing and labelling of platelets

Platelets were isolated from blood* and washed as previously described[7]. Washed platelets were specifically labelled, as previously shown[7], in their terminal sialic acid residues by the sodium metaperiodate + NaB^3H_4 method and in their protein moieties by the lactoperoxidase + iodine-125 method. Labelled platelets (10^9

* Of informed consenting adult donors in accordance with the principles embodied in the Helsinki Declaration.

per 0.5 ml; approximately 4 mg/ml) were solubilized in 1% (w/v) Berol 185 in 20 m*M* triethanolamine (pH 7.4). The protein content in the solubilized samples was determined as previously described[7]. Solubilized platelets were agitated regularly at 4°C for 30 min and centrifuged in an Eppendorf microfuge for 10 min. The supernatant material was carefully removed and stored at −70°C.

Anion-exchange chromatography

The starting buffer was 0.5% (w/v) Berol 185 in 0.02 *M* triethanolamine–HCl (pH 7.4) and the final buffer was 0.5% (w/v) Berol 185 in 0.02 *M* triethanolamine–HCl (pH 7.4) containing 1 *M* sodium chloride. The gradient was generated over 25 min at a flow-rate of 1 ml/min. Solubilized labelled platelets were recentrifuged in an Eppendorf microfuge for 10 min before use. Labelled material (4 mg/ml) was loaded into two 500-μl sample loops, connected in series, before being injected into the Mono Q column.

Gel chromatography

Peaks (400 μl) eluted from the Mono Q column were injected into the Superose 12 column via a 500-μl sample loop. Gel chromatography was performed on one Superose 12 column with a mobile phase containing 0.5% (w/v) Berol 185 in 0.1 *M* triethanolamine–HCl (pH 7.4) at a flow-rate of 1 ml/min in a total separation time of 26 min. High- and low-molecular-weight standards were obtained from Boehringer (Mannheim, F.R.G.).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Peaks eluted from the Mono Q column were treated with 2% (w/v) SDS, following the addition of 0.002 *M* N-ethylmaleimide (NEM) and 0.05 *M* Tris–HCl (pH 6.8) for electrophoresis under non-reducing conditions as previously described[2]. Peaks eluted from the Mono Q or Superose 12 columns were treated with 0.04 *M* dithiothreitol (DTT), following the addition of 2% (w/v) SDS and 0.05 *M* Tris–HCl (pH 6.8) for electrophoresis under reducing conditions as previously described[2]. The amounts of SDS, 0.05 *M* Tris–HCl (pH 6.8) and NEM or DTT given above are final concentrations present in treated eluted peaks. Non-reduced and reduced samples were heated at 100°C for 10 min and immediately applied to a 10% SDS-PAGE slab[8,9]. At the end of electrophoresis, the polyacrylamide gels were fixed in 40% methanol–7% acetic acid and then stained with Coomassie G250[7]. Fluorography or indirect autoradiography of stained polyacrylamide gels was carried out by the methods of Bonner and Mills[10] and Laskey and Mills[11]. Protein markers used in SDS-polyacrylamide gels were from obtained Amersham International (Amersham, U.K.).

RESULTS AND DISCUSSION

Washed, whole platelets, surface-labelled in their protein or terminal sialic acid moieties, showed, when solubilized in Berol 185 and chromatographed on a Mono Q column, several major peaks, eluted by a 0 to 1 *M* NaCl gradient (Fig. 1A). The labelled membrane glycoproteins (GP) in these eluted peaks were observed by indirect autoradiography or fluorography of SDS-PAGE performed under non-reducing

(Figs. 1B and 2A) or reducing (Figs. 1C and 2B) conditions. The total number of protein bands in each eluted peak was obtained by staining the SDS-PAGE with Coomassie G250 (Fig. 2C and D). By comparing the fluorograms and indirect autoradiograms from SDS-PAGE, performed under non-reducing or reducing conditions, it was possible to identify a number of membrane glycoproteins (Ib, IIb, IIIb, V and IX) in the eluted peaks. These membrane glycoproteins were identified, on the basis of previously published data[1,2,12,13], according to their M_r (in the presence or absence of disulphide bonds) and according to their surface-labelling characteristics.

Glycoprotein IIIb (96 kdaltons) was eluted (peaks 3, 4, 5 and 6) at an early stage from the Mono Q ion-exchange column (Figs. 1B, 1C, 2A and 2B). As previously observed[1,2,12,13], this glycoprotein (IIIb) was intensely labelled by the periodate–NaB^3H_4 method and showed no alteration in its M_r on disruption of disulphide bonds. Newman and Kahn[6] applied the detergent phase of a Triton X-114 extraction of platelet membranes to a DEAE-cellulose column and also noted that GPIIIb was rapidly eluted from the ion-exchange column. Gel chromatography of peak 4 did not allow the isolation of GPIIIb from other glycoproteins present at a higher or lower M_r (Fig. 3). It is possible, as shown for other glycoproteins (GPIIb–IIIa), that GPIIIb and these glycoproteins, of different M_r, are present on the platelet surface as a complex that is kept intact in a non-ionic detergent but is dissociated in the presence of EDTA or after SDS treatment. Glycoprotein V (71 kdaltons), situated below GPIIIb, migrates with a slightly higher M_r (78 kdaltons) on reduction and may be present in peaks 4, 5, 6 and 7. Glycoprotein V is cleaved from the platelet surface after thrombin treatment[14]. A minor glycoprotein band (37

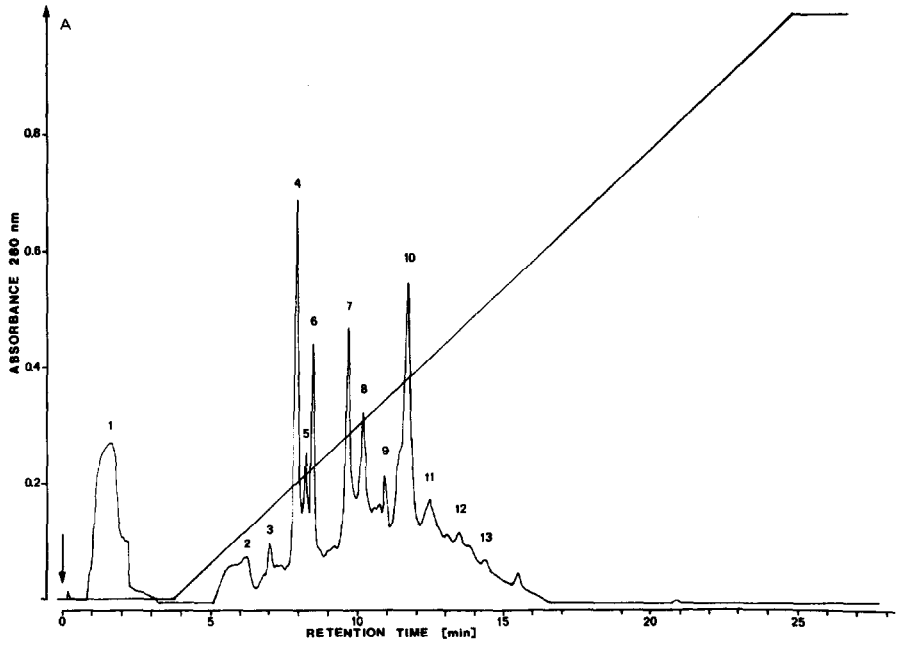


Fig. 1.

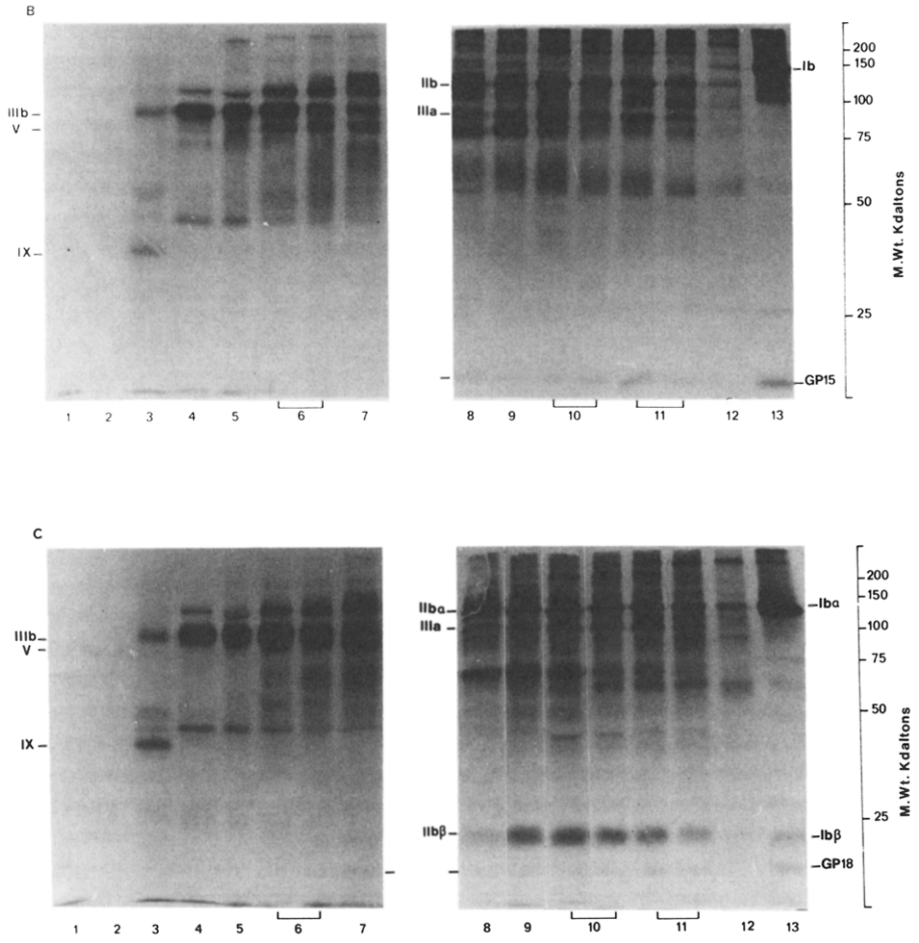
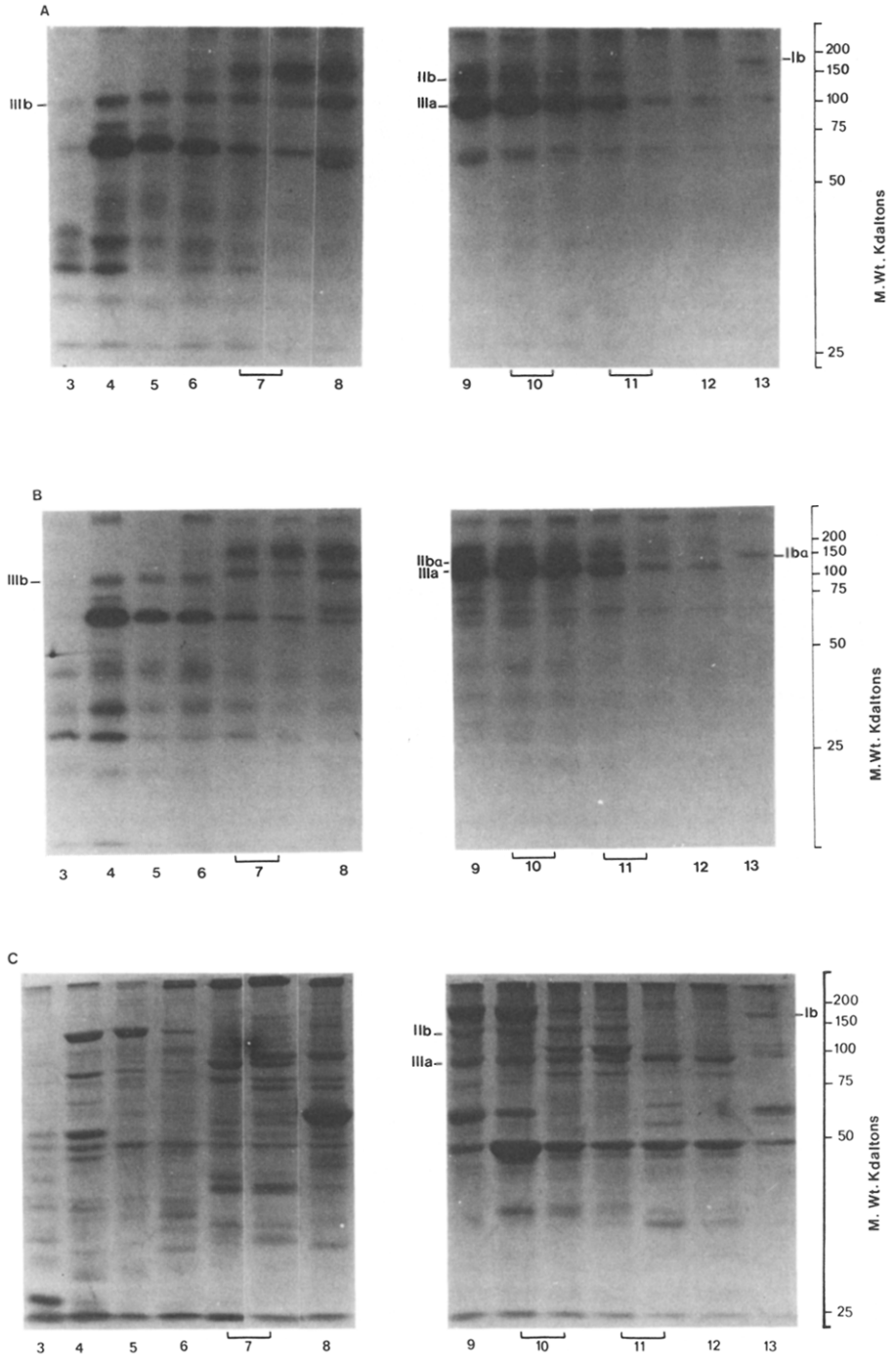


Fig. 1. (A) Chromatographic separation of solubilized human blood platelets, labelled by the periodate–NaB^3H_4 method, on a Mono Q column, connected to an FPLC system. The Berol 185-solubilized platelets (4 mg of proteins per 2 ml) were injected into the column via two 500-μl sample loops, connected in series. The gradient of 0–1 *M* NaCl in 0.5% (w/v) Berol 185, in 0.02 *M* triethanolamine–HCl buffer (pH 7.4), was generated over 25 min at a flow-rate of 1 ml/min. Platelet glycoproteins (labelled in their terminal sialic acid residues) in each eluted peak were rendered visible by fluorography following SDS-PAGE. (B) Fluorograms of Mono Q peaks eluted from a Mono Q column, subjected to electrophoresis under non-reducing conditions on a 10% SDS-PAGE slab. (C) Fluorograms of peaks eluted from a Mono Q column, injected to electrophoresis under reducing conditions on a 10% SDS-PAGE slab. Numbers below each SDS-PAGE lane correspond to peak numbers. Fluorograms in B and C were obtained after exposing pre-flashed Kodak X-omat S film, for 7 days at −70°C, to polyacrylamide gels, treated for fluorography.

kdaltons), present in peak 4, which had an increased M_r on reduction (42 kdaltons), is probably GPIX. Glycoprotein IX is thought to be the platelet HLA antigen[15]. Gel chromatography of peak 7 showed the separation of a number of labelled glycoprotein bands (Fig. 4). The glycoproteins separated from peak 7 probably include GPIIb and GPIIIa and other glycoproteins the identities of which remain to be elucidated.

Glycoprotein IIb (153 kdaltons) is composed of two subunits: a major com-


A
IIIb
IIb
IIIa
Ib
200
150
100
75
50
25
M.Wt. Kdaltons
3 4 5 6 7 8
9 10 11 12 13
B
IIIb
IIba
IIIa
Iba
200
150
100
75
50
25
M.Wt. Kdaltons
3 4 5 6 7 8
9 10 11 12 13
C
IIb
IIIa
Ib
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150
100
75
50
25
M. Wt. Kdaltons
3 4 5 6 7 8
9 10 11 12 13

Fig. 2.

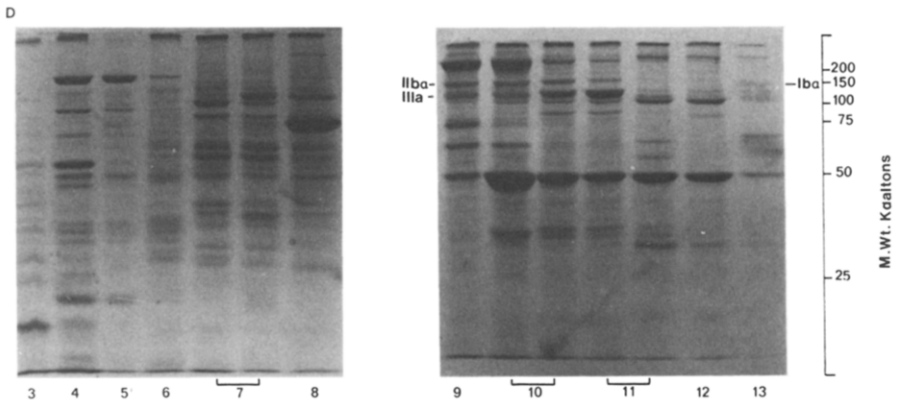

Fig. 2. Chromatographic separation of solubilized human blood platelets, labelled by the lactoperoxidase + iodine-125 method, on a Mono Q column, connected to an FPLC system. The Berol 185-solubilized platelets (4 mg of proteins per 2 ml) were injected into the column via two 500-μl sample loops, connected in series. The gradient of 0–1 *M* NaCl in 0.5% (w/v) Berol 185 in 0.02 *M* triethanolamine–HCl buffer (pH 7.4) was generated over 25 min at a flow-rate of 1 ml/min. Platelet glycoproteins (labelled in their protein moieties) in each eluted peak were rendered visible by indirect autoradiography, following SDS-PAGE. The protein content in each eluted peak was obtained after staining SDS-polyacrylamide gels with Coomassie G250. (A) Indirect autoradiograms of peaks eluted from a Mono Q column, subjected to electrophoresis under non-reducing conditions on a 10% SDS-PAGE slab. (B) Indirect autoradiograms of peaks eluted from a Mono Q column, subjected to electrophoresis under reducing conditions on a 10% SDS-PAGE slab. Numbers below each SDS-PAGE lane correspond to peak numbers. Indirect autoradiograms in A and B were obtained after exposing pre-flashed Kodak X-omat S film for 4 days at −70°C to polyacrylamide gels in the presence of calcium tungstate screens (DuPont, Cronex Lightning Plus intensifying screens). (C) Coomassie G250 staining of SDS-PAGE gel from A. (D) Coomassie G250 staining of SDS-PAGE gel from B.

ponent (IIbα) (130 kdaltons) linked by disulphide bridges to a minor component (IIbβ) (23 kdaltons)[1,12,16]. Glycoprotein IIIa (96 kdaltons) migrates on reduction to a higher M_r (114 kdaltons), owing to the disruption of intra-disulphide bridges[1,12,16]. Changes in the M_r of these two major glycoproteins (IIb, IIIa) and the presence of the IIb$_\beta$ subunit, following disruption of disulphide bridges, clearly indicate that GPIIb and GPIIIa are both eluted in peaks 8–11 (Figs. 1C, 2B). These two glycoproteins, which are extensively labelled in their protein and terminal sialic acid residues (Figs. 1C and 2B), have been shown to be associated on the platelet surface in a calcium-mediated complex[17,18]. In the presence of Berol 185, a non-ionic detergent, GPIIb and GPIIIa are separated on the anion-exchange and gel columns as a complex, which is dissociated after treatment with SDS (Fig. 5). The IIb–IIIa complex was previously shown by crossed immunoelectrophoresis (CIE) or gel chromatography on Sephacryl S-300 to be intact after solubilization of platelets in a buffer containing a non-ionic detergent (Triton X-100) and dissociated in the presence of EDTA or after treatment with SDS[17–19].

Glycoprotein Ib is the most sialylated platelet membrane glycoprotein and is

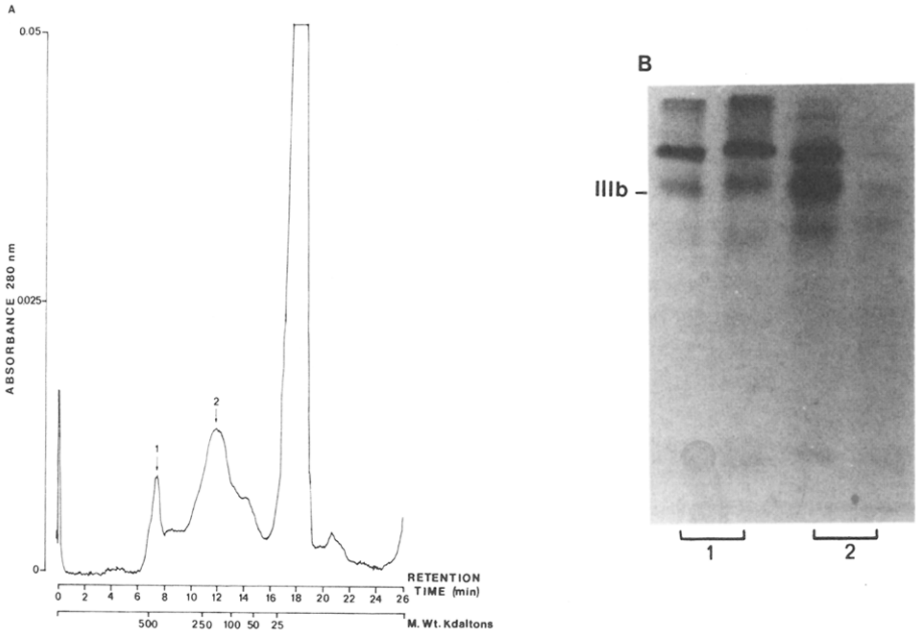

Fig. 3. (A) Gel chromatography on a Superose 12 column of peak 4 eluted from the Mono Q column injected with platelets labelled in their terminal sialic acid residues and solubilized in Berol 185. Peak 4 (approximately 400 μl) was injected into the gel column at a flow-rate of 1 ml/min. A mixture of standards (ferritin, catalase, aldolase, chymotrypsinogen) were separated on the gel column under the same conditions as the peak eluted from the Mono Q column. (B) Fluorograms of peaks eluted from the Superose 12 gel chromatography of peak 4 and subjected to electrophoresis under reducing conditions on a 10% SDS-PAGE slab. Numbers below each lane correspond to peak numbers.

intensively labelled by the periodate–NaB^3H_4 method[1,2,14]. This glycoprotein (Ib) is not well labelled by the lactoperoxidase + iodine-125 method and is poorly stained in SDS-PAGE gels with Coomassie G250[1,2]. Glycoprotein Ib has an M_r of 170 kdaltons and is composed of two subunits: a major component (Ib_α) (150 kdaltons), linked by disulphide bridges to a minor component (Ib_β) (21 kdaltons). The presence of the Ib_β subunit and changes in the M_r of this major glycoprotein (Ib), following disruption of disulphide bridges, indicated that GPIb was mainly eluted in peak 13 and to a much lesser extent in peak 12 (Figs. 1C and 2B). A glycoprotein having a low M_r (15–18 kdaltons) was observed with other glycoproteins to be eluted together with GPIb in peak 13. Patients with a congenital platelet disease, the Bernard Soulier syndrome, have platelets characterized by an absence of GPIb and the 15–18 kdalton glycoprotein[13]. This glycoprotein (15–18 kdaltons) was also reported to be purified with GPIb on an anti-GPIb monoclonal antibody immunoadsorbent column[20]. Another glycoprotein (GPIa) was observed to be immunoprecipitated by a monoclonal

antibody, directed against GPIb[21]. Gel chromatography of peak 13 did not allow the isolation of GPIb from other glycoproteins at a higher or lower M_r (Fig. 6). It is possible that GPIb, in addition to glycoprotein of M_r 15–18 kdaltons or GPIa, may form complexes with other glycoproteins. Gel chromatography of labelled platelets solubilized in Berol 185[22] and crossed immunoelectrophoretic (CIE) experiments[23,24] suggests that GPIb could form complexes of very high M_r with other proteins.

This study has shown that by using tandem chromatography it is possible to separate rapidly platelet membrane proteins and glycoproteins with good resolution. The use of platelet membranes, instead of whole platelets, may allow the isolation of glycoproteins or complexes of glycoproteins, free from cytoplasmic contaminants. Treatment with EDTA and/or SDS of peaks eluted from the anion-exchange column and the presence of this detergent in gel chromatography may be necessary to isolate the glycoproteins. Further studies will be necessary to allow the identification of a majority of the isolated glycoproteins.

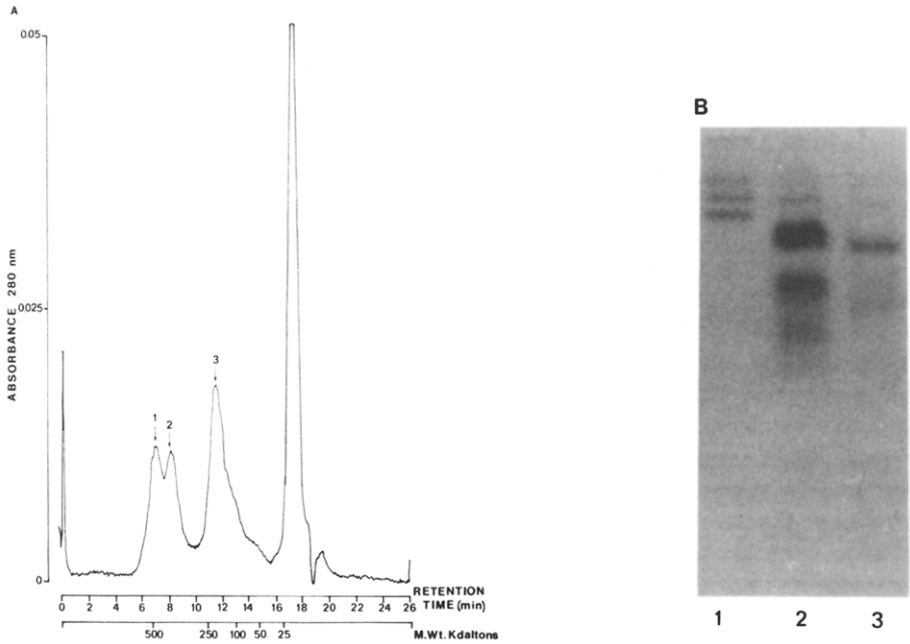

Fig. 4. (A) Gel chromatography on a Superose 12 column of peak 7, eluted from the Mono Q column injected with platelets labelled in their terminal sialic acid residues and solubilized in Berol 185. Peak 7 (approximately 400 μl) was injected into the gel column at a flow-rate of 1 ml/min. A mixture of standards (ferritin, catalase, aldolase, chymotrypsinogen) was separated on the gel column under the same conditions as peaks eluted from the Mono Q column. (B) Fluorograms of peaks eluted from the Superose 12 column chromatography of peak 7 and subjected to electrophoresis under reducing conditions on a 10% SDS-PAGE slab. Numbers below each lane correspond to peak numbers.

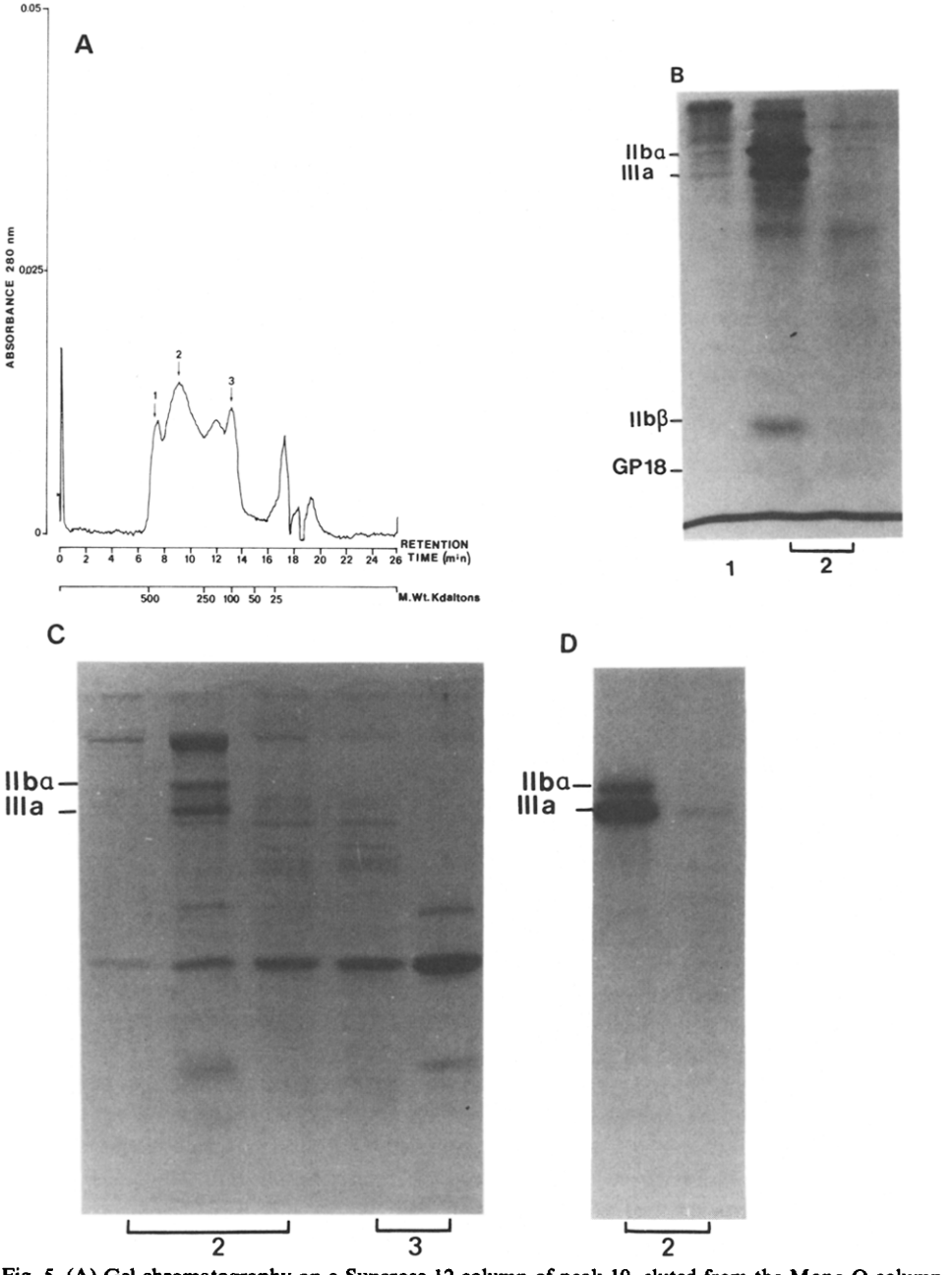

Fig. 5. (A) Gel chromatography on a Superose 12 column of peak 10, eluted from the Mono Q column injected with platelets labelled in their terminal sialic acid residues and solubilized in Berol 185. Peak 10 (approximately 400 μl) was injected into the gel column at a flow-rate of 1 ml/min. A mixture of standards (ferritin, catalase, aldolase, chymotrypsinogen) was separated on the gel column under the same conditions as peaks eluted from the Mono Q column. (B) Fluorograms of peaks eluted from the Superose 12 chromatography of peak 10 and subjected to electrophoresis under reducing conditions on a 10% Laemmli SDS-PAGE slab. Numbers below each lane correspond to peak numbers. (C) Coomassie G250 staining and (D) indirect autoradiograms of peaks eluted from the Superose 12 chromatography of peak 10 (peak 10 is from a separation on a Mono Q column of platelets labelled in their protein moieties and solubilized in Berol 185) and subjected to electrophoresis under reducing conditions on a 10% Laemmli SDS-PAGE slab. Numbers below each lane correspond to peak numbers.

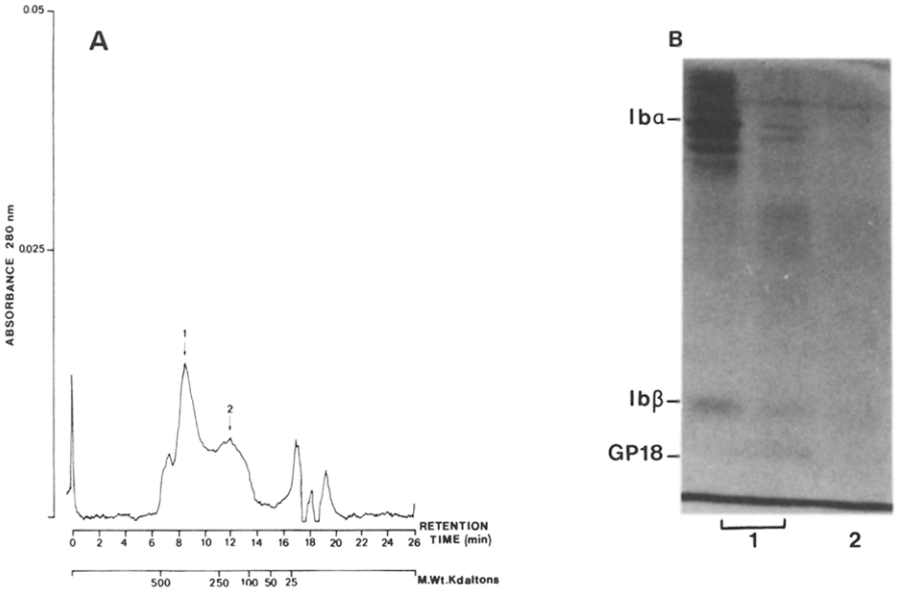

Fig. 6. (A) Gel chromatography on a Superose 12 column of peak 13 eluted from the Mono Q column injected with platelets labelled in their terminal sialic acid residues and solubilized in Berol 185. Peak 12 (approximately 400 μl) was injected into the gel column at a flow-rate of 1 ml/min. A mixture of standards (ferritin, catalase, aldolase, chymotrypsinogen) was separated on the gel column under the same conditions as peaks eluted from the Mono Q column. (B) Fluorograms of peaks eluted from the Superose 12 gel chromatography of peak 12 and subjected to electrophoresis under reducing conditions on a 10% SDS-PAGE slab. Numbers below each lane correspond to peak numbers.

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