Further studies on the topography of human platelet glycoprotein IIb

Localization of monoclonal antibody epitopes and the putative glycoprotein IIIa- and fibrinogen-binding regions

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Glycoprotein IIb (GPIIb) is a major glycoprotein of the human platelet plasma membrane, which together with glycoprotein IIIa (GPIIIa) forms a Ca^{2+}-dependent heterodimer, GPIIb/IIIa, which serves as the major fibrinogen receptor in activated platelets. The precise localization of the epitopes for six anti-GPIIb monoclonal antibodies (M1–M6) has been determined by a combination of enzymic and chemical cleavage procedures, peptide isolation, N-terminal sequence analysis, peptide synthesis and enzyme immunoassay. The following localizations were found: M1, β1-16–36, β2-4–24; M2, α747–755; Mα2, α837–843; M3, α849–857; M4, α143–151; M5, α550–558; M6, α657–665. Besides considerations of the degree of exposure of these epitopes, several remarkable features are readily apparent. The earliest and main chymotryptic cleavage site of GPIIb in whole platelets is between αcysteine-545 and αphenylalanine-551. The epitope for M3 was located within the same sequence (α842–857) as is the epitope for PMI-1 [Loftus, Plow, Frelinger, D'Souza, Dixon, Lacy, Sorge & Ginsberg (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7114–7118] in spite of the fact that the exposure of the latter in whole platelets is EDTA-dependent whereas that in the former is not. The epitope for M5 shares full homology with the 540–548 peptide stretch of the α-subunit of the vitronectin receptor, and this antibody cross-reacts with endothelial cells. The M6 epitope is located in the 25 kDa membrane-bound fragment of GPIIb, which is most resistant to chymotryptic digestion in whole platelets, contrary to what happens in isolated GPIIb in solution, where this epitope is destroyed at an early stage of chymotryptic digestion. This suggests that this region of GPIIb, somewhere between the epitope for M5 (α550–558) and the epitope for M2 (α747–755), may carry the surface of interaction of GPIIb with GPIIIa in the GPIIb/IIIa heterodimer. Finally, the sequence where the epitope for M6 has been located (α657–667) was the only one found to be hydropathically complementary to the γ402–411 peptide of fibrinogen within the amino acid sequence of both GPIIb and GPIIIa. This complementariness, the EDTA- or thrombin-dependence of the exposure of the α657–665 stretch in whole platelets to M6 and the ability of this antibody to inhibit platelet aggregation led us to postulate that this peptide stretch is a putative binding site for fibrinogen in the platelet receptor. The overlap between the M6 epitope and the putative binding site for the γ402–411 peptide sequence of fibrinogen would imply that the unmasking of the α658–667 peptide stretch could be one of the structural changes in GPIIb/IIIa required for the induction of the fibrinogen receptor in activated platelets.

INTRODUCTION

Glycoprotein IIb (GPIIb) is a major platelet plasma-membrane glycoprotein, which together with glycoprotein IIIa (GPIIIa) forms a Ca^{2+}-dependent heterodimer, the GPIIb/IIIa complex, which serves as the inducible receptor for fibrinogen at the surface of activated platelets (Nurden et al., 1986; Marguerie et al., 1987; Phillips et al., 1988). GPIIb (136 kDa) is a bitopic two-chain glycoprotein joined by a single interchain disulphide bond (Calvete & González-Rodríguez, 1986; Poncz et al., 1987; Usobiaga et al., 1987). The heavy chain GPIIbα (114 kDa), also known as GPIIb H, is fully extracellular, whereas the light chain, GPIIbβ (23 kDa), also known as GPIIb L, carries the single transmembrane segment of GPIIb, as predicted from its cDNA-derived amino acid sequence (Poncz et al., 1987). The biochemical composition, amino acid sequence and covalent structure of GPIIb are known, but the tertiary structure and topology in the platelet membrane remain to be determined (Eirin et al., 1986; Calvete et al., 1989a,b; Lam et al., 1989).

Anti-GPIIb monoclonal antibodies have been prepared in several laboratories (McEver et al., 1980; Varon & Karpatkin, 1983; Thorsen et al., 1985; Loftus et al., 1987; Calvete et al., 1989a); however, the precise localization of their epitopes is known for very few of them. The binding of some of these antibodies is EDTA-, thrombin- or RGD peptide-dependent, some are inhibitors of platelet aggregation and/or fibrinogen binding, some cross-react with the α-subunit of the vitronectin receptor in endothelial cells etc. The epitope for PMI-1 has been located between residues α842 and α858 (Loftus et al., 1987), the epitopes for M1, M3 and M4 were found within the sequences β4–24, α826–871 and α115–285 respectively, and finally the epitopes for M5 and M6 were located somewhere between residues α550 and α700 (Calvete et al., 1989a).

Recently considerable interest has been aroused in the localization of the fibrinogen-binding sites in both GPIIb and GPIIIa and some progress has already been made in determining these. RGD peptides become preferentially cross-linked to the peptide

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Abbreviations used: GPIIb, glycoprotein IIb; GPIIbα and GPIIbβ, the α- and the β-subunits respectively of GPIIb; CM-GPIIbα, fully reduced and carboxymethylated α-subunit of GPIIb; GPIIIa, glycoprotein IIIa; Aα and γ, fibrinogen Aα- and γ-chains respectively.

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sequence 109–171 of GPIIIa, in thrombin-stimulated platelets, whereas peptides of the fibrinogen γ-chain C-terminal type label predominantly GPIIb (Santoro & Lawing, 1987; D'Souza et al., 1988). A hydropathic complementarity approach has also been used to predict the binding site for the Aα-chain of fibrinogen in the platelet GPIIb/IIIa complex (Pasqualini et al., 1989).

In the present work, the localization of the epitopes for a set of monoclonal antibodies has been narrowed down, by a combination of chemical and enzymic cleavage procedures, solid-state peptide synthesis and competitive enzyme immunoassay. In addition, we have applied the principle of complementary hydropathy to predict the putative binding sites for fibrinogen in the amino acid sequence of GPIIb.

MATERIALS AND METHODS

Materials

Endoproteinase Lys-C, endoproteinase Glu-C (*Staphylococcus aureus* V8) and tosylphenylalanylchloromethane ('TPCK')-treated trypsin were from Boehringer–Mannheim, Miles Laboratories and Sigma Chemical Co., respectively. The other chemicals and biochemicals were of analytical or chromatographic grade. Chromatographic columns and buffers, as well as the preparation of human platelets, platelet plasma membrane and the isolation of GPIIb and the fully reduced and carboxymethylated forms of GPIIbα (CM-GPIIbα) and GPIIbβ (CM-GPIIbβ) were as previously described (Calvete & González-Rodríguez, 1986; Eirin et al., 1986).

Monoclonal antibody production and purification

Mouse monoclonal antibodies anti-GPIIbα (M2, M3, M4, M5, M6 and Mα2) and anti-GPIIbβ (M1) were prepared by using either whole GPIIb or the isolated subunits according to immunization and fusion protocols and screening assays described previously (Melero & González-Rodríguez, 1984). Antibodies were purified from ascitic fluids after sequential 25%- and 50%-satd.-$(NH_4)_2SO_4$ precipitation. Finally, the 50%-satd.-$(NH_4)_2SO_4$ precipitates were subjected to affinity chromatography on Protein A-Sepharose (Pharmacia) according to the manufacturer's instructions.

Solid-state peptide synthesis

The procedure described by Geysen et al. (1984) was followed, using reagents supplied by Cambridge Research Biochemicals and a polyethylene rod holder with the format and spacing of a micro-titre plate.

Enzyme immunoassay of the natural and synthetic peptides

The criterion for epitope localization was based on the specific recognition by the monoclonal antibodies of natural or synthetic peptides. The assay of synthetic peptides coupled to polyethylene rods (see above) was done as described by Geysen et al. (1984). Anti-(mouse IgG) IgG coupled to horseradish peroxidase was used as the second antibody, with either o-phenylenediamine dihydrochloride or 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) in the developing solution.

The assay of natural peptides was done by a competitive enzyme immunoassay which consisted in the incubation of the natural peptide with the monoclonal antibody of interest for 1 h at 37 °C, followed by the assay of the free antibody in this mixture by an ordinary enzyme immunoassay. Whole glycoprotein or some fragment derived from it was used to coat the walls of the microplate wells. The second antibody and developing solution were the same as those used above for the assay of synthetic peptides.

Other analytical methods

Protein assay was done by the procedure of Markwell et al. (1978). Amino acid and amino sugar analyses were performed with a Biotronik amino acid analyser, after sample hydrolysis at 110 °C in 6 M-HCl for 24 h and in 4 M-HCl for 4 h respectively. N-Terminal sequence analyses were effected in a prototype automated spinning-cup Sequenator, as described previously (Edman & Henschen, 1975). SDS/PAGE was done according to the procedure of Laemmli (1970). Immunoelectroblotting was carried out after gel electrophoresis by transferring the protein bands to nitrocellulose by a standard procedure (Towbin et al., 1979). The monoclonal antibodies were used, either as 50%-satd.-$(NH_4)_2SO_4$ precipitates or after purification by Protein A–Sepharose chromatography (see above). The second antibody was a goat anti-(mouse IgG) IgG coupled to horseradish peroxidase, with 4-chloro-1-naphthol in the developing solution.

Enzymic digestion of the CM-GPIIbα with endoproteinase Glu-C

Isolated CM-GPIIbα (5 mg/ml in 50 mM-sodium phosphate buffer, pH 7.8) was digested with endoproteinase Glu-C at an enzyme/substrate ratio 1:25 (w/w) at 37 °C for 18 h. The reaction was stopped by adding formic acid up to 30% (v/v) final concentration. The digestion products were separated by reverse-phase h.p.l.c., on a C_{18} (pore size 30 nm, particle size 10 μm) Vydac column (25 cm × 0.4 cm) equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solution A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solution B) (90% solution A/10% solution B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 70% of solution B in 120 min.

Enzymic digestion of the 35 kDa N-terminal fragment of CM-GPIIbα with endoproteinase Lys-C

Isolated CM-GPIIbα was cleaved with a 500-fold molar excess of CNBr over its theoretical methionine content in 70% (v/v) formic acid, under N_2 and in the dark. After 24 h at room temperature the mixture was diluted 10-fold with Milli Q water and freeze-dried. The cleavage products were resuspended in 70% formic acid and separated on a Sephacryl S200 column (130 cm × 1 cm), eluted with 10% formic acid as described previously (Calvete et al., 1989b). The isolated 35 kDa CNBr-cleavage fragment, 1.75 mg/ml in 0.1 M-Tris/HCl buffer, pH 7.6, which contains the N-terminal peptide of GPIIbα (α1–285), was further digested with endoproteinase Lys-C, at an enzyme/substrate ratio 1:25 (w/w) for 18 h at 37 °C. The reaction was stopped by adding formic acid up to 30% (v/v) final concentration, and the digestion products were separated by reverse-phase h.p.l.c. on a C_{18} Vydac column (25 cm × 0.4 cm), equilibrated in a mixture of 0.1% trifluoroacetic acid in water (solution A) and 0.1% trifluoroacetic acid in acetonitrile (solution B) (95 solution A/5% solution B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 70% of solution B in 65 min.

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 $5 × 10^9$ 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 min, 15 min, 30 min, 1 h and 2 h, and the digestion was stopped by the addition of phenylmethanesulphonyl fluoride (25 mol/mol of chymotrypsin). 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 obtained by ultracentrifugation (160000 g, $r_{av.}$ 65 mm) for 1 h at 4 °C.

RESULTS

Cleavage susceptibility patterns of the epitopes for monoclonal antibodies M1, M2, Mα2, M3, M4, M5 and M6

As a preliminary strategy to narrow down the location of the epitopes for the set of anti-GPIIb antibodies studied here, we have assessed the susceptibility of these epitopes to different chemical and enzymic cleavage procedures by competitive enzyme immunoassay. The cleavage susceptibility patterns for the different antibodies are summarized in Table 1, from which several guiding conclusions can be drawn. Whereas the epitope for M1 is the only one remaining after tryptic digestion of GPIIb, the epitope for M6 is the only one destroyed by CNBr cleavage of GPIIb, and therefore the primary peptide fragments for the isolation of the epitopes for all the antibodies except for M6 can be obtained after GPIIb cleavage by CNBr. From a consideration of the susceptibility of the epitopes to cleavage by the proteinases used, it is probable that the epitopes for M2, M4, M5 and M6 contain arginine, those for M3 and Mα2 include a lysine residue in their sequence, the epitope for M5 contains some dicarboxylic amino acid and that for M1 is free from dicarboxylic acid, arginine and lysine residues. Finally, the susceptibility of the epitope for M6 to cleavage by CNBr suggests that there is some methionine residue in its sequence.

This information was used subsequently to determine the cleavage procedures employed to isolate the peptide fragments containing the epitopes for these antibodies, and to select those peptide sequences within them that were likely to contain an epitope with a certain cleavage susceptibility pattern. Further assessment of this prediction was done by a combination of solid-state peptide synthesis and enzyme immunoassay, as shown below.

Epitope for M1

The epitope for this anti-GPIIbβ antibody had been found previously.to be at the N-terminus of this subunit, between βPro-4 and Met-24 (Calvete & González-Rodríguez, 1986; Calvete *et al.*, 1989a) by immunoelectroblotting. Now we have confirmed this result using competitive enzyme immunoassay.

Epitopes for M2, M3 and Mα2

Previously we had located the epitope for M3 in the C-terminal 19 kDa CNBr-cleavage product of CM-GPIIbα

Table 1. Chemical and enzymic cleavage susceptibility pattern of the epitopes for a set of anti-GPIIb monoclonal antibodies

The cleavage susceptibility was assessed by competitive enzyme immunoassay, where the different mixtures of cleavage products were made to compete for the different monoclonal antibodies with GPIIb or some fragments derived from it, adsorbed to the microtitration plate. + or − mean antibody epitope conserved or destroyed respectively after chemical or enzymic cleavage.

	Cleavage susceptibility						
	M1	M2	M3	M4	M5	M6	Mα2
CNBr	+	+	+	+	+	−	+
Endoproteinase Lys-C	+	+	−	+	+	+	−
Trypsin	+	−	−	−	−	−	−
Endoproteinase Glu-C	+	+	+	−	−	+	+

Table 2. Synthetic peptides designed from the amino acid sequence of platelet GPIIb

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-GPIIb monoclonal antibodies was assessed by enzyme immunoassay (see Fig. 1).

Peptide		Sequence
1	(718)	S F Q L Q I R S K
2		F Q L Q I R S K N
3		Q L Q I R S K N S
4		L Q I R S K N S Q
5		Q I R S K N S Q N
6		I R S K N S Q N P
7	(728)	S Q N P N S K I V
8		Q N P N S K I V L
9		N P N S K I V L L
10		P N S K I V L L D
11	(737)	L L D V P V R A E
12		L D V P V R A E A
13		D V P V R A E A Q
14		V P V R A E A Q V
15		P V R A E A Q V E
16	(745)	E A Q V E L R G N
17		A Q V E L R G N S
18		Q V E L R G N S F
19		V E L R G N S F P
20	(763)	A E E G E R E Q N
21		E E G E R E Q N S
22		E G E R E Q N S L
23		G E R E Q N S L D
24	(773)	L D S W G P K V E
25		D S W G P K V E H
26		S W G P K V E H T
27		W G P K V E H T Y
28	(831)	P V N P L K V D W
29		V N P L K V D W G
30		N P L K V D W G L
31		P L K V D W G L P
32	(849)	I H P A H H K R D
33		H P A H H K R D R
34		P A H H K R D R R
35	(136)	E S G R R A E Y S
36	(143)	Y S P C R G N T L
37	(149)	N T L S R I Y V E
38	(550)	A F L R D E A D F
39		D E A D F R D K L
40		F R D K L S P I V
41	(653)	L P Q G A H Y M R
42		A H Y M R A L S N

(α704–871) by immunoelectroblotting (Calvete *et al.*, 1989a). Now, besides confirming this, we have located the epitopes for M2 and Mα2 in this polypeptide fragment, using competitive enzyme immunoassay. To narrow down the localization of these epitopes further, solid-state peptide synthesis was used to obtain the overlapping peptide sequence which contained all the arginine and lysine residues of the C-terminal region of GPIIbα. These peptides were used in enzyme immunoassay to locate the three epitopes (Table 2). In Fig. 1 we show that M2 recognizes those synthetic peptides whose sequences contain Arg-750, and that M3 and Mα2 recognize those containing Lys-855 and Lys-836 respectively, as expected from the predictions made from their cleavage susceptibility patterns.
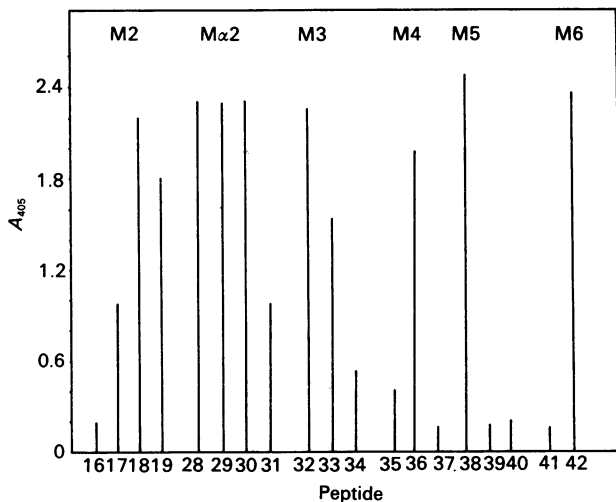
Fig. 1. Localization of the epitopes for monoclonal antibodies M2, Mα2, M3, M4, M5 and M6, using synthetic peptides and enzyme immunoassay

The assessment of the degree of overlapping between the sequences of the synthetic peptides listed in Table 2 and those of the epitopes for the anti-GPIIb monoclonal antibodies was done by enzyme immunoassay according to the procedure of Geysen et al. (1984). The antibodies were used at the following dilutions: M2 (1:2000), Mα2 (1:80000), M3 (1:10000), M5 (1:1000) and M6 (1:5000).

as efficiently as the whole 35 kDa CNBr-cleavage (Fig. 2b). The N-terminal amino acid sequence and the amino acid analyses of this fraction led us to identify this peptide with the α125–164 peptide stretch in the amino acid sequence of GPIIb. When peptide synthesis combined with enzyme immunoassay was used to narrow down the location of the M4 epitope it was found between α143 and α151, as shown in Fig. 1 and Table 2. It therefore contains an arginine residue Arg-147, as expected.

Epitope for M5

Given the fact that M5 cross-reacts with endothelial cells (Muñiz et al., 1990), and therefore with the α-subunit of the vitronectin receptor in these cells, the epitope for M5 should be within an amino acid sequence identical or similar in both platelet GPIIb and the α-subunit of the vitronectin receptor. Using immunoelectroblotting we had previously located the M5 epitope within a 35 kDa membrane-bound chymotryptic product of digestion of GPIIb in whole platelets, which contained the peptide stretch between residues α550 and α682 (Calvete et al., 1989a). The only sequence in this region common to the platelet GPIIb and the α-subunit of the vitronectin receptor is α550–565 (Fig. 3). This sequence contains Arg-553 and several dicarboxylic amino acid residues, as expected from the cleavage susceptibility pattern referred to above (Table 1). Peptide α550–580 was isolated by reverse-phase h.p.l.c. as described previously (see Fig. 1 in Calvete et al., 1989b) and found, by competitive enzyme

Epitope for M4

The N-terminal fragment (α1–285, 35 kDa), liberated by CNBr cleavage of GPIIbα, was isolated on Sephacryl S200 and further digested with endoproteinase Lys-C. Among the cleavage products separated by reverse-phase h.p.l.c. (Fig. 2a) is one (fraction 5) that is capable of inhibiting the binding of M4 to GPIIb half

GPIIbα: $^{(550)}$ AFLRDEADFRDKLSPIVLSLNVSL——PPTEAGM . . .

VNRα: $^{(540)}$ AYLRDESEFRDKLTPITIFMEYRLDYRTAADTTGL . . .

Fig. 3. Comparison of the amino acid sequence α550–580 of platelet GPIIb (Poncz et al., 1987) with the homologous sequence in the α-subunit of the vitronectin receptor (Fitzgerald et al., 1987)
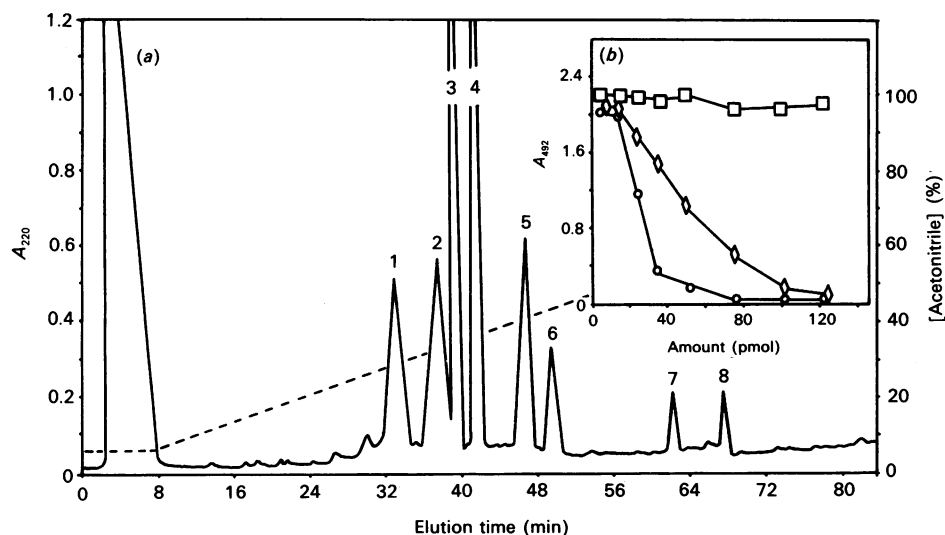

Fig. 2. Localization of the epitope for the monoclonal antibody M4 in the peptide sequence α125–164 of GPIIb

(a) Reverse-phase h.p.l.c. isolation of the products of digestion with endoproteinase Lys-C of the fully reduced and carboxymethylated 35 kDa CNBr-cleavage fragment of GPIIb (α1–285), prepared as described by Calvete et al. (1989b). A 175 μg portion of the CM-35 kDa CNBr-cleavage fragment in 100 μl of 0.1 M-Tris/HCl buffer, pH 7.6, was digested with endoproteinase Lys-C at a peptide/enzyme ratio 25:1 (w/w) for 18 h at 37 °C, and the reaction was stopped with formic acid at 30 % (v/v) concentration. The digestion products were separated on a C_{18} (pore size 30 nm, particle size 10 μm) Vydac column (25 cm × 0.4 cm) equilibrated in 0.1 % trifluoroacetic acid in water (solution A) and 0.1 % trifluoroacetic acid in acetonitrile (solution B) (95 % solution A/5 % solution B), and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear acetonitrile gradient from 5 % to 70 % solution B in 75 min. ——, A_{220}; ----, [acetonitrile]. (b) Competitive enzyme immunoassay of the CM-35 kDa CNBr-cleavage fragment of GPIIb (○), peptide fraction 5 (α125–164) from the column in (a) (◇) and the rest of the column fractions (□), using 1 μg of CM-GPIIbα adsorbed on each micro-titre plate well, and monoclonal antibody M4 at 1:10000 (v/v) dilution.

immunoassay, to compete very effectively with GPIIb for binding of M5. When peptide synthesis combined with enzyme immunoassay was used to narrow down the location of this epitope, it was found to be between α550 and α558 (Fig. 1 and Table 2).

Epitope for M6

This epitope, which had also previously been located by immunoelectroblotting within the 35 kDa membrane-bound chymotryptic product of digestion of GPIIb in whole platelets (Calvete et al., 1989a), was now isolated by reverse-phase h.p.l.c. after digestion of CM-GPIIα with V8 proteinase (Fig. 4a). Fraction 18 was able to inhibit the binding of M6 to CM-GPIIbα, adsorbed on the wells in the titration plates, almost as efficiently as CM-GPIIbα itself (Fig. 4b). The N-terminal amino acid sequence and amino acid analyses of this fraction allowed us to identify it with the α649–667 peptide stretch, which contains αMet-660 and Arg-661 within it, as predicted by the cleavage susceptibility pattern shown by this epitope (Table 1). Again, when peptide synthesis combined with enzyme immunoassay was used to narrow down the location of this epitope further, it was found to be between α657 and α665 (Table 2 and Fig. 1).

Localization of the epitope for M6 in the most resistant region of GPIIbα to chymotryptic digestion of whole platelets

We had found previously that digestion of whole platelets for 5 min with α-chymotrypsin liberates a cleavage product of apparent molecular mass 95 kDa into the soluble fraction. This contains the N-terminal amino acid sequence of GPIIbα, the first five intrachain disulphide bonds and the epitope for M4 (Calvete et al., 1989a). The GPIIb cleavage products, which remain cell-bound, were recognized by M1, M5 and M6. Here, further analysis of the membrane-bound GPIIb products of chymo-

tryptic digestion for longer periods was carried out. In Fig. 5(a) the immunoblotting analysis, after reduction, of the cell-bound chymotryptic products after 5 and 15 min digestion is shown. Both M5 and M6 recognize products of apparent molecular mass 70, 65, 35 and 27 kDa, but the epitopes for M3 and Mα2 are not found after 5 min digestion, and that for M1 is lost after 15 min.

When the digestion of whole platelets is continued up to 30 min, the only membrane-bound epitope remaining is that for M6, which is found in a 25 kDa fragment (Figs. 5b and 5c). After a 1 h digestion, even the M6 epitope is lost (Fig. 5b). The 25 kDa product, containing the M6 epitope, remains membrane-bound even after reduction with dithioerythritol, alkylation and washing of the digested platelets, as we had seen before for the whole α-subunit of GPIIb (Calvete & González-Rodríguez, 1986; Calvete et al., 1989a). Thus in whole platelets the N- and C-termini of GPIIbα and the N-terminus of GPIIbβ are easily digested, leaving the 25 kDa product, which contains the epitope for M6, bound to the membrane.

Localization in the GPIIb sequence of the antipeptide complementary to fibrinogen γ402–411 peptide

We have searched in the sequence of GPIIb for the antipeptide sequences complementary to those fibrinogen peptide stretches putatively involved in the GPIIb/IIIa fibrinogen interaction: the γ-chain C-terminus (γ400–411) and the Aα RGD-containing peptides (Aα568–579 and Aα91–99). The sequence of the antipeptide encoded on the DNA sequence complementary to the coding strand for the γ400–411 peptide (Rixon et al., 1985) was translated in both the 3'–5' and 5'–3' directions, the following antipeptides being obtained:

3'–5' H_2N-V V D P P R F V R P L Q-CO_2H
5'–3' HO_2C-V V Q P S G F L S S V N-NH_2
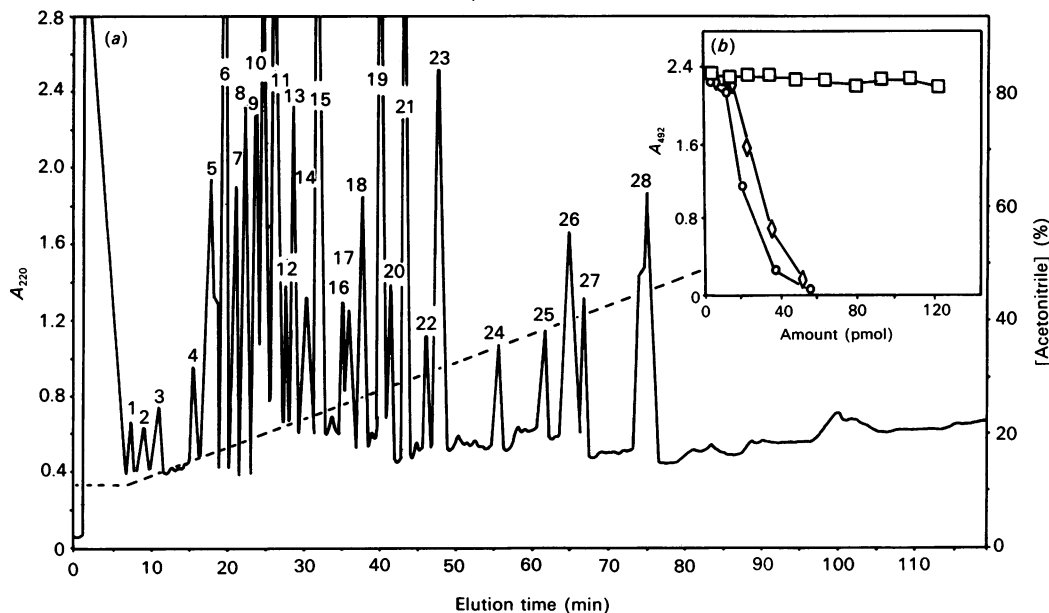

Fig. 4. Localization of the epitope for the monoclonal antibody M6 in the peptide sequence α649–667 of GPIIb

(a) Reverse-phase h.p.l.c. isolation of the products of digestion of CM-GPIIα with endoproteinase Glu-C. A 700 μg protein of CM-BPIIbα in 140 μl of 50 mM-phosphate buffer, pH 7.8, was digested with endoproteinase Glu-C at a substrate/enzyme ratio 25:1 (w/w) for 18 h at 37 °C. The reaction was stopped with formic acid at 30% (v/v) final concentration, and the digestion products were separated on a C_{18} column, as in Fig. 2(a), equilibrated in 0.1% trifluoroacetic acid in water (solution A), and 0.1% trifluoroacetic acid in acetonitrile (solution B) (90% solution A/10% solution B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear acetonitrile gradient from 10% to 70% solution B in 120 min. ——, A_{220}; ----, [acetonitrile]. (b) Competitive enzyme immunoassay of CM-GPIIbα (○), peptide fraction 18 (α649–667) from column in (a) (◇) and the rest of the column fractions (□), using 1 μg of CM-GPIIbα adsorbed on each micro-titre plate well and monoclonal antibody M6 at 1:5000 dilution.

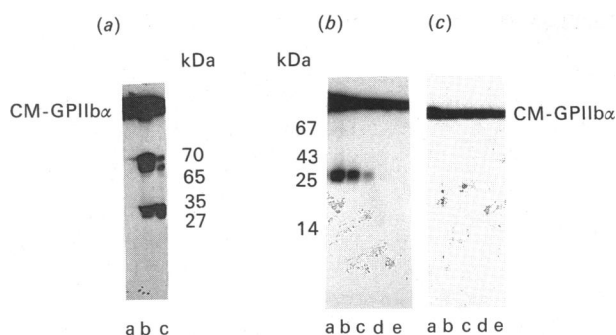Fig. 5. Analysis by immunoelectroblotting of the membrane-bound products of digestion of GPIIb in whole platelets, using monoclonal antibodies M5 and M6

(a) Analysis with monoclonal antibody M5 (1:1000) and M6 (1:2000) of the membrane-bound chymotryptic products of digestion of GPIIb in whole platelets. The particulate fraction was isolated as described in the Materials and methods section. The immuno-electroblotting was carried out after SDS/10%-PAGE of 100 µg samples of the particulate fraction after reduction with dithio-erythritol (a 2000 molar excess over the half-cystine content of estimated GPIIb and GPIIIa), carboxymethylation, washing and SDS solubilization. Lane a, control sample; lane b, sample from the 5 min platelet digestion; lane c, sample from the 15 min platelet digestion. (b) Analysis with the monoclonal antibody M6 of the GPIIb membrane-bound products, as in (a), after 30, 45, 60, 75 and 90 min platelet digestion in lanes a to e respectively. The rest of the experimental conditions as in (a), except that a 12% polyacrylamide gel was used. (c) Analysis with the monoclonal antibody M5 of the GPIIb membrane-bound products, as in (b) after 30, 45, 60, 75 and 90 min of platelet digestion, in lanes a to e respectively.

They have nearly the same hydropathy profiles (Fig. 6), as has been found previously for other antipeptides (Markus et al., 1989). If we do the same for fibrinogen Aα568–579 and Aα91–99 peptides, which contain the RGD sequence, the following

antipeptides, again with the same hydropathy profile, are obtained:

$$3'-5' \quad H_2N\text{-}CSMLSPLRCKLS\text{-}CO_2H$$
$$5'-3' \quad HO_2C\text{-}PTVVSSVGCKFA\text{-}NH_2$$

for the Aα568–579 and

$$3'-5' \quad H_2N\text{-}YL\ STOP\ NSPLKRSR\text{-}CO_2H$$
$$5'-3' \quad HO_2C\text{-}HFNQSAIKG\ STOP\ R\text{-}NH_2$$

for the Aα91–99 (Rixon et al., 1983).

If we now search for sequences in GPIIb hydropathically similar to the six antipeptides identified above we find that the α658–667 sequence is the only one that has a hydropathy profile similar to that of the antisense peptides for the fibrinogen γ402–411 peptide, and a strong hydropathic complementarity to the fibrinogen γ402–411 peptide (Fig. 6). It happens that this GPIIb sequence stretch is the same as that which was found to contain the epitope for M6 (α657–665). Finally, the α821–825 sequence is hydropathically complementary to the fibrinogen Aα94–98 peptide (Fig. 6b), whereas we have not found any hydropathic complementarity in the GPIIb sequence for the fibrinogen Aα568–579 peptide.

DISCUSSION

In Fig. 7 we outline the information obtained in the present work on the precise localization of the epitopes for six anti-GPIIbα and one anti-GPIIbβ monoclonal antibodies. Also shown are the putative binding sites for GPIIIa to form the GPIIb/IIIa complex, and for the γ-chain C-terminal peptide (γ402–411) and Aα94–98 peptide of fibrinogen. These assignments are based on the cDNA-predicted amino acid sequence (Poncz et al., 1987; Uzan et al., 1988) and the chemically determined disulphide bond and the glycosylation patterns of GPIIb (Calvete et al., 1989b).
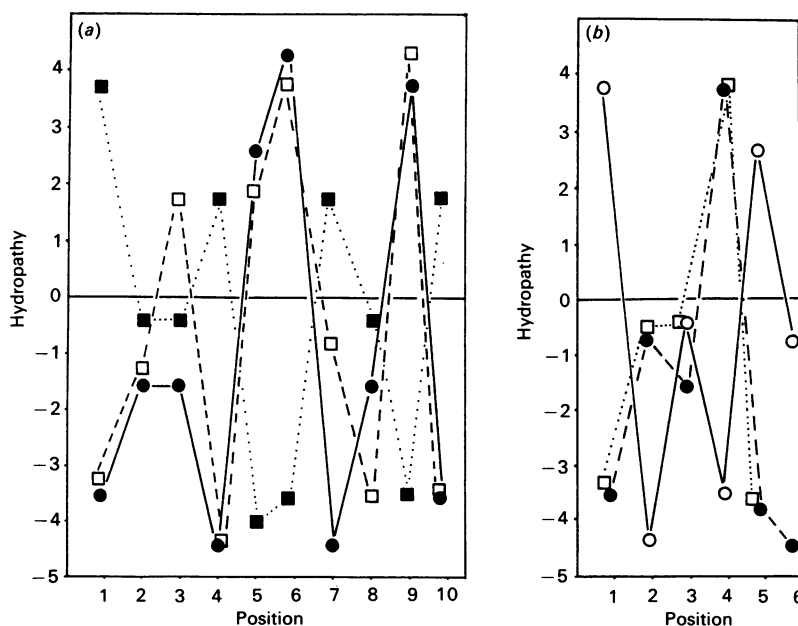

Fig. 6. Hydropathy plots of GPIIb peptide sequences hydropathically complementary to fibrinogen γ402–411 and Aα RGD peptides

(a) Comparison of the hydropathy profile of the human fibrinogen γ402–411 peptide (LGGAKQAGDV) (■) with those of its antipeptide encoded in the DNA sequence complementary to the coding strand for γ402–411 peptide (Rixon et al., 1985) translated in the 3'–5' direction (NH₂-DPPRFVRPLQ-CO₂H) (●), and the GPIIbα658–667 peptide sequence (HYMRALSNVE) (□). (b) Comparison of the hydropathy profile of the human fibrinogen Aα94–98 peptide (LRGDFS) (○) with those of its antipeptide (NH₂-NSPLKR-CO₂H) (●) (Rixon et al., 1983) and the GPIIbα821–825 peptide sequence (QGGLQ) (□).
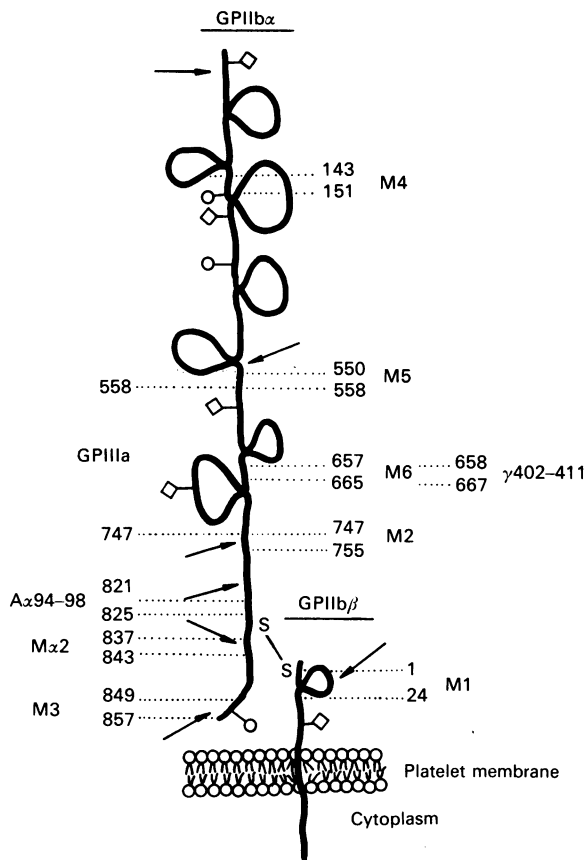
Fig. 7. Outline of the precise localization in GPIIb of the epitopes for monoclonal antibodies M1, M2, Mα2, M3, M4, M5 and M6, and of the putative binding regions found for GPIIIa and fibrinogen

Key to symbols: ◇-, N-glycosylation points; ○-, O-glycosylation points; γ402–411, Aα94–98 and GPIIIa, putative binding regions for the fibrinogen γ402–411 and Aα94–98 peptide sequences and GPIIIa respectively; ——→, chymotryptic cleavage points in whole platelets. The disulphide bond and glycosylation patterns were taken from Calvete et al. (1989b).

Assignment of epitopes for monoclonal antibodies in the structure of GPIIb

We have previously found that the epitope for M1 is located at the N-terminus of GPIIbβ (Calvete et al., 1989a) and that this subunit is heterogeneous in size with two apparent forms, β1, the larger, and β2 (Calvete et al., 1989a,b). Loftus et al. (1987) found that the heterogeneity is due to alternative proteolytic processing of GPIIb to yield the two chains of the mature glycoprotein, and that GPIIbβ1 has a blocked N-terminus of 12–15 residues upstream from the reported N-terminus of GPIIbβ2 (Charo et al., 1986; Calvete et al., 1989a). Chemical analysis of GPIIbβ1 has enabled us to locate the position and identify the nature of its blocked N-terminal residue, pyroglutamic acid (Calvete et al., 1990). Taking this into consideration, we must now refer to the position of the epitope for M1 either as β1-Pro-16–Met-36 or as β2-Pro-4–Met-24. Consideration of the lack of susceptibility of this epitope to attack by endoproteinase Glu-C leads us to suggest that the epitope should be either upstream or downstream of β1-Asp-22 and β2-Asp-10 in GPIIbβ1 and GPIIbβ2 respectively.

Three epitopes are located in the C-terminal region of GPIIbα. That for M2 is localized between residues α747 and α755, upstream from αCys-826, the GPIIbα residue that forms with β2-

Cys-9 the single interchain disulphide bond of GPIIb (Calvete et al., 1989a). That for Mα2 is located between residues α837 and α843, downstream from αCys-826, but very close to it. The third epitope, for M3, is located very close to the C-terminus of GPIIbα, between residues α849 and α857. This is within the same segment (α842–858) as the epitope for the RGD- and/or EDTA-dependent monoclonal anti-GPIIb antibody PMI-1, found by Loftus et al. (1987).

The epitope for M4, previously found to be within the N-terminal region of GPIIbα (α1–185), has now been located precisely between residues α143 and α151, with αArg-147 within it. As this epitope is easily accessible in resting platelets (see below), it can be deduced that this peptide stretch is fully exposed.

The epitope for M5, which is a good marker for endothelial cells (Muñiz et al., 1990), is located in the α550–558 stretch, which has a sequence similar to the 540–548 peptide sequence of the α-subunit of the vitronectin receptor (Fitzgerald et al., 1987). This epitope is at the N-terminus of the fragment of GPIIbα that remains membrane-bound in the early stages of chymotryptic digestion of whole platelets. Therefore both this epitope, which is easily accessible in resting platelets (see below), and the earliest chymotryptic cleavage site are fully exposed and very close to each other.

The epitope for M6 was found to be in the α657–665 peptide stretch, and is located in the membrane-bound fragment of GPIIbα that is most resistant to chymotryptic digestion of whole platelets. However, when isolated GPIIb in solution is subjected to chymotryptic digestion, the epitope for M6 is lost at an early stage. It is thus most probably situated in that region of GPIIb that carries the surface of interaction with GPIIIa in the GPIIb/IIIa complex. The interest of this epitope derives from two facts. First, its exposure in whole platelets is EDTA- or thrombin-dependent (E. Muñiz, C. Castellarnau & J. González-Rodríguez, unpublished work). Secondly its sequence coincides with that found for the putative binding site of the γ-chain C-terminal (γ402–411) peptide of fibrinogen in GPIIb (see below).

Preliminary experiments using immunofluorescence microscopy and fluorescence-activated cell sorting have shown that antibodies M1, M2, Mα2, M3, M4 and M5 label resting platelets readily in physiological medium (E. Muñiz, C. Castellarnau & J. González-Rodríguez, unpublished work). These observations, together with the ease with which the GPIIbα C-terminal and the GPIIbβ N-terminal regions are cleaved at the early stages of chymotryptic digestion of whole platelets and with which the single interchain disulphide bond is reduced, both in solution and in whole platelets (Calvete et al., 1989a), allow us to outline the following features about this region of GPIIb. First, the majority of the GPIIbα C-terminal and GPIIbβ N-terminal regions, which include not only the epitopes for M1, M2, Mα2 and M3 and the single interchain disulphide bond, but also one N- and one O-glycosylation point at β2-Asn-60 and αSer-845 and/or -847 respectively (J. Calvete, A. Henschen & J. González-Rodríguez, unpublished work), seem to be well exposed to the platelet external medium. Second, the epitope for PMI-1 probably has its localization restricted to the α842–849 stretch, because its exposure is RGD- and/or EDTA-dependent, whereas the α849–857 sequence, which corresponds to the M3 epitope, is permanently exposed.

Putative GPIIIa- and fibrinogen-binding sites in GPIIb

We have previously shown that, within the first 5 min of chymotryptic digestion of whole platelets, a soluble product of apparent molecular mass 95 kDa is liberated, which begins at the 17th residue of GPIIb and contains the first five interchain

disulphide bonds of this subunit together with the epitope for M4. The remaining cell-bound products carry the epitopes for M1, M5 and M6 (Calvete *et al.*, 1989*a*). In addition to confirming this, we have now found that the epitopes for Mα2 and M3 at the *C*-terminus of GPIIb are lost within the first 5 min of digestion, while those for M1 and M5 are lost after 15 and 30 min, respectively. The epitope for M6, which is lost only after 60 min of digestion, is found in a membrane-bound fragment of apparent molecular mass of 25 kDa, even after reduction and alkylation of the digested platelets.

Several features of GPIIb structure can be deduced from these observations. First, it seems reasonable to propose that the main surface of GPIIb, which interacts with GPIIIa in the GPIIb/IIIa complex, is somewhere between the epitopes for M5 and M2, i.e. between residues α558 and α747. This is based on the high resistance of the 25 kDa membrane-bound fragment to chymotryptic attack in whole platelets, compared with the ease with which the *N*-terminal 95 kDa product and the *C*-terminus of GPIIbα and the *N*-terminus of GPIIbβ are cleaved, as well as on the EDTA- or thrombin-dependence of the epitope exposure for M6. However, not all this area is hidden, and residues such as Asn-570 and Asn-680, which are glycosylated, should be exposed. Secondly, it is remarkable that none of the predicted Ca^{2+}-binding sites (Poncz *et al.*, 1987) are within this putative binding region for GPIIIa, in spite of the fact that Ca^{2+} is required for the formation of the GPIIb/IIIa complex. However, Ca^{2+} + binding studies have shown the appearance of a new high-affinity Ca^{2+}-binding site in the GPIIb/IIIa complex, compared with the isolated glycoproteins (G. A. Rivas & J. González-Rodríguez, unpublished work), which may well be derived from both glycoproteins and located in this area. Thirdly, as pointed out above, the 95 kDa soluble product contains the αCys-490–Cys-545 disulphide bond, but not the epitope for M5 (α550–558); therefore it can be deduced that the earliest and major chymotryptic cleavage point must be somewhere between αCys-545 and αPhe-551, and very close to the epitope for M5, and that this area of GPIIb must be fully exposed to the external medium in whole platelets.

Finally, it has been shown that an inverse correlation exists between the hydropathic coefficients of amino acids coded by complementary DNA strands in the same reading frame (Blalock & Smith, 1984) and that this is the case for several naturally occurring peptides (Bost *et al.*, 1985; Mulchahey *et al.*, 1986; Elton *et al.*, 1988). As a result, a strong hydropathic complementarity (pairing of hydropathic with hydrophilic residues) is obtained when the antipeptide resulting from reading the DNA non-coding strand in the 3′-to-5′ direction is aligned parallel to the natural peptide (from the DNA coding strand) or if the antisense peptide resulting from reading in the 5′-to-3′ direction is aligned antiparallel to it (Markus *et al.*, 1989). Recently, this approach has been applied to the interactions between two macromolecules, such as the fibronectin–fibronectin receptor pair or the interaction of platelet GPIIb/IIIa complex with fibronectin or the Aα-chain of fibrinogen (Brentani *et al.*, 1988; Pasqualini *et al.*, 1989). When the GPIIb amino acid sequence was searched for antipeptides complementary to the fibrinogen γ-chain *C*-terminal (γ400–411) peptide and to the fibrinogen Aα RGD-containing peptides (Aα568–579 and Aα91–99), we found a high hydropathic complementarity only between the GPIIbα658–667 and fibrinogen γ402–411 sequences and between the GPIIbα821–825 and fibrinogen Aα94–98 sequences. We therefore conclude that these areas of GPIIbα may be involved in the binding of fibrinogen to the functional GPIIb/IIIa complex in whole platelets. There are three other observations that suggest that α658–667, in particular, may be a fibrinogen-binding site. First, although both γ400–411 and RGD peptides

were found cross-linked to both GPIIb and GPIIIa, the γ400–411 type binds preferentially to GPIIb, whereas the RGD-type binds mainly to GPIIIa (Santoro & Lawing, 1987; D'Souza *et al.*, 1988). Secondly, when we searched for γ400–411 hydropathically complementary stretches in the amino acid sequence of GPIIIa, we found none, but Pasqualini *et al.* (1989) and we (Calvete *et al.*, 1991) have found several peptide sequences in GPIIIa complementary to these RGD peptides of the fibrinogen Aα chain. Thirdly, it is remarkable that the epitope for M6 (α657–665), a monoclonal antibody that inhibits platelet aggregation and whose binding to platelets is EDTA- or thrombin-dependent (E. Muñiz, C. Castellarnau, & J. González-Rodríguez, unpublished work), overlaps the putative binding region for the fibrinogen γ400–411 peptide. Such overlapping implies that the unmasking of the α658–667 peptide sequence is one of the structural changes required for induction of the fibrinogen receptor in activated platelets.

REFERENCES

Blalock, J. E. & Smith, E. M. (1984) Biochem. Biophys. Res. Commun. **121**, 203–207

Bost, K. L., Smith, E. M. & Blalock, J. E. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1372–1375

Brentani, R. R., Ribeiro, S. F., Patocnjak, P., Pasqualini, R., Lopes, J. D. & Nakaie, C. R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 364–367

Calvete, J. J. & González-Rodríguez, J. (1986) Biochem. J. **240**, 155–161

Calvete, J. J., Alvarez, M. V., Rivas, G., Hew, C. L., Henschen, A. & González-Rodríguez, J. (1989*a*) Biochem. J. **261**, 551–560

Calvete, J. J., Henschen, A. & González-Rodríguez, J. (1989*b*) Biochem. J. **261**, 561–568

Calvete, J. J., Schäfer, W., Henschen, A. & González-Rodríguez, J. (1990) FEBS Lett. **272**, 37–40

Calvete, J. J., Arias, J., Alvarez, M. V., López, M. M., Henschen, A. & González-Rodríguez, J. (1991) Biochem. J., in the press

Charo, I. F., Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Bekeart, L. S. & Phillips, D. R. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8351–8355

D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. & Plow, E. F. (1988) Science **242**, 91–93

Edman, P. & Henschen, A. (1975) in Protein Sequence Determination, 2nd edn. (Needleman, S. B., ed.), pp. 232–279, Springer-Verlag, Berlin

Eirin, M. T., Calvete, J. J. & González-Rodríguez, J. (1986) Biochem. J. **240**, 147–153

Elton, T. S., Dion, L. D., Bost, K. L., Oparil, S. & Blalock, E. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2518–2522

Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C., Jr., Bennett, J. S. & Phillips, D. R. (1987) Biochemistry **26**, 8158–8165

Geysen, H. M., Meloen, R. H. & Barteling, S. J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3998–4002

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lam, S. C.-T., Plow, E. F. & Ginsberg, M. H. (1989) Blood **73**, 1513–1518

Loftus, J. C., Plow, E. F., Frelinger, A. L., III, D'Souza, S. E., Dixon, D., Lacy, J., Sorge, J. & Ginsberg, M. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7114–7118

Marguerie, G. A., Ginsberg, M. H. & Plow, G. F. (1987) in Platelets in Biology and Pathology (MacIntyre, D. E. & Gordon, J. L., eds.), pp. 95–125, Elsevier, Amsterdam

Markus, G., Tritsch, G. L. & Parthasarathy, R. (1989) Arch. Biochem. Biophys. **272**, 433–439

Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) Anal. Biochem. **87**, 206–210

McEver, R. P., Baenziger, N. L. & Majerus, P. W. (1980) J. Clin. Invest. **66**, 1311–1318

Melero, J. A. & González-Rodríguez, J. (1984) Eur. J. Biochem. **141**, 421–427

Mulchahey, J. J., Neill, J. D., Dion, L. D., Bost, K. L. & Balalock, E. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9714–9718

Muñiz, E., Castellarnau, C., Ribera, A., Madoz, P. & González-Rodríguez, J. (1990) Blood **75**, 318–319

Nurden, A. T., George, J. N. & Phillips, D. R. (1986) in Biochemistry of Platelets (Phillips, D. R. & Shuman, M. A., eds.) pp. 159–224, Academic Press, New York

Pasqualini, R., Chamone, D. F. & Brentani, R. R. (1989) J. Biol. Chem. **264**, 14566–14570

Phillips, D. R., Charo, I. F., Parise, L. V. & Fitzgerald, L. A. (1988) Blood **71**, 831–843

Poncz, M., Eisman, R., Heindenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E. & Bennett, J. S. (1987) J. Biol. Chem. **262**, 8476–8482

Rixon, M. W., Chan, W. Y., Davie, E. W. & Chung, D. W. (1983) Biochemistry **22**, 3237–3244

Rixon, M. W., Chung, D. W. & Davie, E. W. (1985) Biochemistry **24**, 2077–2086

Santoro, S. A. & Lawing, W. J. (1987) Cell **48**, 867–873

Thorsen, L. I., Guadernack, G., Brosstad, F., Olsen, T. M. & Solum, N. O. (1985) Thromb. Haemostasis **54**, 182

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Usobiaga, P., Calvete, J. J., Saiz, J. L., Eirin, M. T. & González-Rodríguez, J. (1987) Eur. J. Biophys. **14**, 211–218

Uzan, G., Frachet, P., Lajmanovich, A., Prandini, M. H., Dernarier, D., Duperray, A., Loftus, J., Ginsberg, M., Plow, E. & Marguerie, G. (1988) Eur. J. Biochem. **171**, 87–93

Varon, D. & Karpatkin, S. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6992–6995