Separation of important new platelet glycoproteins (GPIa, GPIc, GPIc*, GPIIa and GMP-140) by f.p.l.c.

Characterization by monoclonal antibodies and gas-phase sequencing

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A large number of membrane glycoproteins (around 40) are present on the surface of human blood platelets. Some of these glycoproteins are expressed in relatively small amounts, and their functions, as well as their structure, remain to be elucidated. The aim of the present study was to separate rapidly, under non-denaturing conditions, and characterize minor glycoproteins such as Very Late Antigens (VLA) (GPIa, GPIc, GPIc* and GPIIa) and GMP-140 (also known as PADGEM). VLAs and GMP-140 are respectively members of the integrin and selectin families. Platelet membrane glycoproteins were separated by wheat-germ agglutinin lectin affinity and Mono Q anion-exchange f.p.l.c. Peaks bearing isolated glycoproteins were electrophoresed on one- or two-dimensional SDS/polyacrylamide gels, Western blotted on to Immobilon poly(vinylidene difluoride) membranes and gas-phase-sequenced. The identity of isolated glycoproteins was also obtained by the use of monoclonal or polyclonal antibodies and tryptic peptide maps. Five minor [GPIa, GPIc, GPIc*, GPIIa and GMP 140 (PADGEM)], as well as a major (GPIIb) glycoprotein, were eluted at low salt concentrations. GPIIb-IIIa and GPIb were eluted at high salt concentrations. The *N*-terminal sequence of platelet GPIa was identical with that obtained by Takada & Hemler [(1989) *J. Cell Biol.* **109**, 397–407]. However, the *N*-terminal sequence of platelet GPIc + Ic* and GPIIa were found to differ from those deduced from cDNA sequences isolated from human placenta or umbilical-vein endothelial-cell cDNA libraries. The combined use of f.p.l.c. and gas-phase sequencing techniques provides a very powerful tool to separate and characterize rapidly platelet or other cellular proteins for structural, immunological and functional studies.

INTRODUCTION

A large number of glycoproteins have been detected on the surface of human platelets through the use of two-dimensional O'Farrell gel electrophoresis combined with radiolabelling techniques. Major membrane glycoproteins, such as GPIb, GPIIb and GPIIIa, have been shown to play a vital role in human blood platelet functions [1,2]. Moreover, a number of these platelet glycoproteins are members of the integrin, selectin or immunoglobulin superfamily of cell-surface adhesion receptors [2]. However, the function and structure of a majority of the minor platelet glycoproteins remain to be elucidated.

Several chromatographic procedures have been used to isolate major platelet membrane glycoproteins. GPIIb and IIIa were purified either by lectin-affinity chromatography [3,4], gel filtration of Triton X-100-extracted platelet membranes [5] or by anti-GPIIb/IIIa monoclonal-antibody (MAb) affinity chromatography [6,7]. Using lectin-affinity chromatography, Clemetson *et al.* [8] isolated GPIb and GPIa by a tandem technique using wheat-germ agglutinin (WGA) and *Abrus precatorius* lectin (abrin) columns. Tsuji & Osawa [9] purified GPIIIb (also known as GPIV or CD36) in a denatured state using successive columns of *Lens culinaris* (lentil) lectin-Sepharose and wheat-germ lectin-Sepharose followed by preparative SDS/PAGE. Skinner *et al.* [10] recently purified GMP-140 by using a concanavalin A column followed by MAb affinity chromatography.

The use of h.p.l.c. has allowed the purification of GPIIb/IIIa from Triton X-114 platelet extracts [11]. McGregor *et al.* [12] purified GPIIIb, under non-denaturing conditions, from Triton

X-114-extracted platelets, by tandem anion-exchange and size-exclusion f.p.l.c. Rapid separation by f.p.l.c. of minor platelet glycoproteins, some migrating very closely in two-dimensional O'Farrell SDS/polyacrylamide gels, has yet to be performed for functional and structural studies. In the present study, minor as well as major platelet glycoproteins were isolated, under non-denaturing conditions, using f.p.l.c. techniques and characterized by MABs, tryptic-peptide maps and *N*-terminal amino acid sequencing.

MATERIALS AND METHODS

Membrane extraction in sodium deoxycholate

Platelet membranes were prepared from human platelet concentrates as previously described [12]. Platelet membranes were treated for 30 min at 4 °C with 1 % (w/w) sodium deoxycholate (DOC) (Merck, Darmstadt, Germany) in 10 mM-Tris/HCl (pH 8.2)/5 mM-EDTA/leupeptin (0.5 µg/ml). Extracted platelet membranes were then ultracentrifuged at 4 °C for 30 min at 100000 *g* and the supernatant loaded on to a lectin-affinity column.

Lectin-affinity and anion-exchange chromatography

WGA-Sepharose 6MB (Pharmacia, Uppsala, Sweden) was packed into a glass column HR10/10 (100 mm × 10 mm) (Pharmacia), connected to a f.p.l.c. (Pharmacia) and equilibrated in the loading buffer [10 mM-Tris/HCl (pH 8.2) 0.5 % DOC/5 mM-EDTA]. The supernatant of DOC-extracted platelet membranes was then loaded on to the column (flow rate

Abbreviations used: WGA, wheat-germ agglutinin; DOC, (sodium) deoxycholate; PVDF, poly(vinylidene difluoride); GP, glycoprotein (in GPI, GPIIa etc.); MAb, monoclonal antibody.

Fig. 1. Elution profile of WGA-Sepharose 6MB-extracted platelet glycoproteins subjected to f.p.l.c. on a Mono Q anion-exchange column

The WGA-Sepharose glycoprotein extract (see the text) was run on a Mono-Q column equilibrated with 0.1% Lubrol PX. A NaCl (0–1 M) gradient eluted six major peaks whose contents were identified after SDS/7.5%-PAGE under non-reducing or reducing conditions and Coomassie Blue staining. In the inset, lanes 1–6 represent peaks 1–6 electrophoresed under non-reducing (NR) or reducing (R) conditions. GPIIb and GMP140 were present in peaks 1–3, GPIa, GPIc, GPIc* and GPIIa in peaks 2 and 3, GPIIb/IIIa in peaks 3–6, and GPIb in peak 6.

0.5 ml/min), which was then extensively washed with the loading buffer. Glycoproteins bound to the WGA-Sepharose 6MB were eluted with 2.5% (w/v) *N*-acetylglucosamine (Sigma, St. Louis, MO, U.S.A.). Eluted glycoproteins were dialysed overnight against 10 mM-Tris/HCl (pH 7.4)/0.1% Lubrol PX (Sigma) and then run on a Mono Q anion-exchange column (HR5/5; 50 mm × 5 mm; Pharmacia) as previously described [12].

Immunoaffinity chromatography and Western blot

Glycoproteins eluted from the Mono Q column were further isolated and identified by immunoaffinity chromatography, using MAbs LYP22 (specific for GPIIa [13]), or LYP4 (specific for GPIIb/IIIa [14]) and by Western blotting using an anti-GMP-140 MAb (LYP20) [15].

One- and two-dimensional SDS/PAGE

Peaks eluted from the Mono Q column were electrophoresed in one [16]- or two [17]-dimensional non-reducing/reducing SDS/polyacrylamide gels. Two-dimensional non-reducing/reducing O'Farrell SDS/PAGE was performed as previously described [18]. Fixed gels were silver-stained by the method of Morrissey [19].

Two-dimensional tryptic-peptide map analysis of isolated proteins

Isolated proteins were excised from two-dimensional non-reducing/reducing SDS/polyacrylamide gels and their tryptic maps produced as previously described [20].

Protein blotting and sequencing

Isolated proteins were electrophoresed on SDS/polyacrylamide gels using either the modification of Hunkapillar & Hood [21] or the one introduced by Moos *et al.* [22]. Electrophoresed proteins were transferred to a poly(vinylidene difluoride) membrane (Immobilon PVDF, Millipore, Bedford, MA, U.S.A.) by the method of Matsudaira [23]. Proteins immobilized on PVDF membranes were Ponceau S (Sigma)-stained, excised and sequenced by using an automated gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

By using a 0–1 M-NaCl gradient, six major peaks were eluted from the Mono Q column loaded with the WGA-Sepharose 6MB column eluate (Fig. 1). Peak 1 (fractions 11–13), eluted at 0.15 M-NaCl, contained three glycoproteins (GP) bands: GP85/85 kDa (apparent molecular mass under non-reducing/reducing conditions), GP128/132 kDa and GP110/115 kDa (Fig. 1, Fig. 2a and Table 1). Peak 2 (fractions 14 and 15), eluted at 0.3 M-NaCl, contained three glycoproteins bands present in peak 1, with three additional bands: GP150/155 kDa, GP150/130 kDa and GP130/135 kDa (Fig. 1, Fig. 2b and Table 1). Peak 3 (fractions 16 and 17), eluted at 0.3 M-NaCl, contained the six glycoproteins bands present in peak 2 with two additional bands: GP140/115 kDa and GP85/98 kDa (Fig. 1, Fig. 2c and Table 1). Peaks 4 (fraction 21), 5 (fractions 22 and 23) and 6 (fraction 24), eluted respectively at 0.5, 0.6 and 0.7 M-NaCl,

Fig. 2. Two-dimensional non-reducing/reducing SDS/PAGE separations of peaks eluted from the Mono Q anion-exchange column
Peaks 1–4 were subjected to two-dimensional non-reducing/reducing SDS/PAGE. The first dimension (non-reduced) was run in a 6 % (w/v) gel and the second dimension (reduced) in an 8 % gel. The two-dimensional gel was silver-stained; peaks 1–4 are shown as (a)–(d).

Table 1. Glycoproteins eluted from a Mono Q column loaded with WGA–Sepharose 6MB column eluate

Peak	[NaCl] (M)	Eluted glycoproteins
1	0.15	GPIIb (CD36) GMP-140 (CD62) GP110/115
2	0.30	GPIa (CD49b) GPIc (CD49e) GPIc* (CD49f) GPIIa (CD29) GPIIb (CD36) GMP-140 (CD62) GP110/115
4	0.50	GPIIb (CD41a) GPIIIa (CD41b) Traces of: GPIa (CD49b) GPIc (CD49e) GPIc* (CD49f) GPIIa (CD29)
5	0.60	GPIIb (CD41a) GPIIIa (CD41b)
6	0.70	GPIIb (CD41a) GPIIIa (CD41b) GPIb (CD42b)

showed the GP140/120 kDa and GP85/98 kDa bands (Fig. 1, Fig. 2d and Table 1). In addition, a band of GP160/140 kDa was eluted in peak 6 (Fig. 1 and Table 1).

Fig. 3. Western blot using an anti-GMP-140 MAb (LYP20) against peaks eluted from the Mono Q anion-exchange column
Peaks 1 to 6, electrophoresed under non-reducing conditions on SDS/10 % -polyacrylamide gels, were transferred on to an Immobilon (PVDF) membrane that was incubated with LYP20. Western blots were developed with a goat anti-mouse antibody conjugated to horseradish peroxidase.

GMP-140

The GP128/132 kDa band present in peaks 1, 2 and 3 was recognized as GMP-140 in Western blotting by MAb LYP20 [15] (anti-GMP-140) (Fig. 3). This band (GP128/132) migrates just below GPIIa on two-dimensional non-reducing/reducing SDS/

Fig. 4. Two-dimensional non-reducing/reducing SDS/PAGE separations of Very Late Antigens (GPIa, GPIc, GPIc* and GPIIa) isolated on an anti-GPIIa immunoaffinity column (LYP22)

Peaks 1–4 eluted from a Mono Q column were run on a LYP22–Sepharose 4B column. The specially bound fraction was eluted and analysed by two-dimensional non-reducing/reducing SDS/PAGE. The first dimension (non-reduced) was run in a 6% gel and the second dimension (reduced) in a 7.5% gel. The spots were silver-stained and identified, by their molecular masses, as GPIa, GPIc, GPIc*, GPIIa and GPI110/115 (a). Glycoprotein IIb/IIIa isolated on a LYP4–Sepharose 4B column was added to the eluate of LYP22 and separated in a manner identical with that used in (a). Positions of the Very Late Antigens in relation to GPIIb/IIIa are shown in (b).

PAGE (Fig. 2). GMP-140, also known as PADGEM or CD62, is an integral alpha-granule membrane glycoprotein [24] that is exposed on the platelet surface after platelet activation and secretion [6,25]. The cDNA of GMP-140 has been recently sequenced [26] and found to have an important sequence similarity with those of ELAM-1 [27] and Mel-14 [28] and constitute a new family of adhesion proteins [29] known as selectins. The function of GMP-140 remains to be elucidated, but it has been shown to act in platelet–monocyte [30], platelet–neutrophil [31], neutrophil–endothelial cell [32] and platelet–platelet interaction [13].

GPIa/IIa, GPIc/IIa and GPIc*/IIa

Three glycoproteins α -subunits (GPIa, GPIc, GPIc*) form heterodimers, with a common β_1 -subunit (GPIIa), on the surface of platelets and other cells and belong to the integrin family [33]. The GPIa/IIa complex (also known as $\alpha_2\beta_1$ or VLA-2) has recently been found to promote platelet adhesion to collagen [34,35]. GPIc/IIa (also known as $\alpha_5\beta_1$ or VLA-5) and GPIc*/IIa (also known as $\alpha_6\beta_1$ or VLA-6) have been recognized as two

Fig. 5. Two-dimensional O'Farrell SDS/PAGE separations of Very Late Antigens (GPIa, GPIc, GPIc* and GPIIa) isolated on an anti-GPIIa immunoaffinity column (LYP22)

Peaks 1–4 eluted from a Mono Q column were run on a LYP22–Sepharose 4B column. The specially bound fraction was eluted and analysed by two-dimensional O'Farrell SDS/PAGE. The first-dimension run was a separation by pI and the second-dimension (7.5% gel) run was a separation by molecular mass (M). The spots were silver-stained and identified, by their pI and molecular mass, as α_2 (GPIa), $\beta_1 + \alpha_5$ (GPIIa + GPIc), α_6 (GPIc*) and GPI110/115.

distinct heterodimers involved respectively in mediating platelet adhesion to fibronectin [36] and laminin [37].

Several lines of evidence indicate that the GPI150/155, GPI150/130 and GPI130/135 bands (the kDa suffix is hereafter dispensed with), present mainly in peaks 2 and 3, are GPIa, GPIc, GPIc* and GPIIa. (1) Affinity chromatography with an anti-GPIIa MAb (LYP22), known to immunoprecipitate the GPIa/IIa, GPIc/IIa and GPIc*/IIa complexes, bound all three glycoprotein bands (Fig. 4a). (2) Two-dimensional non-reducing/reducing SDS/PAGE gels showed that these glycoprotein bands migrated with the same electrophoretic mobility as GPIa/IIa, GPIc/IIa and GPIc*/IIa [38]. (3) Moreover, when mixed with isolated GPIIb/IIIa, these LYP22, immunopurified bands migrated at exactly the same position, with respect to GPIIb-IIIa, as genuine GPIa, GPIc, GPIc* and GPIIa (Fig. 4b). One should note that, on two-dimensional non-reducing/reducing gels, GPIc and GPIc* migrate at exactly the same position [38]. (4) Analysis by two-dimensional O'Farrell gel electrophoresis of LYP22-immunopurified bands clearly showed, as expected, GPIc and GPIc* as having distinct pI values, but similar apparent molecular masses (Fig. 5). Moreover, under reducing conditions, GPIc had a pI and an apparent molecular mass similar to that of GPIIa. These results are the first to confirm those obtained by Hemler *et al.* [38]. (5) The *N*-terminal sequence of one of the bands (GPI150/155), cut out from a two-dimensional non-reducing/reducing gel, was identical with that obtained by Takada & Hemler [39] for GPIa (Tables 2 and 3). Platelet GPIc and GPIc*, located on the same spot on non-reducing/reducing two-dimensional SDS/PAGE, appear to have identical *N*-terminal sequences, since the Edman degradation produced one amino acid phenylthiohydantoin derivative at each cycle (Tables 2 and 3). The GPIc + GPIc* sequence obtained in the present study was similar (11 among 13 residues were identical) to a band immunoaffinity-purified by Takada *et al.* [39,40], using an anti-GPIIa MAb (A-1A5) migrating to the same position as GPIa. Reasons for such discrepancy may be due to the co-purification with GPIIa of different α -subunits which migrate in the same range of molecular mass on one-dimensional SDS/PAGE. Surprisingly, the *N*-terminal sequence of platelet GPIc + GPIc* obtained in the present study was found to differ from the amino acid sequence deduced from the cDNA sequences of the fibronectin receptor [41,42] isolated respectively from

Table 2. *N*-Terminal sequences of presently isolated platelet glycoproteins and sequences previously published

Platelet glycoprotein	<i>N</i> -Terminal sequence	
	Present f.p.l.c. isolation	Previously published
GPIa (CD49b)	YNVGLPEAKIFSGP	FNLDTXEDNVFRGP [40] YNVGLPEAKIFSGP [39]
GPIc (CD49e) (CD49f)	FNLDTREDNVIRK	FNLD AEA PAVLSG [41,42]
GPIIb (CD36)	GXDRNXGLIAGAVIGAVLAXFG	GXDRNXGLIAGAVIGAVLAVFG [45]
GPIIb (CD41a)	LNLDPVQLTFY	LNLDPVQLTFYAGP [48]
GPIIIa (CD41b)	GPNIXTTRGV	GPNICTTRGVSSCQ [49]
GPIIa (CD29)	XNLDEAG	QTDENRCLKANAK [41]

Table 3. Edman degradation of platelet glycoproteins GPIa, GPIc, GPIIa and a minor product

Abbreviations used: ND, not determined; PTH, amino acid phenylthiohydantoin derivative; the one-letter code for amino acid residues is used.

Edman cycle no.	GPIa		GPIc		GPIIa		Minor product	
	Residue	PTH yield (pmol)	Residue	PTH yield (pmol)	Residue	PTH yield (pmol)	Residue	PTH yield (pmol)
1	Y	15.7	F	43.0	X (S?)	ND		
2	N	9.6	N	30.9	N	1.5		
3	V	11.0	L	41.5	L	4.3	F	1.3
4	G	11.2	D	24.7	D	2.8		
5	L	12.6	T	33.4	E	2.1	F	1
6	P	9.9	R	6.7	A	3.1		
7	E	8.3	E	15.9	G	1.7	E	ND
8	A	12.4	D	14.1				
9	K	6.4	N	11.2				
10	I	7.6	V	14.3				
11	F	7.9	I	16.3				
12	S	ND	R	4.2				
13	G	5.8	K	1.6				
14	P	5.1						

human placental and human umbilical-vein endothelial cells. In addition, the five *N*-terminal amino acids obtained from platelet GPIIa (Tables 2 and 3) were found to differ from the sequence of the β -subunit of the fibronectin receptor [41]. Further work is needed to investigate the possibility that GPIc, GPIc* and GPIIa in platelets may be slightly different from those of other cell types.

GP110/115, present in peaks 1, 2 and 3 (Fig. 2), was also immunoaffinity-purified with GPIa/IIa, GPIc/IIa and GPIc*/IIa by LYP22 (Fig. 4a). Amounts of isolated GP110/115, which has a pI on two-dimensional O'Farrell gel similar to that of GPIIa and GPIc (Fig. 5), varied from one preparation to another. This glycoprotein (GP110/115) could be a new α -subunit complexed with GPIIa. However, it is most likely a GPIIa degradation product, since it is recognized by LYP22 (an anti-GPIIa MAb) in Western blots (results not shown) and has a tryptic-peptide map similar to that of GPIIa (Fig. 6). Additional spots present in the GP110/115 tryptic-peptide map could be contaminants originating from a degraded form of GPIc co-migrating with GP110/115. A band with a molecular mass similar to that of GP110/115 was also reported to be present in trypsin-treated endothelial-cell lysates immunoprecipitated with a MAb directed against VLA-2 [43] (GPIa/GPIIa). Further work is needed to

identify the epitope on GP110/115 that binds LYP22. The tryptic peptide maps of GPIa, GPIc and GPIIa were performed after a separation on two-dimensional non-reduced/reduced SDS/PAGE. The tryptic peptide map of GPIc + GPIc* obtained in the present study (Fig. 6) is the first to be available. The tryptic-peptide maps of GPIa and GPIIa share similar patterns, but also show differences from those previously published [19], which were produced from peptides cut out from O'Farrell polyacrylamide gels.

GPIIb

The molecular mass, under non-reducing or reducing conditions, of the 85 kDa protein present in peaks 1, 2 and 3 was similar to that of GPIIb (also known as GPIV or CD36). Results indicating that the 85 kDa band is GPIIb were obtained by tryptic-peptide-map analysis [12] (Fig. 5) and *N*-terminal sequencing (Tables 2 and 4). An anti-GPIIb MAb (OKM5) and a polyclonal antibody also respectively showed, in e.l.i.s.a. and Western blots, that the GP85 band was GPIIb (results not shown). GPIIb in peak 1 could be purified to homogeneity through an additional step involving gel filtration, performed after anion-exchange chromatography. GPIIb was recently reported to be a platelet receptor of thrombospondin [11,44].

Fig. 6. Tryptic peptide maps of glycoproteins (GMP-140, GPIa, GP1c + GP1c*, GPIIa, GP110/115 and GPIIb) isolated by two-dimensional non-reducing/reducing SDS/PAGE

Glycoproteins were excised from two-dimensional non-reducing/reducing SDS/PAGE gels. The iodinated-tryptic-peptide maps were separated in the first dimension by high-voltage electrophoresis and in the second dimension by t.l.c.

Table 4. Edman degradation of platelet glycoproteins GPIIb, GPIIb and GPIIIa

For abbreviations, see Table 3.

Edman cycle no.	GPIIIb		GPIIb		GPIIIa	
	Residue	PTH yield (pmol)	Residue	PTH yield (pmol)	Residue	PTH yield (pmol)
1	G	15.2	L	50.5	G	100.0
2	X		N	44.4	P	70.5
3	D	6.2	L	60.7	N	46.7
4	R	4.0	D	35.0	I	48.0
5	N	2.9	P	44.8	X	
6	X		V	38.0	T	40.0
7	G	3.9	Q	37.0	T	35.0
8	L	5.6	L	36.7	R	9.0
9	I	5.4	T	36.5	G	33.26
10	A	5.9	F	29.0	V	28.0
11	G	4.8	Y	26.2	S	ND
12	A	5.8				
13	V	4.0				
14	I	2.7				
15	G	4.1				
16	A	2.5				
17	V	2.9				
18	L	2.9				
19	A	4.0				
20	V	1.8				
21	F	2.8				
22	G	2.6				

Moreover, GPIIIb may act as a primary receptor for collagen and mediate platelet adhesion to collagen [45,46]. GPIIIb has recently been shown to be a receptor of *Plasmodium falciparum*-infected red blood cells [47]. GPIIIb has been isolated here by a new WGA-affinity and anion-exchange f.p.l.c. technique that

could be used to study the structural and functional properties of this glycoprotein.

GPIIb/IIIa and GPIb

GP140/120 and GP85/98 present in peaks 3, 4, 5 and 6 were identified as GPIIb and GPIIIa by affinity chromatography with MAb LYP4 and by gas-phase sequencing. Their *N*-terminal sequences were found to be identical with those previously published [48,49] (Tables 2 and 4). The GP160/130 band present in peak 6 was recognized as GPIb by Western blotting using a MAb directed against GPIb (result not shown). GPIb is the most sialylated platelet membrane glycoprotein and has been previously shown to be eluted at the end of the NaCl gradient [50].

In the present study a number of important new minor platelet glycoproteins were, for the first time, rapidly separated under non-denaturing conditions by using WGA-affinity and anion-exchange f.p.l.c. and characterized by gas-phase sequencing and MABs. Further work is needed, using the same techniques, to characterize other glycoproteins present on the platelet surface.

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