Further studies on the topography of the *N*-terminal region of human platelet glycoprotein IIIa

Localization of monoclonal antibody epitopes and the putative fibrinogen-binding sites

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The precise localization of the epitopes for six monoclonal antibodies specific for the *N*-terminal region of human platelet glycoprotein IIIa (GPIIIa) was determined. The epitope for P₃₇, a monoclonal antibody that inhibits platelet aggregation, was found at GPIIIa 101–109, flanked by the epitopes for P₂₃₋₃ (GPIIIa 16–28), P₂₃₋₄ (GPIIIa 83–91), P₂₃₋₅ (GPIIIa 67–73), P₂₃₋₇ (GPIIIa 114–122) and P₄₀ (GPIIIa 262–302), and very close to the early chymotryptic cleavage site of GPIIIa in whole platelets (Phe-100). When the amino acid sequence of GPIIIa was searched for peptide sequences hydrophatically complementary to the fibrinogen γ -chain C-terminal (γ 400–411) and A α -chain RGD-containing peptides, none was found for the γ 400–411, two (GPIIIa 128–132 and 380–384) were found complementary to fibrinogen A α 571–575 and two (GPIIIa 109–113 and 129–133) were found for A α 94–99. Two of these putative fibrinogen-binding sites overlap with each other, and a third one overlaps with the epitope for P₃₇. These findings reinforce the earlier suggestion that the *N*-terminal region of GPIIIa is involved in fibrinogen binding, and suggest the existence in GPIIIa of either multiple or alternative RGD-binding sites or one RGD-binding domain with several moieties. Finally, early chymotryptic cleavage of GPIIIa in whole platelets liberates to the soluble fraction the peptide stretch Ser-101–Tyr-348, which carries the epitope for P₃₇ and the putative binding sites for fibrinogen. The rest of the molecule, together with the GPIIb-resistant moiety, remains membrane-bound. This leads us to propose that the fibrinogen-binding domain of GPIIIa is not involved in the binding to GPIIb to form the Ca²⁺-dependent GPIIb–GPIIIa complex.

INTRODUCTION

Glycoprotein IIIa (GPIIIa) is a major platelet plasma-membrane protein, which together with glycoprotein IIb (GPIIb) forms a Ca²⁺-dependent heterodimer, the GPIIb–GPIIIa complex, which serves as the inducible fibrinogen receptor at the surface of activated platelets, and plays a primary role in platelet aggregation (Nurden *et al.*, 1986; Marguerie *et al.*, 1987; Phillips *et al.*, 1988). GPIIIa (92 kDa), whose cDNA-derived amino acid sequence predicts a single transmembrane segment that joins the short cytoplasmic C-terminal region to the large extracellular *N*-terminal domain, is a bitopic membrane glycoprotein, highly cross-linked by 28 disulphide bonds (Nurden *et al.*, 1986; Eirin *et al.*, 1986; Usobiaga *et al.*, 1987; Fitzgerald *et al.*, 1987; Rosa *et al.*, 1988). As with GPIIb, the biochemical composition and covalent structure of GPIIIa are mostly known; however, the higher-order structures and topology in the platelet plasma membrane are largely unknown (Eirin *et al.*, 1986; Calvete *et al.*, 1988, 1991b; Niewiarowski *et al.*, 1989; Beer & Coller, 1989).

Several laboratories have reported the preparation of monoclonal antibodies specific for GPIIIa, some of them with properties such as inhibition of adhesive protein binding or platelet aggregation and/or granule secretion, recognition of platelets only after EDTA and/or thrombin treatment, cross-reaction with endothelial or other cells etc. (Di Mino *et al.*, 1983; Thurlow *et al.*, 1983; Kornecki *et al.*, 1984; Melero & González-Rodríguez, 1984; Newman *et al.*, 1985, 1987). However, the precise localization of the epitopes for these antibodies is largely

unknown, except for P₃₇, an inhibitor of platelet aggregation whose epitope was located within the first 170 amino acid residues of the *N*-terminal region of GPIIIa (Calvete *et al.*, 1988).

Recently, it was found that RGD peptides become preferentially cross-linked to GPIIIa, somewhere between residues 109 and 171, in thrombin-stimulated platelets (Santoro & Lawing, 1987; D'Souza *et al.*, 1988). A hydrophatic complementarity approach was also used to predict the binding site for the A α -chain of fibrinogen to the GPIIb–GPIIIa complex, identifying the tetrapeptide NLGT (residues 133–136 of GPIIIa) as the putative sequence responsible for the binding of fibrinogen A α -chain to GPIIIa (Pasqualini *et al.*, 1989).

In the present paper a combination of chemical and enzymic cleavage procedures, solid-state peptide synthesis and enzyme immunoassay have led us to the precise localization of the epitopes for five monoclonal antibodies specific for the *N*-terminal region of GPIIIa. Further, the application of the principle of complementary hydrophathy (Blalock & Smith, 1984; Bost *et al.*, 1985) was used to predict the putative binding sites for fibrinogen in the amino acid sequence of GPIIIa.

MATERIALS AND METHODS

Materials

Chymotrypsin and tosylphenylalanylchloromethane ('TPCK')-treated trypsin were from Sigma Chemical Co. The other chemicals and biochemicals were of analytical or

Abbreviations used: GPIIb and GPIIIa, glycoproteins IIb and IIIa respectively; GPIIb α and GPIIb β , α - and β -subunits respectively of GPIIb; CM-GPIIIa, fully reduced and carboxymethylated GPIIIa; A α and γ , fibrinogen A α - and γ -chains respectively.

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chromatographic grade. Chromatographic columns and buffers, as well as the preparation of human platelets, platelet plasma membranes and the isolation of GPIIIa and the fully reduced and carboxymethylated form of GPIIIa (CM-GPIIIa), were as described previously (Eirin *et al.*, 1986). Solid-state peptide synthesis was performed by the procedure of Geysen *et al.* (1984), with reagents supplied by Cambridge Research Biochemicals (Cambridge, U.K.).

Monoclonal antibody production and purification

Mouse monoclonal antibodies P₆, P₃₇ and P₄₀ were produced as described previously (Melero & González-Rodríguez, 1984). Monoclonal antibodies P₂₃₋₃, P₂₃₋₄, P₂₃₋₅ and P₂₃₋₇ were prepared by using the *N*-terminal 23 kDa product of digestion of GPIIIa (Calvete *et al.*, 1988), according to immunization and fusion protocols and screening assays described previously (Melero & González-Rodríguez, 1984). Antibodies were purified from ascitic fluids after sequential 25%-saturated (NH₄)₂SO₄ and 50%-saturated (NH₄)₂SO₄ precipitation. Finally, the 50%-saturation-(NH₄)₂SO₄ precipitates were subjected to affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Analytical methods

Protein assay (Markwell *et al.*, 1978), amino acid and amino sugar analyses, SDS/PAGE (Laemmli, 1970), peptide electroelution from SDS/PAGE band (Hunkapiller *et al.*, 1983), peptide blotting from SDS/PAGE gels on to poly(vinylidene difluoride) membranes (Matsudaira, 1987), immunoelectroblotting (Towbin *et al.*, 1979) and enzyme immunoassay of natural and synthetic peptides were effected as described in a previous paper (Calvete *et al.*, 1991a). *N*-Terminal sequence analyses were effected either in a prototype automated spinning-cup sequencer (Edman & Henschen, 1975) or in an Applied Biosystems 470 gas-phase sequencer, and the phenylthiohydantoin derivatives of the amino acids were analysed by reverse-phase h.p.l.c. (Henschen, 1986).

Digestion of whole platelets with chymotrypsin

Platelets were washed in 150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, containing either 1 mM-EDTA or 1 mM-CaCl₂ and 2 µg of apyrase (Sigma grade V)/ml, and resuspended at 5 × 10⁹ platelets/ml in the same buffer. The platelet suspension was incubated at 37 °C with 0.2 mg of chymotrypsin/ml. Samples were taken at 5, 15, 30, 60 and 360 min, and the digestion stopped by addition of phenylmethanesulphonyl fluoride (20 mol/mol of chymotrypsin). The digested platelets were centrifuged at 10000 *g* (*r*_{av.} 75 mm) for 10 min at 4 °C. The pellet was resuspended and sonicated in the same buffer, and the particulate fraction was obtained by ultracentrifugation at 160000 *g* (*r*_{av.} 65 mm) for 1 h at 4 °C.

Early tryptic digestion and chemical cleavage of isolated GPIIIa and CM-GPIIIa

Pure GPIIIa or CM-GPIIIa (2 mg/ml) in 50 mM-NH₄HCO₃/0.1% (v/v) *N*-ethylmorpholine buffer, pH 8.0 were treated with trypsin at an enzyme/glycoprotein ratio of 1:250 (w/w) at 37 °C, samples were taken at 5, 15, 45, 60 and 300 min, and proteolysis was stopped with a 25-fold molar excess of phenylmethanesulphonyl fluoride over trypsin (Calvete *et al.*, 1988).

CNBr (100 mg/ml) cleavage of CM-GPIIIa (10 mg/ml) was performed in 70% (v/v) formic acid under N₂ and in the dark. After 4 h at room temperature, the mixture was diluted with Milli Q water, freeze-dried, then suspended in 0.2 M-NH₄HCO₃

for 2 h at 37 °C, and finally freeze-dried again (Gross & Witkop, 1962).

RESULTS

Localization of the early site of chymotryptic cleavage of GPIIIa in whole platelets

When whole platelets are digested with chymotrypsin for periods of time ranging from 5 to 360 min, two main membrane-bound chymotryptic products are observed by SDS/PAGE and immunoblotting analyses of the digested platelet membrane fraction. The earliest product has a 120 kDa apparent molecular mass and is detectable with antibodies P₆, P₂₃₋₃, P₂₃₋₄, P₂₃₋₅, P₂₃₋₇, P₃₇ and P₄₀, and disappears after 30 min of digestion (Fig. 1a, lanes c and d). After 5 min of digestion, a 60 kDa product is already detectable by all those monoclonal antibodies except P₂₃₋₇, P₃₇ and P₄₀ (Fig. 1a, lane d). Two reduction fragments of the 120 kDa product are obtained: a large one of 93 kDa apparent molecular mass, detectable only with P₆, P₂₃₋₇, P₃₇ and P₄₀ (Fig. 1a, lanes e and f), and a small one of 14 kDa, detectable only with P₂₃₋₃, P₂₃₋₄ and P₂₃₋₅ (Fig. 1b, lane b). On the other hand, reduction of the 60 kDa product splits it into two new fragments: a large one of 65 kDa apparent molecular mass, recognized only by P₆ (Fig. 1a, lane f), and a small one of 14 kDa, detectable only by P₂₃₋₃, P₂₃₋₄ and P₂₃₋₅ (not shown), apparently the same as the small reduction fragment of the 120 kDa early product.

When the 93 kDa reduction fragment was electroblotted on to a poly(vinylidene difluoride) membrane, the *N*-terminal sequence determined was SIQV, which corresponds to a fragment beginning at Ser-101 in the amino acid sequence of GPIIIa. The differences in both the reduction products and the antibody recognition patterns between the 120 kDa and the 93 kDa products, together with the fact that the P₂₃ series of monoclonal antibodies were raised against the 23 kDa *N*-terminal tryptic fragment of GPIIIa (Calvete *et al.*, 1988), indicate that the epitopes for P₂₃₋₃, P₂₃₋₄ and P₂₃₋₅ are within the Gly-1-Phe-100 peptide stretch of GPIIIa (Fig. 2).

Localization of the early sites of tryptic cleavage of GPIIIa in solution and the epitope for P₄₀

We had seen before that early tryptic digestion of pure GPIIIa in solution effects a single cleavage, which after selective reduction of a single disulphide bond splits the molecule into two fragments: one of 23 kDa, which carries the *N*-terminal sequence of GPIIIa and the epitope for P₃₇, and a large fragment of 80 kDa, recognized by P₆ and P₄₀ (Calvete *et al.*, 1988). When the 80 kDa fragment was isolated by SDS/PAGE and electroelution, the *N*-terminal sequence determined was DAPEGGF, which corresponds to a peptide starting at Asp-217 in the amino acid sequence of GPIIIa. If the digestion continues, two new tryptic products are observed: the 17 kDa fragment, which still carries the epitopes for P₃₇, and the 70 kDa fragment, which carries the epitopes for P₆ and P₄₀ (Calvete *et al.*, 1988) (Fig. 2).

When the 70 kDa fragment was isolated by SDS/PAGE and electroelution, the *N*-terminal sequence determined was LAGIVQ, which corresponds to a peptide beginning at Leu-262 in the GPIIIa amino acid sequence. Further digestion leads to degradation of the 17 kDa fragment into small peptides and loss of the P₃₇ epitope, and the 70 kDa fragment is cleaved further, giving rise to a new product of 52 kDa, which has lost the epitope for P₄₀, but still preserves the epitope for P₆. When this 52 kDa product is isolated by SDS/PAGE electrophoresis and electroelution, the *N*-terminal amino acid sequence determined was NINLIF, which corresponds to a peptide beginning at Asn-303 in the amino acid sequence of GPIIIa (Fig. 2).

Fig. 1. Analysis by immunoelectroblotting of the membrane-bound early chymotryptic products of digestion of GPIIIa on whole platelets

The 5 min digestion with chymotrypsin of whole platelets and the preparation of the particulate fraction were as described in the Materials and methods section. Monoclonal antibodies were used individually at the following dilutions: P_6 (1:5000), P_{23-3} (1:3000), P_{23-4} (1:3000), P_{23-5} (1:5000), P_{23-7} (1:5000), P_{37} (1:2000) and P_{40} (1:1000). (a) The immunoelectroblotting analysis was carried out after SDS/PAGE (7% polyacrylamide) of 50 μ g of total protein of the particulate fraction, either solubilized with SDS (lanes c and d) or reduced, carboxymethylated, washed and solubilized with SDS (lanes e and f). Lane c, products recognized by P_{23-3} , P_{23-4} , P_{23-5} , P_{23-7} , P_{37} and P_{40} . Lane e, products detected by P_{23-7} , P_{37} and P_{40} . Lanes d and f, products recognized by P_6 . Lanes a and h, non-reduced and reduced molecular-mass standard proteins respectively: Gal (135 kDa), β -galactosidase; Ph (92 kDa), phosphorylase b; BSA (68 kDa), bovine serum albumin; Ovo (43 kDa), ovalbumin. Lanes b and g, non-reduced and reduced GPIIIa (5 μ g) respectively. Lanes a, b, g and h were stained with Amido Black. (b) The immunoblotting analysis of the reduced and carboxymethylated particulate fraction was carried out after SDS/PAGE (15% polyacrylamide). Lanes a and b, fragments recognized by P_{37} and P_{23-4} respectively.

Fig. 2. Outline of the early cleavage points and products of tryptic and chymotryptic digestion of GPIIIa in solution and in whole platelets respectively

Proteolytic cleavage points are indicated by arrows, numbered according to the time course of the appearance of the corresponding cleavage products, and located in the amino acid sequence of GPIIIa by the position (when known) of the C-terminal side of the cleavage point. The potential CNBr-cleavage points at the N-terminal region of GPIIIa are indicated by the position of the methionine residues in the sequence. The depicted disulphide bond, which joins the N-terminal region to the rest of the molecule, has been assigned (Calvete *et al.*, 1991b). (+), Determined by Niewiarowski *et al.* (1989). Abbreviations: TRY, trypsin; CHY, chymotrypsin; r- before a fragment means peptide obtained after reduction of the corresponding proteolytic product; amino acid residues are referred by one-letter symbols.

The information above indicates that the epitope for P₄₀ is between Leu-262 and Asn-303, that the epitope for P₆ must be upstream of Asn-303 and that the epitopes for P₂₃₋₇ and P₃₇ must be located between Phe-100 and Asp-217.

Localization of the epitopes for P₂₃₋₃, P₂₃₋₄, P₂₃₋₅, P₂₃₋₇ and P₃₇

Immunoelectroblotting analysis shows that all these antibodies recognize the reduced and carboxymethylated 23 kDa and 17 kDa tryptic products of digestion of GPIIIa in solution (Fig.

3). When CM-GPIIIa was cleaved with trypsin or with CNBr and the cleavage mixture assayed by competitive enzyme immunoassay, it was found that the epitopes for P₂₃₋₃, P₂₃₋₅, and P₃₇ were destroyed by trypsin digestion, but not by CNBr treatment, and vice versa for the epitopes for P₂₃₋₃ and P₂₃₋₇.

The 17 kDa peptide contains the first 150 amino acid residues of GPIIIa, and therefore four methionine residues (Fig. 2) and 11

Table 1. Synthetic peptides designed from the amino acid sequence of platelet GPIIIa

The peptides were synthesized by the procedure of Geysen *et al.* (1984), and the degree of overlapping of their sequences with those of the epitopes for a set of anti-GPIIIa monoclonal antibodies was assessed by enzyme immunoassay (see Fig. 4).

Peptide	Sequence
1	(2) PNICTTRGV
2	ICTTRGVSS
3	TTRGVSSCQ
4	(16) CLAVSPMCA
5	AVSPMCAWC
6	SPMCAWCSD
7	(31) LPLGSPRCD
8	LGSPRCDLK
9	SPRCDLKEN
10	RCDLKENLL
11	DLKENLLKD
12	KENLLKDNC
13	NLLKDNCAP
14	LKDNCAPES
15	(56) FPVSEARVL
16	VSEARVLED
17	EARVLEDRP
18	(66) DRPLSDKGS
19	PLSDKGSGD
20	SDKGSGDSS
21	(81) TQVSPQRIA
22	VSPQRIALR
23	PQRIALRLR
24	RIALRLRPD
25	ALRLRPDDS
26	LRPDDSKNF
27	PDDSKNFSI
28	DSKNFSIQV
29	NFSIQVRQV
30	SIQVRQVED
31	QVRQVEDYP
32	(112) VDIYYLMDL
33	IYYLMDLSY
34	YLMDLSYSM
35	MDLSYSMKD
36	LSYSMKDDL
37	YSMKDDLWS
38	(131) IQNLGTKLA
39	NLGTKLATQ
40	GTKLATQMR
41	KLATQMRKL
42	ATQMRKLTS
43	QMRKLTSNL
44	KLTSNLRIG
45	TSNLRIGFG
46	NLRIGFGAF
47	(159) KPVSPYMYI
48	VSPYMYISP
49	PYMYISPPE

Fig. 3. Analysis by immunoelectroblotting of the N-terminal tryptic digestion products of isolated GPIIIa in solution recognized by P₂₃₋₃, P₂₃₋₄, P₂₃₋₅, P₂₃₋₇ and P₃₇

GPIIIa (2 mg/ml) in 50 mM-NH₄HCO₃/0.1 % N-ethylmorpholine buffer, pH 8.0, was digested with trypsin at a glycoprotein/trypsin ratio of 250:1 (w/w). The immunoelectroblotting analysis was done after SDS/PAGE (10% polyacrylamide) of the reduced and carboxymethylated tryptic products at different digestion times. Lanes a and c, 15 min and 45 min digestion products respectively recognized by antibodies P₂₃₋₃, P₂₃₋₄, P₂₃₋₅, P₂₃₋₇ and P₃₇, used separately at the same dilutions as in Fig. 1; lane b, GPIIIa control.

Fig. 4. Localization of the epitopes for P₂₃₋₃, P₂₃₋₄, P₂₃₋₅, P₂₃₋₇ and P₃₇ with the use of synthetic peptides and enzyme immunoassay

The assessment of the degree of overlapping between the sequences of the synthetic peptides listed in Table 1 and those of the epitopes for the GPIIIa N-terminal-specific monoclonal antibodies was done by enzyme immunoassay according to the procedure of Geysen *et al.* (1984). The antibodies were used at the following dilutions: P₂₃₋₃ (1:3000), P₂₃₋₄ (1:3000), P₂₃₋₅ (1:5000), P₂₃₋₇ (1:5000) and P₃₇ (1:2000).

Fig. 5. Hydropathy plots of GPIIIa peptide sequences hydropathically complementary to fibrinogen A α -chain RGD peptides

(a) Comparison of the hydropathy profile of fibrinogen A α 571-575 peptide (NRGDS) (■) with those of its anti-peptide, encoded in the DNA sequence complementary to the coding strand for A α 571-575 peptide (Rixon *et al.*, 1983) and translated in the 3'→5' direction (LSPLR) (●), and the GPIIIa 128-132 (LWSIQ) (□) and GPIIIa 380-384 (IPGLK) (○) peptide sequences. (b) Comparison of the hydropathy profile of fibrinogen A α 94-99 peptide (LRGDFS) (○) with those of its 3'→5' anti-peptide (NSPLK) (Rixon *et al.*, 1983) (●) and the GPIIIa 109-113 (DYPVD) (□) and GPIIIa 129-133 (WSIQN) (■) peptide sequences.

putative tryptic-cleavage sites (Calvete *et al.*, 1988). So, to reach a high resolution in the localization of the epitopes for these antibodies, all the peptides around these methionine residues and tryptic-cleavage sites were synthesized, and the synthetic peptides (Table 1) assayed by enzyme immunoassay (Fig. 4). In this way we were able to determine the precise localization for all these antibodies: P₂₃₋₃, Cys-16-Asp-28; P₂₃₋₄, Val-83-Arg-91; P₂₃₋₅, Arg-67-Gly-73; P₂₃₋₇, Ile-114-Tyr-122; P₃₇, Ser-101-Asp-109.

Localization in the GPIIIa sequence of the anti-peptides complementary to the fibrinogen A α -chain RGD peptide sequences

We have searched in the sequence of GPIIIa for the presence of the anti-peptide sequences hydropathically complementary to those fibrinogen peptide stretches putatively involved in the GPIIb-GPIIIa-fibrinogen interaction, i.e. the γ -chain C-terminal end (γ 400-411) and the A α -chain RGD-containing peptides (A α 568-579 and A α 91-99).

The sequences of the anti-peptides encoded in the DNA sequence complementary to the coding strand for the γ 400-411 (Rixon *et al.*, 1985) and A α 568-579 and A α 91-99 (Rixon *et al.*, 1983; Kant *et al.*, 1983) were translated both in the 3'→5' and the 5'→3' directions (see Calvete *et al.*, 1991a). If we now search for sequence stretches in the GPIIIa amino acid sequence hydropathically similar to these six anti-peptides, we find no complementary sequence for the γ 400-411 anti-peptides, two complementary sequences for the A α 571-575 anti-peptides, GPIIIa 128-132 and GPIIIa 380-384, and two for the A α 94-99 anti-peptides, GPIIIa 109-113 and GPIIIa 129-133 (Figs. 5a and 5b).

Fig. 6. Outline of the precise localization in the N-terminal region of GPIIIa of the epitopes for monoclonal antibodies P₂₃₋₃, P α 23-4, P α 23-5, P α 23-7, P α 37 and P α 40 and the putative binding regions found for fibrinogen

Key to symbols: ◇—, N-glycosylation points; A α 94-99 and A α 571-575, putative binding regions for the fibrinogen A α -chain; ←, early chymotryptic-cleavage points in whole platelets; S—S, disulphide bond joining the N-terminal end to the proteinase-resistant core of GPIIIa; black thickened segments, sequences of highest similarity among the integrin β -subunits.

DISCUSSION

In Fig. 6, and on the basis of the cDNA-derived amino acid sequence (Fitzgerald *et al.*, 1987; Rosa *et al.*, 1988), we outline the information gathered in the present work on the precise localization of the early chymotryptic cleavage sites of GPIIIa in whole platelets, the epitopes for six monoclonal antibodies specific for the N-terminal region of GPIIIa and the putative binding sites for the A α -chains of fibrinogen in GPIIIa.

Early chymotryptic cleavage sites

The pattern of chymotryptic digestion of GPIIIa in whole platelets is the same as that reported before (Kornecki *et al.*, 1985; Calvete *et al.*, 1988; Beer & Coller, 1989). The late-appearing 60 kDa fragment, being the same as that reported by Kornecki *et al.* (1985), must contain two N-terminal sequences, as found by Niewiarowski *et al.* (1989), one that corresponds to the 14 kDa reduction product and is identical with that in intact GPIIIa, and the sequence beginning at Gly-349, which corresponds to the 65 kDa reduction product. The early chymotryptic-cleavage point was determined here by N-terminal amino acid sequence analysis of the 120 kDa fragment and found at Phe-100, which does not confirm previous estimations made by Beer & Coller (1989), who had located it somewhere between residues 156 and 166 of GPIIIa. We have not seen the small product (22 kDa) of reduction of the 120 kDa fragment,

which Beer & Collier (1989) observed, because under our experimental conditions it must be degraded very rapidly to the 14 kDa fragment, which is the only one that we detect. From the present information and our earlier findings on the selective cleavage of the single disulphide bond joining the *N*-terminal region (the first 17 kDa from the *N*-terminal end) to the rest of the molecule of GPIIIa, it can be concluded that the other cysteine residues forming this disulphide bond must be upstream of Gly-349.

Epitopes for the monoclonal antibodies specific for the GPIIIa *N*-terminal domain

In previous work (Calvete *et al.*, 1988), the epitope for P₃₇, an antibody that inhibits platelet aggregation, had been located within the first 17 kDa from the *N*-terminal of GPIIIa. At the same time, RGD peptides had been found cross-linked to this region of GPIIIa, both in the GPIIb–GPIIIa complex of activated platelets (D'Souza *et al.*, 1988) and in the placental vitronectin receptor in solution (Smith & Charesh, 1988).

Now we have located the epitope for P₃₇ between residues 101 and 109 of GPIIIa, immediately close to two very accessible sites in the topography of this glycoprotein in whole platelets, namely Asn-99, an *N*-glycosylation point in mature GPIIIa (J. J. Calvete, A. Henschen & J. González-Rodríguez, unpublished work), and Phe-100, the early chymotryptic cleavage site of GPIIIa in whole platelets. The peptide loop carrying these three fully accessible sites in whole platelets is flanked by non-accessible peptide stretches in resting platelets (E. Muñoz, C. Castellarnau & J. González-Rodríguez, unpublished work), at its *N*-terminal site the epitopes for P₂₃₋₄ (GPIIIa 83–91) and P₂₃₋₅ (GPIIIa 67–73), and at its *C*-terminal site the epitope for P₂₃₋₇ (GPIIIa 114–122), which becomes exposed only after EDTA treatment or thrombin activation.

Monoclonal antibody P₂₃₋₃, whose epitope (GPIIIa 16–28) is not accessible in resting platelets (E. Muñoz, C. Castellarnau & J. González-Rodríguez, unpublished work), is a good marker for the *N*-terminal cysteine-rich domain of GPIIIa, which is joined to the cysteine-rich proteinase-resistant core of GPIIIa by a single disulphide bond (Calvete *et al.*, 1991b). On the other hand, P₄₀, whose epitope (GPIIIa 262–302) exposure in whole platelets is EDTA-dependent, cross-reacts with the β -subunit of vitronectin receptor in endothelial cells (E. Muñoz, C. Castellarnau & J. González-Rodríguez, unpublished work), as P₃₇ and P₂₃₋₇ do, suggesting that the topography of GPIIIa in the platelet GPIIb–GPIIIa complex is different from that in the endothelial vitronectin receptor.

Putative binding sites in GPIIIa for the A α -chains of fibrinogen

The sequence GPIIIa 128–132 (LWSIQ), hydrophatically complementary to the fibrinogen A α 571–575 peptide, is within the peptide stretch where D'Souza *et al.* (1988) found an RGD peptide cross-linked to GPIIIa, and very close to the epitope (GPIIIa 101–109) for P₃₇, an antibody that inhibits platelet aggregation (Melero & González-Rodríguez, 1984), and to the epitope for the anti-WTVPTA antibody (an inhibitor of platelet aggregation), which Pasqualini *et al.* (1989) located tentatively in the GPIIIa 133–136 sequence. The sequence GPIIIa 380–384 (IPGLK), also hydrophatically complementary to the fibrinogen A α 571–575 peptide, is very close to the binding site for the anti-WTVPTA/anti-GAVSTA antibody in rat and human respectively, fibronectin receptor (Brentani *et al.*, 1988), this antibody being an inhibitor of the binding of these receptors to fibronectin matrices.

Of the GPIIIa sequence stretches found to be hydrophatically complementary to the fibrinogen A α 94–99 peptide, the sequence

GPIIIa 129–133 (WSIQN) overlaps the GPIIIa 128–132 sequence, which is complementary to the fibrinogen A α 571–575 peptide, as we have seen above, whereas the GPIIIa 109–113 sequence (DYPVD) overlaps the *C*-terminal end of the epitope for P₃₇.

The results from the hydrophatic complementarity approach (Pasqualini *et al.*, 1989; Calvete *et al.*, 1991a; and the present work) reinforce the importance of the *N*-terminal region of GPIIIa for the binding of the A α chains of fibrinogen, as the localization of the epitopes for the antiaggregating antibodies and the cross-linking binding sites for peptide inhibitors had already suggested. They also point to the existence in GPIIIa of either multiple and alternative RGD-binding sites or one fibrinogen-binding domain with several moieties.

Clues on the binding sites in GPIIIa for GPIIb when forming the GPIIb–GPIIIa complex

Several clues suggest that the area of GPIIIa involved in the binding to GPIIb is located outside the sequence in whole platelets early chymotryptic-cleavage sites of GPIIIa in whole platelets (Phe-100–Tyr-348). The access to the epitopes for P₄₀ and P₂₃₋₇ suggests that both glycoproteins should be dissociated for these epitopes to be exposed, or some thrombin-induced rearrangement has to take place before P₂₃₋₇ binds to whole platelets (E. Muñoz, C. Castellarnau & J. González-Rodríguez, unpublished work). This could lead to postulate that GPIIIa 114–303 sequence (carrying the epitopes for P₂₃₋₇ and P₄₀) could be involved in the formation of the surfaces of interaction between GPIIb and GPIIIa, as we postulated for the GPIIb α 570–748 sequence carrying the epitope for M6 (Calvete *et al.*, 1991a), whose exposure is also EDTA- or thrombin-dependent. However, whereas the GPIIb α 570–748 sequence is the region of GPIIb α most resistant to chymotryptic digestion in whole platelets, the opposite happens to the GPIIIa 100–348 sequence. This is lost once the late-appearing 60 kDa fragment is formed and the induced fibrinogen-binding capacity is lost, and while the most resistant region of GPIIb α still remains membrane-bound.

Therefore there seems to be mounting evidence for a prominent role of the GPIIIa 100–348 domain in the binding of fibrinogen to its platelet receptor, as well as some clues on its lack of involvement in the binding of GPIIb to GPIIIa in the Ca²⁺-dependent complex. This domain includes the most highly conserved sequence stretches among all the integrin β -subunits. The other three domains of GPIIIa would be the *N*-terminal cysteine-rich domain, the proteinase-resistant core, which is bound to the *N*-terminal domain by a single disulphide bond, and the *C*-terminal domain, comprising the transmembrane and the short cytoplasmic subdomains.

J.A. has been a recipient of a Research Action training contract (Biotechnology Programme) of the Commission of the European Communities. We thank Dr. J. A. Melero for his assistance at the fusion stage of the monoclonal antibody production and Dr. G. Rivas for his help in the preparation of GPIIIa. We also thank Mrs. H. Gross, Mrs. G. Pinillos and Mrs. B. Rinke for technical assistance, Miss S. Salado and Mr. F. Romera for typing the manuscript, and the Blood Banks of Centro Ramón y Cajal, La Paz and Doce de Octubre (Madrid) for providing us with the outdated platelet concentrates. This work was supported by the Secretaría de Estado para Universidades e Investigación (ID 87077 S.E.U.I.; PM-022 S.E.U.I.) and an Acción Integrada Hispano-Germana (1989–1990 43 A).

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Received 21 May 1990/20 August 1990; accepted 3 September 1990