Proteolytic dissection of the isolated platelet fibrinogen receptor, integrin GPIIb/IIIa

Localization of GPIIb and GPIIIa sequences putatively involved in the subunit interface and in intrasubunit and intrachain contacts

Juan J. CALVETE,*† Karlheinz MANN,† María V. ALVAREZ,* María M. LÓPEZ* and José GONZÁLEZ-RODRÍGUEZ*‡

*Instituto de Química Física, C.S.I.C., Serrano 119, 28006 Madrid, Spain, and †Max-Planck-Institut für Biochemie, W-8033 Martinsried, München, Federal Republic of Germany

Human platelet glycoproteins IIb (GPIIb) and IIIa (GPIIIa) form the subunits of the Ca^{2+} -dependent heterodimer GPIIb/IIIa, which belongs to the integrin family of phylogenetically related receptors mediating a wide variety of cell–cell and cell–substratum interactions. GPIIb/IIIa plays a central role in haemostasis as a receptor for fibrinogen and other adhesive proteins at the surface of activated platelets. The covalent structure of the subunits is largely known; however, the tertiary and quaternary structures of the heterodimer remain to be determined. To this end, our approach consisted of limited proteolysis of the isolated heterodimer with proteinases of different specificities, followed by protein-chemical and immunochemical analyses of the peptide fragments within each isolated proteolytic product. From the information obtained, we have drawn a rudimentary map which outlines the demarcation of compact domains and the subunit peptide stretches carrying the sequences putatively involved in intrachain, intrasubunit and intersubunit non-covalent connectivity in the heterodimer. Three compact domains have been well defined: one in the heavy (H) chain of GPIIb [GPIIbH-(600–700)], and two in GPIIIa, the *N*-terminal [GPIIIa-(1–52)] and the core [GPIIIa-(423–622)] domains. Between the latter two domains there is a proteolysis-susceptible region, which is partly involved in ligand binding [GPIIIa-(100–220)] and partly implicated as being in the subunit interface of the heterodimer. Contrary to GPIIIa, GPIIbH is highly susceptible to proteolysis all along its sequence. Equally susceptible are the extracellular end of the transmembrane segment of both GPIIIa and the light (L) chain of GPIIb (GPIIbL), and the *N*-terminal end of GPIIbL. Three sequence stretches along the C-terminal half of GPIIbH, one sequence stretch in GPIIbL and three sequence stretches within the GPIIIa-(217–421) region were putatively involved in the subunit interface of the heterodimer. Most likely, the *N*-terminal end of GPIIbL is folded over the *N*- and C-terminal regions of GPIIbH, and the *N*-terminal end of GPIIbH is folded against the GPIIbH-(600–700) domain. This map of GPIIb/IIIa does not fit the current accommodation of the amino acid sequence of GPIIb and GPIIIa in the head/two-tails image of the heterodimer obtained by metal-rotary-shadowing electron microscopy.

INTRODUCTION

Glycoprotein IIb (GPIIb or α_{IIb} ; 136 kDa) is a two-chain bitopic membrane protein which, together with the single-chain bitopic membrane glycoprotein IIIa (GPIIIa or β_3 ; 92 kDa) (Phillips & Agin, 1977; Calvete & González-Rodríguez, 1986; Usobiaga *et al.*, 1987), forms the Ca^{2+} -dependent heterodimer GPIIb/IIIa at the surface of the human platelet (Kunicki *et al.*, 1981; Rivas & González-Rodríguez, 1991). GPIIb/IIIa, also known as integrin $\alpha_{\text{IIb}}\beta_3$, is the major intrinsic protein at the platelet plasma membrane, where it serves as the inducible receptor for fibrinogen and other adhesive proteins, and plays a central role in platelet aggregation and adhesion (Plow & Ginsberg, 1989; Kieffer & Phillips, 1990). GPIIb/IIIa is the integrin that has been most extensively characterized, and together with the vitronectin receptor (integrin $\alpha_v\beta_3$) forms the β_3 integrin subfamily (Ginsberg *et al.*, 1988). The complete amino acid sequences of GPIIb and GPIIIa have been derived by cDNA cloning (Fitzgerald *et al.*, 1987; Poncz *et al.*, 1987), and the genomic organization of both genes has been established (Heidenreich *et al.*, 1990; Lanza *et al.*, 1990; Zimrin *et al.*, 1990).

Much of the covalent structure of mature GPIIb/IIIa is known (Calvete *et al.*, 1989, 1990, 1991b), and its topography is being gradually revealed (D'Souza *et al.*, 1988, 1990, 1991; Calvete *et al.*, 1988, 1991a,c; Lam *et al.*, 1989; Loftus *et al.*, 1990; Charo *et al.*, 1991; J. J. Calvete, W. Schäfer, K. Mann, A. Henschen & J. González-Rodríguez, unpublished work). However, the tertiary and quaternary structures of the heterodimer remain to be determined. To this end, our approach here consists of limited proteolysis of the isolated heterodimer in non-denaturing conditions, followed by protein-chemical and immunochemical analyses of the peptide fragments within the isolated proteolytic products. The information obtained was used to draw a rudimentary map which outlines the demarcation of proteinase-resistant and/or compact domains and the subunit sequences involved in putative intrachain, intrasubunit and intersubunit non-covalent connectivity in the heterodimer in solution.

MATERIALS AND METHODS

Materials

1-Chloro-4-phenyl-3-L-tosylamidobutan-2-one (Tos-Phe-

Abbreviations used: GPIIb, GPIIIa and GPIIb/IIIa, glycoproteins IIb, IIIa and the heterodimer formed between them; GPIIbH and GPIIbL, the heavy (H) and the light (L) chains of GPIIb; GPIIbL₁ and GPIIbL₂, the large and the small forms of GPIIbL; Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

‡ To whom correspondence and reprint request should be sent.

CH₂Cl; TPCK)-treated trypsin, α -chymotrypsin and *n*-octyl β -D-glucopyranoside (> 98 % pure) were from Sigma (St. Louis, MO, U.S.A.); endoproteinase Glu-C (*Staphylococcus aureus* V8 proteinase) was from Worthington (Freehold, NJ, U.S.A.); TSK-SW 2000 and 3000 (7.5 mm $\times$ 600 mm) size-exclusion columns were from LKB (Bromma, Sweden); *N*-hydroxysuccinimidyl-4-azidosalicylic acid was from Pierce (Rockford, IL, U.S.A.). GPIIb/IIIa was prepared as described previously (Rivas *et al.*, 1991a). The other chemicals and biochemicals were of analytical or chromatographic grade.

Analytical methods

Amino acid analyses were performed with a Biotronik amino acid analyser after sample hydrolysis at 110 °C in 6 M-HCl for 24 h. SDS/PAGE was carried out according to Laemmli (1970). Immunoelectroblotting after gel electrophoresis and competitive enzyme immunoassay were done as described previously (Calvete *et al.*, 1991a,c) using a collection of mouse monoclonal antibodies specific for GPIIb (M₁, M₂, M₂₋₂, M₃, M₄, M₅ and M₆) and GPIIIa (P₂₃₋₇, P₃₇ and P₄₀) whose epitopes have been precisely assigned in the amino acid sequence of these glycoproteins (Calvete *et al.*, 1991a,c). *N*-Terminal sequence analyses were effected either in a Beckman 890 spinning-cup sequencer or in an Applied Biosystems 473A gas-liquid phase sequencer, following the manufacturers' instructions.

Proteolytic digestion of GPIIb/IIIa and isolation and characterization of the products of proteolysis

Freeze-dried GPIIb/IIIa was dissolved at 4 mg/ml (final concentration) in 0.1 M-Tris/HCl/0.025 % (w/v) Na₂S₂O₃/0.1 mM-CaCl₂, pH 7.8 (digestion buffer) and digested at 37 °C with trypsin (Tos-Phe-CH₂Cl-treated), α -chymotrypsin or V8 proteinase at enzyme/substrate ratios of 1:250, 1:250 and 1:100 (w/w) respectively. The digestion was stopped at the selected time by addition of a 25-fold molar excess over the enzyme concentration of phenylmethanesulphonyl fluoride. The digested samples were cleared by centrifugation at 20 000 *g* for 10 min and the supernatants were subjected to size-exclusion chromatography on a TSK-SW 3000 column equilibrated and eluted with 10 mM-Tris/HCl/0.1 % *n*-octyl β -D-glucopyranoside/0.025 % Na₂S₂O₃/0.15 M-NaCl/0.1 mM-CaCl₂, pH 7.0, at a flow rate of 0.1 ml/min. The collected fractions were concentrated by centrifugation using a 10 kDa cut-off cartridge (Millipore, Bedford, MA, U.S.A.) and rechromatographed on either a TSK-SW 3000 or a TSK-SW 2000 column, depending on the size of the proteolytic products, under the same conditions as above. Alternatively, the GPIIb/IIIa digestion mixtures were incubated with EDTA (10 mM final concentration) at 37 °C for 30 min, and then subjected to size-exclusion chromatography as above, but replacing CaCl₂ by 1 mM-EDTA. The isolated proteolytic products were designated by the initial of the proteinase used for digestion (T for trypsin, C for chymotrypsin and V for V8 endoproteinase) followed by the apparent molecular mass (kDa) determined by size-exclusion chromatography.

The isolated proteolytic products were subjected to amino acid, *N*-terminal sequence and immunochemical analyses, and afterwards were dialysed overnight against Milli-Q water, freeze-dried and dissolved in 70 % (v/v) formic acid. The dissociated peptide fragments were isolated by reverse-phase h.p.l.c. on a C4 (pore size 30 nm; particle size 10 μ m) Vydac column (25 cm $\times$ 0.4 cm) equilibrated in a mixture of 0.1 % (v/v) trifluoroacetic acid in water (A) and 0.1 % trifluoroacetic acid in acetonitrile (B) (100 % A/0 % B) and eluted at 1 ml/min. Elution was isocratic for 5 min, followed by a linear gradient up to 70 % B in 70 min. The isolated peptides were subjected to amino acid, *N*-terminal sequence and immunochemical analyses.

Binding and cross-linking of RGD and AKQAGDV peptides to the proteolytic products of GPIIb/IIIa

The peptides CKRKRKRKRGRDV and CYHHLGGAK-QAGDV were synthesized using a semi-automated Labortec SP 640 peptide synthesizer (Labortec ARG, Bubendorf, Switzerland). A fluorescent reporter group (eosin 5'-iodoacetamide) and a photoactivatable cross-linker group (*N*-hydroxysuccinimidyl-4-azidosalicylic acid) were subsequently attached to the *N*-terminal cysteine residue (J. J. Calvete, W. Schäfer, K. Mann, A. Henschen & J. González-Rodríguez, unpublished work). The derivatized peptides were then used to assay the capacity of the different GPIIb/IIIa proteolytic mixtures to bind and cross-link them. Samples of the GPIIb/IIIa proteolytic mixtures, in which the proteinase was inhibited by phenylmethanesulphonyl fluoride, were incubated with 0.1 mM derivatized peptide for 1 h at 37 °C in the dark, followed by irradiation with a 250 W u.v. lamp for 15 min. The irradiated samples were then subjected to size-exclusion chromatography as described above. Cross-linking of the synthetic peptides to the isolated proteolytic products was estimated from the absorption at 280 and 530 nm, using the following extinction coefficients: ϵ_{280} 1.1 ml $\cdot$ mg⁻¹ $\cdot$ cm⁻¹ for protein content and ϵ_{530} 85 000 M⁻¹ $\cdot$ cm⁻¹ for eosin (Usobiaga *et al.*, 1987).

Hydropathy plots

Hydropathic profiles of peptide sequence stretches were generated using the hydrophilic/hydrophobic scale of Kyte & Doolittle (1982). The GPIIb and GPIIIa amino acid sequences used were those derived by cDNA cloning (Heidenreich *et al.*, 1990; Zimrin *et al.*, 1990). The search for GPIIIa sequence stretches that are hydropathically complementary to selected GPIIb sequence stretches was carried out by the method of Blalock & Smith (1984).

RESULTS

Pattern of fragmentation of GPIIb/IIIa by tryptic digestion

When GPIIb/IIIa in digestion buffer was cleaved with Tos-Phe-CH₂Cl-treated trypsin at an enzyme/substrate ratio of 1:250 (w/w) for 1 h at 37 °C (see the Materials and methods section), five fractions were separated by size-exclusion chromatography on a TSK-SW 3000 column (Fig. 1a). These were designated according to their apparent molecular mass (kDa), as follows: T-100, T-60, T-45, T-20 and T-18. Each fraction was rechromatographed on either a TSK-SW 3000 or a TSK-SW 2000 column, depending on its molecular mass, in the presence of 0.1 mM-Ca²⁺ or of 1 mM-EDTA after incubation in 10 mM-EDTA at 37 °C. It was found that all of the fractions were homogeneous in size and the proteolytic product(s) within each of them were stable, both with and without Ca²⁺. Amino acid, *N*-terminal sequence and immunochemical analyses of each fraction allowed us to assign their subunit origin and, to a certain extent, the size of the proteolytic fragments which constituted each of them, as well as establishing that each fraction contained a single proteolytic product. Thus it was found that T-100, T-20 and T-18 were primary proteolytic products of GPIIb/IIIa, whereas T-60 and T-45 were secondary products of T-100. It was also found that both T-100 and T-20 contained peptide fragments derived from GPIIbH, GPIIbL and GPIIIa, that T-60 contained fragments from both GPIIbL and GPIIIa, and that the T-45 and T-18 products contained fragments from only GPIIIa and GPIIbH respectively. Moreover, T-100 contained only the epitopes for antibodies P₂₃₋₇ [GPIIIa-(114-122)] and P₃₇ [GPIIIa-(101-109)], T-20 contained only the epitopes for antibodies P₄₀ [GPIIIa-(262-303)] and M₁ [GPIIbL₂-(1-24)], and no epitope was found

Fig. 1. Isolation by size-exclusion chromatography of the proteolytic products of digestion of GPIIb/IIIa in solution

(a) Size-exclusion pattern of the tryptic products after 1 h of digestion of GPIIb/IIIa (see the Materials and methods section). (b) Size-exclusion pattern of the proteolytic products of digestion of GPIIb/IIIa with V8 endoproteinase for 5 h (—) and 24 h (·····). (c) Size-exclusion pattern of the chymotryptic products of GPIIb/IIIa after 3.5 h (—) and 24 h (·····) of digestion. Separation was done on a TSK-SW 3000 column (0.75 cm × 60 cm) equilibrated in 10 mM-Tris/HCl/0.1% n-octyl β-D-glucopyranoside/0.015% NaN₃/0.15 M-NaCl/0.1 mM-CaCl₂, pH 7.0, and eluted at a flow rate of 0.1 ml/min. The elution positions of the standards used for size calibration of the column are given at the top of the figure: a, Dextran Blue (2000 kDa); b, IgG (150 kDa); c, BSA (63 kDa); d, ovalbumin (43 kDa); e, soybean trypsin inhibitor (23 kDa); f, horse heart cytochrome c (12.5 kDa); g, ADP (427 Da).

in the other three tryptic products for any of the antibodies tested.

Size-exclusion chromatography of each proteolytic product, in buffer where n-octyl β-D-glucopyranoside was replaced by SDS, revealed that the constituent fragments of each proteolytic product were non-covalently associated. Furthermore, solubilization of each proteolytic product in 70% formic acid, followed by separation of the constituent fragments by reverse-phase h.p.l.c. (Fig. 2) and amino-acid, N-terminal sequence and immunochemical analyses of the isolated peptides, confirmed the non-covalent nature of the associations and the lack of disulphide bond exchange among the fragments within each proteolytic product, and provided additional data for a more precise assignment of the size of each peptide fragment (Tables 1 and 2). In two out of five experiments, the peptide stretch GPIIbL₂- (125–137) was found associated with the T-18 product, but in the

Table 1. Products of digestion of GPIIb/IIIa with Tos-Phe-Ch₂Cl-treated trypsin (250:1, w/w) for 1 h at 37 °C

Digestion products	Peptide fragments within each product	
	N-Terminal sequence	cDNA sequence (residue nos.)
T-100 GPIIIa	G P N I X T T R G V	1–150
	N I N L I F A V T E	303–689
	S X V L P Q T K	472–479
	T P V S X F N I Q	480–553
GPIIbL ₂	N A Q I G I A M L V	696–734
	G Q R A M V T V L A	27–75
T-60 GPIIIa	G P N I X T T R G V	1–62
	V R G X P Q E K E K	403–422
	D S L I V Q V T F D	423–622
	G A L H D E N T X N	623–689
GPIIbL ₂	G Q R A M V T V L A	27–75
	A M V T V L A F L W	30–75
T-45 GPIIIa	G P N I X T T R G V	1–62
	V R G X P Q E K	403–410
	D S L I V Q V T F D	423–622
	G A L H D E N T X N	623–689
T-20 GPIIIa	D A P E G G F D A I	217–235
	L A G I V Q P N D G	262–298
GPIIbH	Q G L G A S V V S W	91–139
	V E H T Y E L H N N	780–836
GPIIbL ₂	L Q D P V L V S X D	1–26
T-18 GPIIbH	L N L D P V Q L T F	1–32
	I V L D X G E D D V	598–661
	L I X N Q K	672–677
	V V L X E L G N P M	684–694

other experiments this peptide was not found in any of the isolated products.

Tryptic digestion of GPIIb/IIIa for only 15–30 min provided a fragmentation pattern similar to that described above for a 1 h digestion, except that part of GPIIb/IIIa remained undegraded, the peptide bond in GPIIbH between residues 479 and 480 was still maintained, and the T-60 and T-45 products were minor species. On the other hand, after 2 h of tryptic digestion T-100 itself represented only a minor fraction, whereas the secondary products T-60 and T-45 were major species, being the peptide GPIIbH 91–139, within the T-20 kDa product, cleaved between residues 118 and 119, but the tryptic products were maintained together by the GPIIb 107–130 disulphide bond.

Pattern of fragmentation of GPIIb/IIIa on digestion with V8 endoproteinase

Cleavage of GPIIb/IIIa with V8 endoproteinase was carried out at an enzyme/substrate ratio of 1:100 (w/w) for 5 or 24 h at 37 °C. The 5 h digestion products were separated by size-exclusion chromatography into two major fractions, designated V-100 and V-20. The 24 h digestion products were also distributed in two major fractions, designated V-60 and V-16 (Fig. 1b). All of these fractions were homogeneous in size and were stable, both with and without Ca²⁺, upon size-exclusion rechromatography. Amino-acid, N-terminal sequence and immunochemical analyses of the undissociated fractions and of their constituent peptides, separated by reverse-phase h.p.l.c. after dissociation in

Fig. 2. Isolation by reverse-phase h.p.l.c. of the constituent peptide fragments of the T-100 product of GPIIb/IIIa

T-100 (fraction 1 in Fig. 1a) was dissociated in 70% (v/v) formic acid. The peptide composition is given in Table 1. Peaks 1–5 correspond to GPIIbL₂-(25–75), GPIIbL₂-(30–75), GPIIIa-(1–150)-S-S-(303–689), GPIIbH-(696–734) and GPIIbH-(472–479)-S-S-(480–553) respectively. The separation was carried out on a C4 (pore size 30 nm, particle size 10 µm) Vydac column (25 cm × 0.4 cm) equilibrated in a mixture of 0.1% (v/v) trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) (100% A/0% B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 70% B in 70 min.

70% formic acid (see the Materials and methods section), allowed us to assign the subunit and chain origins and the sizes of the proteolytic fragments within each fraction, and to establish that each fraction contained a single proteolytic product, which is produced by non-covalent association of peptides (Tables 2 and 3). It was found that V-100 and V-20 were the primary proteolytic products of GPIIb/IIIa, whereas V-60 and V-16 were secondary products of V-100 and V-20 respectively. It was also found that V-100 contained peptide fragments from GPIIbH, GPIIbL and GPIIIa and the epitopes for the antibodies P₂₃₋₇, P₃₇ and M₅ [GPIIbH-(550–558)], V-60 contained sequences from GPIIbL

and GPIIIa, V-20 contained sequences from GPIIbH and GPIIbL and the epitopes for the antibodies M₁ and M₆ [GPIIbH-(657–665)], and V-16 contained peptide fragments only from GPIIbH and the epitope for antibody M₆.

Pattern of fragmentation of GPIIb/IIIa on chymotryptic digestion

Digestion of GPIIb/IIIa with chymotrypsin was carried out at an enzyme/substrate ratio of 1:250 (w/w) for 3.5 and 24 h. The 3.5 h digestion products were separated by size-exclusion chromatography into three main fractions, designated C-90, C-60 and C-20 (where numbers denote molecular mass in kDa). The 24 h products were distributed in three chromatographic fractions, designated C-70, C-45 and C-18 (Fig. 1c). All of these fractions, as with the tryptic and V8 endoproteinase fractions, were both homogeneous in size and stable, with and without Ca²⁺, upon size-exclusion rechromatography. Amino-acid, N-terminal sequence and immunochemical analyses of each undissociated fraction and of their constituent peptides, separated by reverse-phase h.p.l.c. after solubilization in 70% formic acid, allowed us to assign the subunit and chain origins and the sizes of the proteolytic fragments within each fraction, as well as establishing that each size-exclusion fraction contained a single proteolytic product (Tables 2 and 4). Thus it was found that C-90 and C-20 were primary chymotryptic products of GPIIb/IIIa, and C-70, C-60, C-45 and C-18 were secondary products of C-90. It was also found that C-45 and C-18 contained peptide fragments from only GPIIIa and GPIIbH respectively, C-20 contained sequences from GPIIbH and GPIIbL and the epitopes for the antibody M₁, C-70 and C-60 contained sequences from both GPIIbL and GPIIIa, and C-90 was the only product which contained peptide fragments from GPIIbH, GPIIbL and GPIIIa, and also carried the epitopes for antibodies M₂ [GPIIb-(747–755)], M₅ and M₆.

Photocross-linking of the RGD and KQAGDV peptides to the digestion products of GPIIb/IIIa

A sample of each GPIIb/IIIa digestion mixture, after stopping proteolysis at the desired time by addition of phenylmethanesulphonyl fluoride, was incubated with the derivatized synthetic peptides for 1 h at 37 °C, followed by u.v. irradiation (see the Materials and methods section). The reaction mixtures were then subjected to size-exclusion chromatography to isolate

Table 2. Distribution of the epitopes for a set of GPIIb- and GPIIIa-specific monoclonal antibodies (mAbs) among the digestion products of GPIIb/IIIa

Digestion products were isolated as shown in Fig. 1. Peptide compositions are given in Tables 1, 3 and 4. + and – indicate the presence and the absence respectively of the epitope in the corresponding digestion product.

Digestion product	mAb...	P ₂₃₋₇	P ₃₇	P ₄₀	M ₁	M ₂	M ₂₋₂	M ₃	M ₄	M ₅	M ₆
T-100		+	+	–	–	–	–	–	–	–	–
T-60		–	–	–	–	–	–	–	–	–	–
T-45		–	–	–	–	–	–	–	–	–	–
T-20		–	–	+	+	–	–	–	–	–	–
T-18		–	–	–	–	–	–	–	–	–	–
V-100		+	+	–	–	–	–	–	–	+	–
V-60		–	–	–	–	–	–	–	–	–	–
V-20		–	–	–	+	–	–	–	–	–	+
V-16		–	–	–	–	–	–	–	–	–	+
C-90		–	–	–	–	+	–	–	–	+	+
C-70		–	–	–	–	–	–	–	–	–	–
C-60		–	–	–	–	–	–	–	–	–	–
C-45		–	–	–	–	–	–	–	–	–	–
C-20		–	–	–	+	–	–	–	–	–	–
C-18		–	–	–	–	–	–	–	–	–	–

Table 3. Digestion of GPIIb/IIIa with V8 endoproteinase

V8 endoproteinase was present at a substrate/enzyme ratio of 100:1 (w/w), and incubations were carried out at 37 °C for (a) 5 h or (b) 24 h.

Digestion products	Peptide fragments within each product	
	N-Terminal sequence	cDNA sequence (residue nos.)
(a)		
V-100		
GPIIIa	G P N I X T T R G V	1-126
	L I P G T T V G V L	324-692
GPIIbH	A G F S S V V T Q A	169-247
	V G R V Y L F L Q P	325-365
	G L R S R P S Q V L	398-560
	L G N P M K K N A Q	689-740
GPIIbL₂	M A R G Q R A M V T	24-89
V-20		
GPIIbH	L N L D P V Q L T F	1-136
	Q T R I V L D X G E	595-670
	R L I X N Q K K E X	671-688
	I Q P Q G G L Q X F	818-838
GPIIbL₁	Z I F L P E P E Q P	1-35
GPIIbL₂	L Q D P V L V S X D	1-23
(b)		
V-60		
GPIIIa	G P N I X T T R G V	1-52
	L S L S F X A T X L	366-378
	V I P G L K S X M G	379-647
	T G K D A V X X T Y	648-692
GPIIbL₂	M A R G Q R A M V T	24-77
V-16		
GPIIbH	Q T R I V L D X G E	595-628
	L Q M D A A N E G E	633-646
	A E L A V H L P Q G	647-670
	R L I X N Q K K E X	671-688

the different digestion products of GPIIb/IIIa described above, to establish the peptide distribution present and to quantify the peptide cross-linking. Eosin was not found in any of the chromatographic fractions, except in those containing undegraded GPIIb/IIIa. Therefore no photocross-linking of the RGD and KQAGDV peptides to the proteolytic products had taken place, in spite of the fact that some of the products still maintained, partially or totally intact, peptide sequences which had previously been found to be photocross-linked to intact GPIIb/IIIa (Tables 1, 3 and 4; J. J. Calvete, W. Schäfer, K. Mann, A. Henschen & J. González-Rodríguez, unpublished work): GPIIbH-(696-724) and GPIIIa-(63-87) and -(303-305) for the RGD peptide; and GPIIbH-(752-768) and GPIIIa-(9-37) for the KQAGDV peptide.

DISCUSSION

Proteolytic enzymes have often been used for demarcation of structural and/or functional domains in proteins, since flexible loops, high-mobility regions, segments connecting structural domains and extensions on the protein surface are usually more accessible and, therefore, more susceptible to limited proteolysis than the folded domains (Wilson, 1991). Thus, although limited proteolysis in combination with protein-chemical and immunochemical analyses does not provide unequivocal evidence of the domains of a protein, it represents a simple although laborious first step which yields additional information on the fragmen-

Table 4. Digestion of GPIIb/IIIa with chymotrypsin

Chymotrypsin was present at a substrate/enzyme ratio of 250:1 (w/w), and incubations were carried out at 37 °C for (a) 3.5 h or (b) 24 h.

Digestion products	Peptide fragments within each product	
	N-Terminal sequence	cDNA sequence (residue nos.)
(a)		
C-90		
GPIIIa	G P N I X T T R G V	1-100
	S E L I P G T T V G	322-694
GPIIbH	S V A V G E F D G D	238-419
	N I Q M X V G A T G	486-719
	Q L Q I R S K N S Q	720-776
GPIIbL₂	A R G Q R A M V T V	25-81
C-60		
GPIIIa	G P N I X T T R G V	1-100
	S I E A K V R G X P	398-421
	K D S L I V Q V T F	422-694
GPIIbL₂	A R G Q R A M V T V	25-81
C-20		
GPIIbH	R A E G G Q	97-113
	N V L E K T E E A E	114-143
	A Q A P V A D I F S	196-237
	S L R G A V D I D D	420-485
GPIIbL₁	I L D I Q P Q G G	815-839
	Z I F L P E P E Q P	1-36
GPIIbL₂	L Q D P V L V S X D	1-24
(b)		
C-70		
GPIIIa	G P N I X T T R G V	1-100
	G L K I G D T V S F	388-694
GPIIbL₂	A R G Q R A M V T V	25-81
C-45		
GPIIIa	G P N I X T T R G V	1-100
	S I E A K V R G X P	398-414
	V T F D X D X A X Q	429-694
C-18		
GPIIbH	S V A V G E F D G D	238-288
	V L D X G E D D V	599-645
	I X N Q K K E X E T	673-703
	V A A E E G E R E Q	761-776

tation patterns obtained with proteinases of different specificities, the topography of the protein and the intrachain, intrasubunit and intersubunit non-covalent connections. This information lays down the basis for further work, leading to a more precise demarcation of structural and functional domains. This was our approach in the present work on the structure of the isolated GPIIb/IIIa heterodimer in non-denaturing conditions.

The patterns of proteolytic cleavage of GPIIb/IIIa indicate the presence of particular regions along the amino acid sequences of GPIIIa, GPIIbH and GPIIbL which show either high susceptibility or high resistance to proteolysis by trypsin, chymotrypsin and V8 endoproteinase. Thus the major susceptible regions of GPIIIa are within the sequences of GPIIIa comprising residues 100-150, 308-324 and 689-694. The first two regions are located within the so-called ligand-binding domain, identified previously in isolated GPIIIa, and the other region is found immediately adjacent to the single transmembrane sequence of GPIIIa (Calvete *et al.*, 1991b). The two proteolysis-resistant domains of GPIIIa are identical to the cysteine-rich regions already identified in isolated GPIIIa: the N-terminal domain

Scheme 1. Outline of the cleavage points and peptide fragments within the GPIIb/IIIa proteolytic products isolated in Fig. 1

Peptide compositions are given in Tables 1, 3 and 4. The proteolytic products are designated by the initial of the proteinase used for GPIIb/IIIa digestion (T, trypsin; V, V8 endoproteinase; C, chymotrypsin) followed by the apparent molecular mass (in kDa) determined by size-exclusion chromatography. Peptide fragments and interpeptide disulphide bridges are represented by bold and thin lines respectively. Numbers represent the positions of the *N*- and *C*-terminal ends of the peptide fragments in the amino acid sequences of GPIIIa, GPIIbH and GPIIbL₂ (Heidenreich *et al.*, 1990; Zimrin *et al.*, 1990).

(residues 1–52) and the core (residues 423–622), which are disulphide-bonded between themselves (Calvete *et al.*, 1991b). GPIIbL has two highly proteolysis-susceptible regions: GPIIbL₂-(24–27), which determines the location of the *N*-terminal end of GPIIbL in those fragments carrying GPIIbH residue Cys-826 (Calvete *et al.*, 1989), and GPIIbL₂-(75–89), which is adjacent to the transmembrane segment of GPIIbL. Finally, and contrary to the case with GPIIIa, GPIIbH is highly susceptible to proteolysis all along its sequence, in good agreement with previous structural and immunochemical studies (Calvete *et al.*, 1991a). Thus trypsin degrades very rapidly the long stretch GPIIbH-(140–472), which carries the epitope of the monoclonal antibody M₄ and the four Ca²⁺-binding sites (Calvete *et al.*, 1991a; Rivas & González-Rodríguez, 1991) and cleaves the rest of the GPIIbH chain at several highly susceptible sites, some of which [GPIIbH-(136–

143), -(595–598), -(660–700) and -(818–838)] are also susceptible to digestion by chymotrypsin and V8 endoproteinase. The latter proteinases do not degrade GPIIbH as extensively as does trypsin, so that most of the sequences missing from their 90 and 100 kDa products respectively are found in their 20 kDa products (Scheme 1). This observation, together with the high reproducibility of the fragmentation patterns obtained in five different experiments and the lack of disulphide bond exchange, indicates that, most probably, no rearrangement of fragments within and between proteolytic products has taken place.

Sequences of GPIIb and GPIIIa putatively involved in the subunit interface in the GPIIb/IIIa heterodimer

From the proteolytic patterns of the GPIIb/IIIa heterodimer, three sets of GPIIb and GPIIIa sequences can be outlined as

being putatively involved in the subunit interface of the heterodimer:

- (a) GPIIIa-(217–235)-S { GPIIbH-(91–139),
GPIIIa-(262–298)-S { GPIIbH-(780–836)-S
GPIIbL₂-(1–26)-S
- (b) GPIIIa-(403–421)/GPIIbL₂-(30–75)
- (c) GPIIIa-(324–366)/GPIIbH-(486–553)
and GPIIbH-(696–734)

This suggests that GPIIbL as well as GPIIbH contributes to the subunit interface (Lam *et al.*, 1989; Calvete *et al.*, 1991a).

The first putative subunit interface is easily inferred from the peptide composition of the T-20 product (Table 1 and Scheme 1). Although the actual pattern of peptide interaction within this product is still unknown, some information is available. Thus intrachain and/or intrasubunit interactions between GPIIbH-(91–139) and GPIIbH-(780–836) and/or GPIIbL₂-(1–26) can be inferred from the presence of these sequences, in all or most of their length, in other products such as C-20 and V-20 (Scheme 1). Furthermore, the lack of any GPIIIa sequence and of the GPIIbH-(780–814) stretch in the latter two products points to an interaction between the GPIIIa-(217–235)-S-S-(269–298) and the GPIIbH-(780–814) sequences. On the other hand, hydropathy plots show a high hydropathic complementarity (pairing of hydrophobic and hydrophilic residues) between GPIIIa-(225–235) and GPIIbH-(783–793) (Fig. 3a) and between GPIIbH-(103–110) and GPIIbL₂-(7–14) (Fig. 3b). If all these hypotheses prove to be correct, then the pattern of peptide interactions within the T-20 product (and probably within GPIIb/IIIa) would be GPIIIa-(225–235)/GPIIbH-(783–793) and GPIIbH-(103–110)/GPIIbL₂-(7–14).

This pattern of peptide interactions agrees with previous immunochemical and molecular genetic studies. Thus an anti-serum against the GPIIIa-(217–231) stretch did not recognize its epitope in the heterodimer (Cook *et al.*, 1992). On the other hand, an epitope on the C-terminus of the α -subunit precursor of Mac-1, whose equivalent localization in GPIIb would be within the GPIIbH-741–GPIIbL₂-28 stretch, becomes hidden in the heterodimer (Arnaout *et al.*, 1988).

The second subunit interface may be inferred by comparison of the peptide compositions of the T-60 and C-60 products with those of the T-45 and C-45 products respectively (Tables 1, 3 and 4; Scheme 1). The latter two products lack the GPIIIa-(403–422) and GPIIbL₂-(30–75) sequences; we thus propose that these two sequences form part of the interface between GPIIb and GPIIIa in the heterodimer. Alternatively, the GPIIbL₂-(30–75) sequence could be interacting with other regions of GPIIIa which are affected by the structural change induced in the remains of GPIIIa (the 45 kDa products) by the cleavages within the GPIIIa-(410–415) stretch, or the GPIIbL₂-(30–75) sequence itself could be extensively degraded at this late stage of proteolysis of GPIIb/IIIa. However, the high hydropathic complementarity found between the GPIIIa-(406–413) and GPIIbL₂-(29–36) sequences (Fig. 3c) favours the first interpretation. Moreover, since the GPIIIa-(415–428) stretch is missing from the C-45 product, part of this stretch may also contribute to the second subunit interface.

The sequences GPIIIa-(324–365), GPIIbH-(486–553) and GPIIbH-(696–734) were present in the T-100, C-90 and V-100 products of GPIIb/IIIa digestion, but were not found in the T-60, C-60 or V-60 products (Tables 1, 3 and 4; Scheme 1). We therefore propose that the GPIIIa-(324–365) sequence is involved, together with GPIIbH-(486–553) and/or GPIIbH-(696–734), in the subunit interface of the heterodimer, in good agreement with previous structural studies of GPIIb/IIIa in whole platelets (Calvete *et al.*, 1991a). An alternative interpretation, based on

Fig. 3. Hydropathy plots of hydropathically complementary peptide sequence stretches of GPIIb and GPIIIa putatively involved in intrachain, intrasubunit and intersubunit non-covalent connectivity in the GPIIb/IIIa heterodimer in solution

(a) GPIIbH-(783–793) (TYELHNNPGT) (●) and GPIIIa-(225–235) (AIMQATVCDEK) (□); (b) GPIIbH-(103–110) (VIVACAPW) (●) and GPIIbL₂-(7–14) (PEQPSRLQ) (□); (c) GPIIbL₂-(29–36) (RAMVTVLA) (●) and GPIIIa-(406–413) (CEQEKEKS) (□); (d) GPIIIa-(328–337) (PGTTVGVLMS) (●) and GPIIbH-(702–711) (AMLVSUGNLE) (□); (e) GPIIIa-(342–350) (VLQLIVDAY) (●) and GPIIbH-(496–504) (HNIPQKISL) (□); (f) GPIIbH-(679–688) (ENETRVVLC) (●) and GPIIbH-(598–607) (IVLDCGEDDV) (□); (g) GPIIbH-(4–11) (DPVQLTFY) (●) and GPIIbH-(630–637) (VLELQMDA) (□).

the structural changes that are proteolytically induced in the remains of both GPIIbH (C-18 and V-16) and GPIIIa (the 60 kDa products) could lead us to other, as yet unidentified, sequences that are involved in the intersubunit interactions taking place in the 90 and 100 kDa products. Hydropathy plots of the proposed interacting sequences identified a high hydrophobic complementarity between the GPIIIa-(328–337) and GPIIbH-(702–711) sequences (Fig. 3d) and between the GPIIIa-(342–350) and GPIIbH-(496–504) sequences (Fig. 3e), which supports our first proposition.

GPIIbH sequences putatively involved in intrachain contacts

The GPIIbH-(595–628), -(633–646), -(647–670) and -(671–688) sequences found in the V-16 product (Table 3; Scheme 1) must form a compact intrachain domain in the GPIIb/IIIa heterodimer. Although the actual interactions between these peptides cannot be inferred from the information available at present, the high hydrophobic complementarity found between the GPIIbH-(598–607) and GPIIbH-(679–688) sequences (Fig. 3f) points to the likelihood of a specific interaction between these two sequences.

The T-18 product (Table 1; Scheme 1) contains the first 32 residues of GPIIbH and sequences found in V-16 extended six residues upstream, to residue 694 of GPIIbH, but lacking the GPIIbH-(662–671) and -(678–683) stretches (Scheme 1). The structure of this product confirms the idea that the GPIIbH-(595–690) sequence forms a well-defined domain which remains intact independently of cleavage at different points within this sequence by the three proteases (T-18, C-18 and V-16). Also, the composition of the T-18 product suggests that the *N*-terminal end of the GPIIbH chain is folded against the GPIIbH-(595–690) domain. Further support for this interpretation comes from the high hydrophobic complementarity found between the GPIIbH-(4–11) and GPIIbH-(630–637) sequences, as shown in Fig. 3(g).

The V-20 product comprises the sequences found in V-16, but without cleavage at residues 629, 633 and 647 of GPIIbH, and two additional fragments from both ends of GPIIbH, i.e. GPIIbH-(1–136) and GPIIbH-(818–838), disulphide-bonded to GPIIbL₂-(1–23) (Table 3; Scheme 1). We have already discussed the interaction of the GPIIbH-(91–139) sequence with the *N*-terminal stretch of GPIIbH and the association of the *N*-terminal end of GPIIbH with the GPIIbH-(595–690) region. Therefore the GPIIbH-(1–136) stretch most probably interacts at its *N*-terminal end with the GPIIbH-(595–690) region and at its C-terminal end with GPIIbH-(818–838)-S-S-GPIIbL₂-(1–23).

Further proof that the GPIIbH-(600–700) sequence forms a packed domain is given by the C-18 product, which comprises the GPIIbH-(599–703) sequence (but lacking residues 646–672), and two additional fragments, GPIIbH-(238–288) and GPIIbH-(761–776) (Table 4; Scheme 1). This suggests that the first Ca²⁺-binding site of GPIIbH (residues 243–254) may be folded against the GPIIbH-(600–700) domain, together with the *N*-terminal end of GPIIbH, although there is no indication of the actual sequences involved in this interaction.

Finally, the C-20 product contains, among others, the GPIIbH-(97–143) and GPIIbH-(815–839)-S-S-GPIIbL₂-(1–24) sequence stretches, in agreement with inferences made above from the structural analysis of other proteolytic products. The other fragments present in this product are GPIIbH-(196–237) and -(420–485). If we take into consideration that the V-100 product (Table 3; Scheme 1) contains the last two sequences but lacks the GPIIbH-(97–143) and GPIIbH-(815–839)-S-S-GPIIbL₂-(1–24) stretches, it seems reasonable to propose that the GPIIbH-(196–237) and -(420–485) sequences interact with each other. At present, there is no indication of how they associate with the other fragments of C-20.

A final aspect which remains to be investigated is how the large fragments of GPIIbH, present in the 90 and 100 kDa products, are associated with GPIIIa and/or with each other.

GPIIb sequences putatively involved in intrasubunit contacts

As discussed above, the GPIIbL₂-(27–75) sequence may be involved in an intersubunit interaction with GPIIIa, while the GPIIbL₂-(1–23) sequence most probably forms intrasubunit contacts through its interaction with the GPIIbH-(103–110) stretch (see above), besides being disulphide-bonded to the C-terminal region of GPIIbH (Calvete *et al.*, 1989). This suggests that the *N*- and C-terminal regions of GPIIbH and the *N*-terminus of GPIIbL are close to each other in the heterodimer in solution. A similar conclusion was reached in a previous study on the localization of the cross-linking sites of the RGD and KQAGDV peptides in GPIIb/IIIa in solution (J. J. Calvete, W. Schäfer, K. Mann, A. Henschen & J. González-Rodríguez, unpublished work).

A model of the GPIIb/IIIa heterodimer in solution

From the above discussion, some features can be identified upon which the emerging model of GPIIb/IIIa in solution can be outlined. (1) The high stability of the proteolytic products of GPIIb/IIIa points to high affinity between the constituent fragments and, most probably, to high specificity of the interactions involved within each product. (2) None of the proteolytic products showed the Ca²⁺-dependent stability characteristic of the GPIIb/IIIa heterodimer (Rivas & González-Rodríguez, 1991). This may be due to destruction of the high-affinity Ca²⁺-binding site, either directly by cleavage or indirectly by the structural change subsequent to proteolysis. Alternatively, the proteolytic cleavages could stabilize the subunit interface structures and make them Ca²⁺-independent, as occurs at low temperature (Rivas *et al.*, 1992). (3) No cross-linking sites of RGD and KQAGDV peptides were found in the proteolytic products, which may be due to direct or indirect destruction of the peptide-binding sites, or because binding took place but not cross-linking. (4) The fragmentation pattern of GPIIb/IIIa and the amino acid sequence putatively involved in the subunit interface and in the intrasubunit and intrachain contacts are consistent with previous structural and immunochemical studies (Calvete *et al.* 1989, 1991a,b,c; Niewiarowski *et al.*, 1989; Beer & Collier, 1989; J. J. Calvete, W. Schäfer, K. Mann, A. Henschen & J. González-Rodríguez, unpublished work). (5) The three main proteolysis-susceptible regions of GPIIIa in the heterodimer allowed us to confirm the existence of two proteolysis-resistant disulphide-bond-rich domains, the *N*-terminal domain residues (1–52) and the core (residues 423–622), which are disulphide-bonded between themselves, and a highly proteolysis-susceptible region, known as the ligand-binding domain (residues 101–400). These three domains had already been identified in isolated GPIIIa in solution (Beer and Collier 1991; Calvete *et al.*, 1991b,c). (6) The extracellular end of the single transmembrane segment of both GPIIIa and GPIIbL, the *N*- and C-termini of GPIIbH and the *N*-terminus of GPIIbL in the heterodimer in solution are easily accessible to the three proteinases used in the present study. (7) The following pairs of sequences were found to be putatively involved in the subunit interface of the heterodimer: (a) GPIIIa-(217–235)-S-S-GPIIIa-(262–298)/GPIIbH-(780–836), (b) GPIIIa-(324–366)/GPIIbH-(486–553) and GPIIbH-(696–734), and (c) GPIIIa-(403–421)/GPIIbL₂-(30–75). It is remarkable that these putative intersubunit regions correspond well to exon boundaries within the gene structure of GPIIb and GPIIIa (Heidenreich *et al.*, 1990; Zimrin *et al.*, 1990). Thus sequences within GPIIIa exons IV–V (residues 180–287), VII–VIII (residues 320–396) and the *N*-terminal part of exon IX

Fig. 4. A rudimentary connectivity map for the GPIIb/IIIa heterodimer in solution

The filled arrows indicate those peptide sequences (hatched stretches) within which putative intersubunit contacts were found. The open arrows indicate those peptide sequences (surrounded by a dotted line) within which putative intrasubunit or intrachain contacts were found. Compact domains [GPIIbH-(600–700), GPIIIa-(1–52), GPIIIa-(423–622)] are surrounded by a dashed line. ◇—, N-Glycosylation site; ○—, O-glycosylation site; C, C-terminal end. Disulphide bond and glycosylation patterns were taken from Calvete *et al.* (1989) and Calvete *et al.* (1991b).

(residues 396–537) may participate in the intersubunit surface, together with the products of GPIIb exons XVI–XVII [H-(485–553)], XXII–XXIII [H-(699–752)], XXV–XXVI [H-(786–857), L₁-(1–19)] and XXVII [L₁-(20–57)]. Such correlations were not apparent from analysis of the linear primary structure of GPIIb and GPIIIa (Poncz *et al.*, 1987; Fitzgerald *et al.*, 1987; Calvete *et al.*, 1989, 1991b). (8) The pair of sequences GPIIbH-(97–113)/GPIIbL₂-(1–23) was found to be putatively involved in intrasubunit interactions in GPIIb. (9) The sequence GPIIbH-(600–700) must form a compact domain. (10) The following pairs of sequences were found to be putatively involved in intrachain contacts: (a) GPIIbH-(1–32)/GPIIbH-(598–694) [which suggests that the N-terminus of GPIIbH is folded over the GPIIbH-(600–700) domain], (b) GPIIbH-(196–237)/GPIIbH-(420–485), (c) GPIIbH-(238–288)/GPIIbH-(761–776), and (d) GPIIbH-(598–607)/GPIIbH-(671–688).

Based on all of these features, a rudimentary connectivity map for the GPIIb/IIIa heterodimer is given in Fig. 4. It is immediately evident that this map does not agree with the current accommodation of the primary structure of GPIIb and GPIIIa within the head/two-tails image of GPIIb/IIIa in solution seen by electron microscopy (Carrell *et al.*, 1985; Rivas *et al.*, 1991b). Thus, according to this interpretation, the tips of the two tails would represent the C-terminal regions of GPIIIa and GPIIbL respectively, and the N-terminal halves of GPIIIa and GPIIbH would represent the globular head (Kieffer & Phillips, 1990). However, the present findings suggest that the N-terminal region

of GPIIbL is folded over the N- and C-terminal regions of GPIIbH, that the GPIIbL₂-(30–75) stretch is folded with the GPIIIa-(403–421) stretch, between the ligand-binding and the core domains of GPIIIa, and that the N-terminal end of GPIIbH is folded over the GPIIbH-(600–700) domain. Therefore one would expect to find all these structures together, most probably in the head of the electron microscopy image of GPIIb/IIIa. Further structural work, together with the use of monoclonal antibodies whose epitopes are well assigned, will lead to a GPIIb/IIIa topography and domain demarcation which is closer to reality.

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REFERENCES

- Arnaout, M. A., Remold-O'Donell, E., Pierce, M. W., Harris, P. & Tenen, D. G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2776–2780
- Beer, J. & Collier, B. S. (1989) *J. Biol. Chem.* **264**, 17564–17573
- Blalock, J. E. & Smith, E. M. (1984) *Biochem. Biophys. Res. Commun.* **121**, 203–207
- Calvete, J. J. & González-Rodríguez, J. (1986) *Biochem. J.* **240**, 155–161
- Calvete, J. J., Rivas, G. A., Maruri, M., Alvarez, M. V., McGregor, J.-L., Hew, C. L. & González-Rodríguez, J. (1988) *Biochem. J.* **250**, 697–704
- Calvete, J. J., Henschen, A. & González-Rodríguez, J. (1989) *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. (1991a) *Biochem. J.* **273**, 767–775
- Calvete, J. J., Henschen, A. & González-Rodríguez, J. (1991b) *Biochem. J.* **274**, 63–71
- Calvete, J. J., Arias, J., Alvarez, M. V., López, M. M., Henschen, A. & González-Rodríguez, J. (1991c) *Biochem. J.* **274**, 457–463
- Carrell, N. A., Fitzgerald, L. A., Steiner, B., Erickson, H. P. & Phillips, D. R. (1985) *J. Biol. Chem.* **260**, 1743–1749
- Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A. & Scarborough, R. M. (1991) *J. Biol. Chem.* **266**, 1415–1421
- Cook, J. J., Trybulec, M., Lasz, E. C., Kahn, S. & Niewiarowski, S. (1992) *Biochim. Biophys. Acta*, in the press
- D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. & Plow, E. F. (1988) *Science* **242**, 91–93
- D'Souza, S. E., Ginsberg, M. H., Burke, T. A. & Plow, E. F. (1990) *J. Biol. Chem.* **265**, 3440–3446
- D'Souza, S. E., Ginsberg, M. H., Matsueda, G. R. & Plow, E. F. (1991) *Nature (London)* **350**, 66–68
- Fitzgerald, L. A., Steiner, B., Rall, S. J., Jr., Lo, S. S. & Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 3936–3939
- Ginsberg, M. H., Loftus, J. C. & Plow, E. F. (1988) *Thromb. Haemostasis* **59**, 1–12
- Heidenreich, R., Eisman, R., Delgrosso, K., Surrey, S., Bennett, J. S., Schwartz, E. & Poncz, M. (1990) *Biochemistry* **29**, 1232–1244
- Kieffer, N. & Phillips, D. R. (1990) *Annu. Rev. Cell Biol.* **6**, 329–358
- Kunicki, T. J., Pidard, D., Rosa, J.-P. & Nurden, A. T. (1981) *Blood* **58**, 268–278
- Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lam, S. C.-T., Plow, E. F. & Ginsberg, M. H. (1989) *Blood* **73**, 1513–1518
- Lanza, F., Kieffer, N., Phillips, D. R. & Fitzgerald, L. A. (1990) *J. Biol. Chem.* **265**, 18098–18103
- Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A., Frelinger, A. L., III & Ginsberg, M. H. (1990) *Science* **249**, 915–918
- Niewiarowski, S., Norton, K. J., Eckardt, A., Lukasiewicz, H., Holt, J. C. & Kornecki, E. (1989) *Biochim. Biophys. Acta* **893**, 91–99
- Phillips, D. R. & Agin, P. P. (1977) *J. Biol. Chem.* **252**, 2121–2126
- Plow, E. F. & Ginsberg, M. H. (1989) *Prog. Thromb. Haemostasis* **9**, 117–156

- Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E. & Bennett, J. S. (1987) *J. Biol. Chem.* **262**, 8476–8482
- Rivas, G. A. & González-Rodríguez, J. (1991) *Biochem. J.* **276**, 35–40
- Rivas, G. A., Calvete, J. J. & González-Rodríguez, J. (1991a) *Protein Expression Purif.* **2**, in the press
- Rivas, G. A., Arnárez, J. A., Usobiaga, P., Sáiz, J. L. & González-Rodríguez, J. (1991b) *Eur. Biophys. J.* **19**, 335–345
- Rivas, G. A., Usobiaga, P. & González-Rodríguez, J. (1992) *Eur. Biophys. J.* **20**, in the press
- Usobiaga, P., Calvete, J. J., Sáiz, J. L., Eirín, M. T. & González-Rodríguez, J. (1987) *Eur. Biophys. J.* **14**, 211–218
- Wilson, J. E. (1991) *Methods Biochem. Anal.* **35**, 207–250
- Zimrin, A. B., Gidwitz, S., Lord, S., Schwartz, E., Bennett, J. S., White, G. C., II & Poncz, M. (1990) *J. Biol. Chem.* **265**, 8590–8595

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