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Interchain and intrachain disulphide bonds in human platelet glycoprotein IIb

Localization of the epitopes for several monoclonal antibodies

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The single interchain disulphide bond in platelet glycoprotein IIb (GPIIb) is accessible to extracellular reductants, and selective cleavage does not liberate GPIIbα from platelet plasma membrane, confirming that non-covalent interactions contribute to maintaining attachment of this subunit to the membrane. Eosin–maleimide labelling of isolated GPIIb after selective cleavage of this interchain disulphide bond, followed by full reduction and alkylation, CNBr cleavage, and analysis of the cleavage products allowed us to establish that this interchain disulphide bridge is formed between GPIIbβ (GPIIb β-subunit) Cys-9 and GPIIbα Cys-826, and this conclusion was confirmed by independent routes. The other two cysteines of GPIIbβ (Cys-14 and Cys-19) form the single intrachain disulphide bond in this subunit. Last, the intrachain disulphides in GPIIbα (GPIIb α-subunit) are distributed in four main peptide domains which are not disulphide-bonded among themselves. The linear epitope for monoclonal antibody M1 is localized between Pro-4 and Met-24 (or Met-31) of GPIIbβ. The linear epitope for M3 is situated between Cys-826 and the *C*-terminus of GPIIbα. The M4 epitope is also linear and localized somewhere between residues 115 and 285 of GPIIbα. Finally, the epitopes for M5 and M6 are somewhere between Cys-608 and Met-704, within a 35 kDa membrane-bound chymotryptic product of digestion of GPIIb in whole platelets. The *N*-terminal amino acid sequences determined for eight different cleavage products of GPIIbα and GPIIbβ agree with the corresponding amino acid sequences predicted by cDNA sequence for human-erythroleukaemic-cell GPIIb [Poncz, Eisman, Heindenreich, Silver, Vilaire, Surrey, Schwartz & Bennett (1987) J. Biol. Chem. **262**, 8476–8482].

INTRODUCTION

Glycoprotein IIb (GPIIb) is a 136.5 kDa major component of platelet plasma membrane (Clemetson & McGregor, 1987; Usobiaga *et al.*, 1987), which together with glycoprotein IIIa (GPIIIa, 91.5 kDa) serves as an inducible receptor for fibrinogen (Marguerie *et al.*, 1987) and other adhesive proteins, and plays a primary role in platelet aggregation (Nurden & Caen, 1974; Phillips *et al.*, 1975). These two glycoproteins have been isolated and characterized both as individual glycoproteins and as a GPIIb–GPIIIa complex (Leung *et al.*, 1981; McEver *et al.*, 1982; Jennings & Phillips, 1982; Eirín *et al.*, 1986). In previous work (Calvete & González-Rodríguez, 1986; Usobiaga *et al.*, 1987) we reported the isolation, the amino acid and sugar composition and the molecular characterization of the α-(GPIIbα, 114 kDa) and β-(GPIIbβ, 22.5 kDa) subunits of GPIIb. We also found that GPIIbα and GPIIbβ are joined by a single disulphide bond and that the remaining half-cystine residues participate in intrachain bonds, six in GPIIbα and one in GPIIbβ.

GPIIb and GPIIIa are derived from the translation of two distinct mRNAs that are found in both human erythroleukaemic (HEL) cells (Bray *et al.*, 1986) and megakaryocytes (Duperray *et al.*, 1987). Increasing structural and immunochemical evidence has led to the hypothesis that the GPIIb–GPIIIa complex belongs to a superfamily of heterodimeric glycoproteins of similar sequence comprising cellular-adhesion receptors (cyto-adhesins), integrins and leukocyte-adhesion receptors (Fitzgerald *et al.*, 1987; Ginsberg *et al.*, 1988; Uzan *et al.*, 1988).

In the present study we established (i) the position in the α- and β-subunits of the half-cystine residues forming the interchain disulphide bridge in GPIIb and its extracellular location, (ii) the position of the single intrachain disulphide bond in GPIIbβ and also that for some of the intrachain disulphide bonds in GPIIbα, and (iii) the localization of the epitopes for several anti-GPIIb

Abbreviations used: GPIIbα and GPIIbβ, the α- and β-subunits of glycoprotein IIb (GPIIb), after selective reduction of the single disulphide joining them and further carboxymethylation; CM-GPIIbα and CM-GPIIbβ, α- and β-subunits of GPIIb at any other degree of reduction, carboxymethylation and association; PMSF, phenylmethanesulphonyl fluoride; HEL, human erythroleukaemic-cell line; GPIIIa, glycoprotein IIIa; PTH, phenylthiohydantoin.

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monoclonal antibodies. We also confirmed previous findings that neither the β- nor the α-subunit is liberated from the membrane after selective cleavage of the interchain disulphide bond (Calvete & González-Rodríguez, 1986).

MATERIALS AND METHODS

Materials

Eosin–maleimide was from Molecular Probes (Junction City, OR, U.S.A.). Chymotrypsin was from Sigma (St. Louis, MO, U.S.A.). All chemicals and biochemicals were of analytical or chromatographic grade. Chromatographic columns and buffers, as well as the preparation of human platelets, platelet plasma membranes and the isolation of GPIIb and the α- and β-subunits of GPIIb in their fully reduced, partially reduced and alkylated and fully alkylated forms, were as described previously (Calvete & González-Rodríguez, 1986; Eirín *et al.*, 1986).

Analytical methods

Assay of proteins (Markwell *et al.*, 1978), amino acid analysis, free-thiol (Ellman, 1959) and disulphide-group (Zahler & Cleland, 1968) determinations, labelling with eosin–maleimide and measurement of bound eosin, and SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), were done as described previously (Calvete & González-Rodríguez, 1986; Eirín *et al.*, 1986). Isolation of peptides by electroelution from SDS/polyacrylamide-gel-electrophoretic bands was carried out as described by Hunkapiller *et al.* (1983).

Immunoelectroblotting

After gel electrophoresis the glycoprotein bands were transferred to nitrocellulose by a standard procedure (Towbin *et al.*, 1979) at 250 mA for up to 5 h. The first antibodies used, all in the form of the 50%-satd.-$(NH_4)SO_4$-precipitated fraction of ascitic fluids, were mouse anti-GPIIbα (M3, M4, M5, M6) and anti-GPIIbβ (M1) monoclonal antibodies, prepared by using either total GPIIb or the isolated subunits, according to immunization and fusion protocols and screening assays described previously (Melero & González-Rodríguez, 1984; Calvete & González-Rodríguez, 1986; Calvete *et al.*, 1986*a*,*b*). The second antibody was goat anti-(mouse IgG) IgG. Detection was effected by autoradiography using X-Omat films (Kodak) after incubation with ^{125}I-protein A.

Automated Edman degradation

Automated Edman degradation was done as described previously (Edman & Henschen, 1975; Calvete *et al.*, 1988). The phenylthiohydantoin (PTH) derivatives of the amino acids were analysed by reverse-phase h.p.l.c. using either a Beckman Ultrasphere PTH column (Hew *et al.*, 1986) or a Spherisorb ODSII column (Henschen, 1986). Thiol modification with 4-vinylpyridine and identification of the *S*-β-4-pyridylethylcysteine during amino acid and sequence analysis was done as described by Henschen (1986).

Reduction and carboxymethylation of whole platelets

Platelets were washed twice in 20 mM-Tris/HCl/150 mM-NaCl, pH 8.0, and resuspended in the same buffer at 2×10^9 platelets/ml. Samples (1 ml) of the platelet suspension were reduced for 1 h at room temperature with increasing molar excesses of dithioerythritol over the theoretical half-cystine contents of GPIIb and GPIIIa in platelets. The following data were used: GPIIb (136.5 kDa, 16 half-cystine residues) and GPIIIa (91.5 kDa, 50 half-cystine residues) represent 1.7 and 1.2% (w/w) respectively of the total platelet proteins (Eirín *et al.*, 1986) Reduction was followed by alkylation for 30 min with a 5-fold molar excess of iodoacetate over reductant. The reduced and alkylated platelets were washed twice, resuspended and sonicated in the same buffer, and the particulate fraction was obtained by high-speed ultracentrifugation at 160000 *g* ($r_{av.}$, 65 mm) for 1 h at 4 °C.

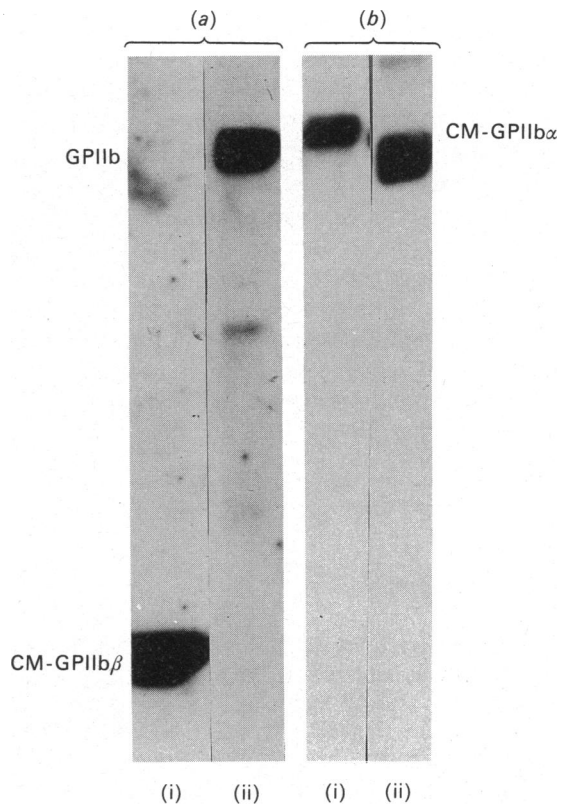


Fig. 1. Analysis by immunoelectroblotting of the cleavage by reduction of the interchain disulphide bond of GPIIb in whole platelets, using anti-GPIIbα (M3) and anti-GPIIbβ (M1) monoclonal antibodies

Reduction and carboxymethylation of whole platelets, isolation of the particulate fraction and SDS/polyacrylamide-gel electrophoresis and immunoelectroblotting of this fraction were carried out as described in the Materials and methods section. Gels containing 10% (w/v) polyacrylamide and monoclonal antibody dilutions of 1:500 (M1) and 1:1000 (M3) were used. (*a*) Identification pattern of GPIIb and GPIIbβ in the particulate fraction of whole platelets reduced with a 100-fold molar excess of dithioerythritol over half-cystine content of GPIIb and GPIIIa in platelets, using M1. Lane (i), reduced platelets; lane (ii), control platelets. (*b*) Identification pattern of GPIIb and GPIIbα in the particulate fraction of whole platelets reduced as in (*a*) using M3. Lane (i), reduced platelets; lane (ii), control platelets.

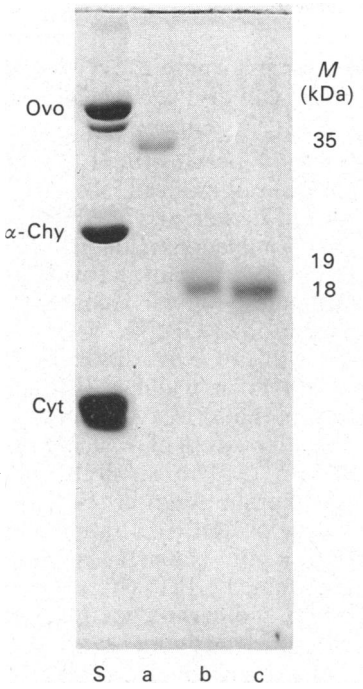


Fig. 2. Electrophoretic analysis of the isolated CNBr products of cleavage of fully reduced and carboxymethylated GPIIbα (after selective eosin-labelling)

Eosin-labelling, full reduction and carboxymethylation, CNBr cleavage, isolation of cleavage products by electroelution, and SDS/polyacrylamide-gel electrophoresis were done as described in the Materials and methods section. Gels containing 15% polyacrylamide and Coomassie Blue staining were used. Abbreviations: Ovo, ovoalbumin; α-Chy, α-chymotrypsin; Cyt, cytochrome *c*; *M*, molecular mass. Key to lanes: lane S, standards; lane a, 35 kDa CNBr fragment; lane b, 19 kDa CNBr fragment; lane c, 18 kDa CNBr fragment.

Digestion of whole platelets with chymotrypsin

Platelets were washed in 10 mM-Tris/HCl/150 mM-NaCl/1 mM-EDTA, pH 7.4, and resuspended at 5×10^9 platelets/ml in the same buffer. The platelet suspension was incubated at 37 °C with chymotrypsin (0.2 mg/ml). After 5 min the digestion was stopped with PMSF (25 mol/mol of chymotrypsin). Digested platelets were centrifuged at 10000 *g* ($r_{av.}$, 75 mm) for 10 min at 4 °C. The supernatant containing the soluble products of digestion was dialysed against Milli Q water at 4 °C and freeze-dried. The digested platelets were resuspended and sonicated in the same buffer, and the particulate fraction was obtained by high-speed ultracentrifugation as above.

Reduction and carboxymethylation of isolated GPIIb

Pure GPIIb (5 mg/ml) in 50 mM-Tris/HCl/2 mM-EDTA/0.1% SDS, pH 8.0, was reduced at room temperature for 2 h with a 5-fold molar excess of dithioerythritol over the theoretical half-cystine content of GPIIb, followed by alkylation with a 1.5-fold molar excess of eosin–maleimide over reductant for 2 h in the dark. The sample was passed first through a Sephadex G-50 column (1 cm × 40 cm) to separate free from bound eosin and afterwards through a Sephacryl S-200 column (1.5 cm × 100 cm) to isolate the eosin-labelled α- and β-subunits (Calvete & González-Rodríguez, 1986). The isolated subunits were further fully reduced with a 150-fold molar excess of dithioerythritol over their theoretical half-cystine contents and alkylated in the same buffer as follows: the α-subunit with a 1.5-fold molar excess of iodoacetate over reducing agent, and the β-subunit with a 1.5 molar excess of 4-vinylpyridine, as described by Henschen (1986). The subunits were finally dialysed against Milli Q water and freeze-dried.

Table 1. Amino acid composition of the 35, 19 and 18 kDa CNBr fragments of cleavage of CM-GPIIbα and of the 110 and 28 kDa reduced chymotryptic soluble products of digestion of GPIIb in whole platelets

The theoretical amino acid composition of the corresponding peptide stretches tentatively assigned for each of these fragments in the cDNA-deduced GPIIbα amino acid sequence is given alongside. The experimental results are an average of two determinations with two different preparations.

	Composition (mol/100 mol of total amino acid)									
Residue	35 kDa	1–285*	19 kDa	703–871*	18 kDa	314–489*	110 kDa	17–570*	28 kDa	115–375*
Lys	2.0	1.8	2.1	3.0	2.6	2.3	3.2	2.3	2.1	1.6
His	2.0	1.8	3.8	4.2	–	0.6	1.4	1.9	1.8	2.0
Arg	5.3	5.4	4.9	4.2	6.6	6.3	5.7	5.9	6.4	6.3
Asx	7.3	7.9	11.6	9.7	8.9	9.1	8.5	8.4	11.7	8.8
Thr	4.8	5.1	1.8	1.2	2.7	2.8	5.4	4.5	6.6	4.4
Ser	9.5	9.8	10.2	9.7	8.3	7.4	7.9	8.6	8.6	9.1
Glx	11.3	11.6	15.6	15.8	7.2	7.4	10.8	9.7	11.3	9.1
Pro	6.6	5.4	12.4	12.1	7.4	8.0	5.8	6.2	4.6	5.1
Gly	11.5	10.8	6.8	7.3	12.1	11.7	12.6	10.8	11.8	11.6
Ala	7.0	7.2	4.1	4.2	8.9	9.7	9.3	8.4	6.6	8.3
Cys	2.0	2.2	0.4	0.6	1.2	1.4	1.7	1.8	1.6	1.2
Val	7.8	8.3	7.6	7.8	9.9	9.7	7.5	8.8	5.5	7.1
Met	0.3	–	–	–	–	–	1.0	0.9	1.0	0.8
Ile	2.6	2.3	4.6	4.8	2.4	2.8	3.8	2.5	2.7	2.4
Leu	11.1	9.8	10.9	11.5	10.8	11.4	8.7	11.3	7.2	11.1
Tyr	4.7	4.7	1.2	1.2	4.2	4.0	3.1	3.7	7.0	6.7
Phe	5.2	5.4	2.3	2.4	4.4	4.0	3.1	4.2	3.4	4.4
Hse	0.4	0.4	–	–	0.5	0.6	–	–	–	–

* HEL cells (Poncz *et al.*, 1987).

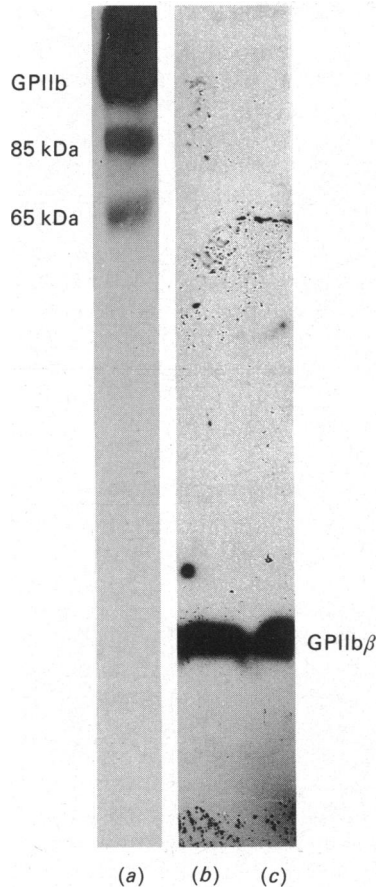


Fig. 3. Analysis by immunoelectroblotting of the particulate products of chymotryptic digestion of GPIIb in whole platelets, using anti-GPIIbα (M5 and M6) and anti-GPIIbβ (M1) monoclonal antibodies

Whole platelet digestion with chymotrypsin, isolation of the soluble and particulate products of digestion, SDS/polyacrylamide-gel electrophoresis and immunoelectroblotting were effected as described in the Materials and methods section. Gels containing 10% polyacrylamide and monoclonal antibodies at 1:1000 dilution were used. (*a*) Identification of the unreduced particulate products of chymotryptic digestion of GPIIb in whole platelets, using monoclonal antibody M5. The same pattern was obtained when M6 was used. (*b*) Control platelets reduced with 1% 2-mercaptoethanol for 2 min at 100 °C, using M1. (*c*) The same as in (*a*), but after reduction with 2-mercaptoethanol and using M1 as in (*b*).

CNBr cleavage of GPIIb and eosin-labelled α- and β-subunits

Pure GPIIb and fully reduced and carboxymethylated eosin-labelled GPIIbα and GPIIbβ (see above) were cleaved with a 500-fold molar excess of CNBr over their theoretical methionine contents in 70% (v/v) formic acid, under N_2 and in the dark. After 24 h at room temperature (unless stated otherwise) the mixture was diluted with Milli Q water and freeze-dried. Then it was suspended in 0.2 M-NH_4HCO_3 for 2 h at 37 °C and finally freeze-dried again (Gross & Witkop, 1962).

RESULTS

Dithioerythrithol access from the outside of the platelet to the GPIIb interchain disulphide bond

Fig. 1 shows the extracellular reduction of GPIIb in intact platelets by dithioerythritol. The supernatant and pellet after sonication of reduced, alkylated and washed platelets (see the Materials and methods section) were analysed by SDS/polyacrylamide-gel electrophoresis, followed by immunoelectroblotting with GPIIb-specific monoclonal antibodies: M1 for recognition of GPIIbβ, and M3 for recognition of GPIIbα. With a 40-fold molar excess of dithioerythritol over the half-cystine content of GPIIb and GPIIIa in platelets (Eirín *et al.*, 1986), GPIIb is already partially cleaved (results not shown). With a 100-fold molar excess of reducing agent, GPIIb is totally cleaved into its two subunits, both of which remain in the membrane fraction (Fig. 1). The electrophoretic mobility of GPIIbα after reduction and carboxymethylation in platelets is lower than the mobilities of unmodified GPIIb (Fig. 1*b*) and of GPIIbα after full reduction and carboxymethylation in solution (Calvete & González-Rodríguez, 1986).

Localization in the β-subunit of the half-cystine residue forming the interchain disulphide bridge in GPIIb and of the single intrachain disulphide bond

When isolated GPIIb is reduced with a 5-fold molar excess of dithioerythritol over total half-cystine residues (determined by amino acid analysis of GPIIb) and further alkylated with a 1.5-fold molar excess of eosin–maleimide (Calvete & González-Rodríguez, 1986), the molar content of eosin in the α- and β-subunits after their separation by size-exclusion chromatography was 1.9 in GPIIbα and 0.95 in GPIIbβ. If eosin-labelled GPIIbβ was then totally reduced with a 150-fold molar excess of dithioerythritol and further alkylated with 4-vinylpyridine (see the Materials and methods section), the *N*-terminal-sequence analysis of this subunit gave the following sequence:

Leu-Gln-Asp-Pro-Val-Leu-Val-Ser-Blank-Asp-
Ser-Ala-Pro-Cys-Thr-Val-Val-Gln-Cys-Asp
| |
Pyr Pyr

where a gap is found in position 9 and *S*-β-4-pyridylethylcysteine residues (Cys-Pyr) are found in positions 14 and 19. This, together with the *N*-terminal sequence predicted for unmodified GPIIbβ (Poncz *et al.*, 1987) allowed us to deduce that Cys-9 is involved in the single interchain disulphide bridge in GPIIb, whereas cysteines 14 and 19 form the single intrachain disulphide bond in GPIIbβ.

Localization in the α-subunit of the half-cystine residue forming the interchain disulphide bridge in GPIIb

Eosin–maleimide distribution among CNBr peptides of GPIIbα. Eosin-labelled GPIIbα isolated as described above (1.9 mol of eosin/nmol of GPIIbα) was further reduced with a 150-fold molar excess of dithioerythritol over half-cystine content, and later carboxymethylated with a 1.5-fold excess of iodoacetate over dithioerythritol. The fully reduced and carboxymethylated sample was cleaved by CNBr and subjected to SDS/polyacrylamide-gel electrophoresis. The three products containing eosin,

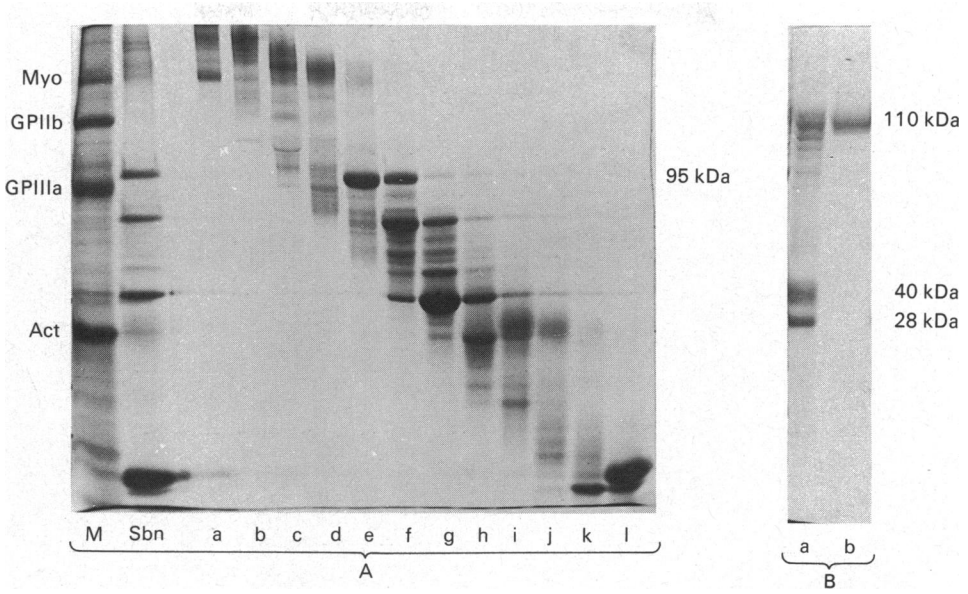


Fig. 4. Chromatographic isolation and electrophoretic analysis of the 95 kDa soluble product of chymotryptic digestion of GPIIb in whole platelets

Digestion of whole platelets with chymotrypsin, isolation of the soluble products of digestion and SDS/polyacrylamide-gel electrophoresis were effected as described in the Materials and methods section. Gels containing 7% polyacrylamide and Coomassie Blue staining were used. Abbreviations: Myo, myosin; Act, actin. Panel A, electrophoretic analysis of the unreduced soluble products of chymotryptic digestion of whole platelets, after size-exclusion chromatography on a column (2.5 cm × 150 cm) of Sephacryl S-300 in 0.1 M-phosphate/0.1% SDS/1 mM-EDTA/2 mM-PMSF/0.025% NaN_3 buffer, pH 6.8. Abbreviations: M, control of platelet membranes; Sbn, soluble products of digestion before fractionation; lanes a–l are samples from fractions along the elution profile of the Sephacryl column. Panel B: lane a, electrophoretic analysis after reduction with 1% 2-mercaptoethanol (2 min, 100 °C) of the 95 kDa product isolated as described in panel A; lane b, analysis of the 110 kDa fragment isolated by electroelution after SDS/polyacrylamide-gel electrophoresis of the 95 kDa product under reducing conditions, as in lane a.

viewed under a u.v. lamp, had apparent molecular masses of 35, 19 and 18 kDa respectively (results not shown). The 35 and 19 kDa fragments are recognized by anti-GPIIbα monoclonal antibodies M4 and M3 respectively. The three peptides were isolated by electroelution (Fig. 2) and their *N*-terminal sequence and amino acid content determined.

The 35 kDa peptide has the same *N*-terminal sequence as that of isolated GPIIbα, namely Leu-Asn-Leu-Asp-Pro. The *N*-terminal sequence of the 19 kDa peptide is Leu-Val-Ser-Val-Gly, which is identical with the sequence from residues 704–708 in the GPIIb amino acid sequence, as predicted from the sequence of identified cDNA clones from HEL cells and megakaryocytes (Poncz *et al.*, 1987; Uzan *et al.*, 1988). Therefore the 19 kDa peptide corresponds tentatively to residues 704 onwards in this predicted sequence. The *N*-terminal sequence for the 18 kDa fragment Glu-Ser-Arg-Ala-Asp, which correspond tentatively to a peptide from residue 314 to 489 in the HEL GPIIb sequence. The amino acid composition of the three peptides (Table 1) further supports the notion that: (i) the 35 kDa fragment corresponds to the peptide stretch from residue 1 to residue 285 in the HEL GPIIb sequence; (ii) the 19 kDa fragment corresponds to the stretch from residue 704 to residue 871; and (iii) the 18 kDa fragment corresponds to the 314–489 stretch.

On the other hand, the eosin contents (mol/mol of fragment) were 1.41, 0.91 and 0.21 in the 35, 19 and 18 kDa fragments respectively. Therefore the 18 kDa fragment can be excluded as carrier of the half-cystine residue involved in the interchain disulphide bridge. Several lines of evidence (see below) pointed to the 19 kDa fragment as the carrier of this cysteine residue.

Localization of the peptide carrying the half-cystine residue involved in the interchain bridge. Platelets digested with chymotrypsin were pelleted at low speed, and the supernatant, containing the soluble products of digestion, was freeze-dried after being dialysed against distilled water (see the Materials and methods section). The digested pellets were further sonicated and the particulate fraction obtained by high-speed differential centrifugation. SDS/polyacrylamide-gel electrophoresis of the unreduced soluble products of platelet digestion, followed by immunoelectroblotting, shows a product, of apparent molecular mass 95 kDa, recognized by M4 (see below). Similar analysis of the unreduced particulate fraction shows two products with apparent molecular masses of 85 and 65 kDa, both of which were recognized by monoclonal antibodies M5 and M6 (Fig. 3*a*), the 85 kDa peptide being recognized, in addition, by M1. After reduction of this peptide, M1 recognizes a product with the same electrophoretic mobility as GPIIbβ (Fig. 3*c*). This tells us that the half-cystine residue involved in the interchain disulphide bridge of GPIIb cannot be in the soluble fragment of 95 kDa liberated in the supernatant after early digestion of whole platelets with chymotrypsin.

The 95 kDa fragment was isolated by a size-exclusion chromatography on Sephacryl 300 (Fig. 4, panel A). The electrophoretic analysis of the isolated peptide under reducing conditions shows a mixture of peptides of

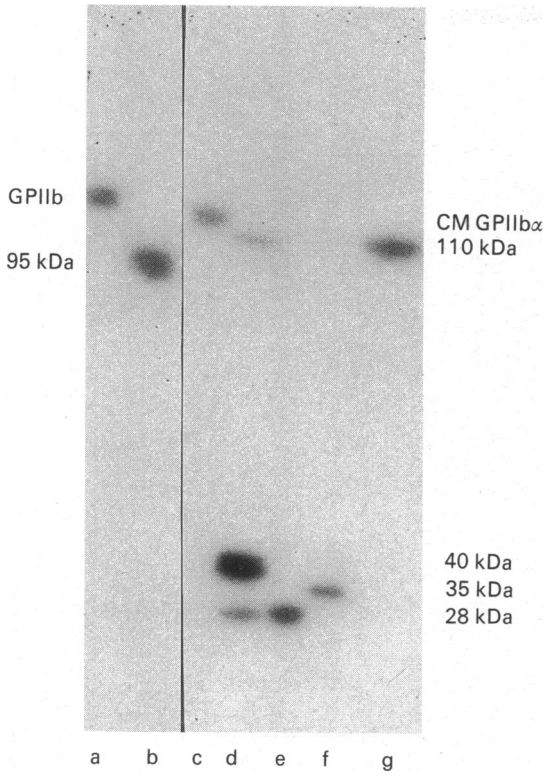


Fig. 5. Analysis by immunoelectroblotting of the soluble products of chymotryptic digestion of GPIIb in whole platelets, using monoclonal antibody M4

The isolation of the 95 kDa soluble product of chymotryptic digestion of GPIIb in whole platelets was done as described in Fig. 4. SDS/polyacrylamide-gel electrophoresis, electroelution and immunoelectroblotting were done as described in the Materials and methods section. Gels containing 7% polacrylamide, M4 at 1:1000 dilution and sample reduction (when indicated) with 1% 2-mercaptoethanol (2 min, 100 °C) were used. Lane a, unreduced GPIIb; lane b, unreduced 95 kDa product; lane c, fully reduced and carboxymethylated GPIIbα; lane d, reduced 95 kDa product; lane e, reduced 28 kDa fragment isolated by electroelution after SDS/polyacrylamide-gel electrophoresis of the 95 kDa product under reducing conditions; lane f, reduced 35 kDa peptide obtained by CNBr cleavage of fully reduced and carboxymethylated GPIIbα and electroelution (see Fig. 2); lane g, reduced 110 kDa fragment isolated by electroelution after SDS/polyacrylamide-gel electrophoresis of 95 kDa fragment under reducing conditions, as in lane b of Fig. 4, panel B.

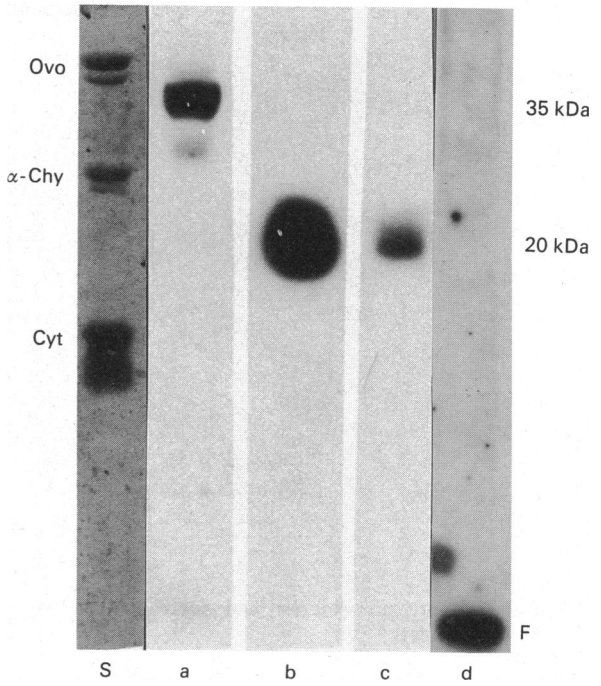


Fig. 6. Analysis by immunoelectroblotting of CNBr products of cleavage of whole GPIIb, using monoclonal antibodies M1, M3 and M4

CNBr cleavage of GPIIb, SDS/polyacrylamide-gel electrophoresis and immunoelectroblotting were carried out as described in the Materials and methods section. Gels containing 15% polyacrylamide, monoclonal antibodies at 1:1000 (M3 and M4) and 1:500 (M1) dilutions, and sample reduction (when indicated) with 1% 2-mercaptoethanol (2 min at 100 °C) were used. Lane S, protein standards as in Fig. 2; Lanes a, b and c, identification patterns of unreduced CNBr products of cleavage of whole GPIIb using M4, M3 and M1 respectively; lane d, the same as lane c, but after reduction of the CNBr products. The periods of electroblotting were 3 h for lanes a, b and c and 15 min for lane d; F, electrophoresis front.

apparent molecular masses mainly 110, 105, 40/45 and 28 kDa (Fig. 4, panel B). Monoclonal antibody M4 recognizes 110, 40/45, and 28 kDa fragments after immunoelectroblotting (Fig. 5). Peptides 110 and 28 kDa were further isolated by electroelution (Fig. 5, lanes g and e) and subjected to Edman degradation. The *N*-terminal sequence obtained for the 110 kDa peptide was Ser-Gln-Phe, which corresponds in the HEL GPIIb sequence to the peptide stretch beginning at residue 17. This would imply that 110 kDa peptide, liberated from the supernatant by chymotrypsin digestion of whole platelets, contains the whole 35 kDa fragment obtained by CNBr cleavage of CM-GPIIbα, except for the *N*-terminal first 16 residues, and therefore this fragment cannot contain the half-cystine residue joining the α- and β-subunits in GPIIb. Thus the only candidate left is the 19 kDa peptide recognized by M3.

The *N*-terminal sequence analysis of the 28 kDa product shows heterogeneity in the cleavage point, Val^{115}-Leu-Glu-Lys-Thr-Glu-Glu-Ala being the major sequence, and Lys^{118}-Thr-Glu-Glu-Ala and Ala^{133}-Gln-Pro-Glu-Ser being minor ones. This, together with the amino acid composition of this product (Table 1), led us to identify it with the peptide stretch beginning at residue 115.

Further evidence on the identification of the α-chain half-cystine residue involved in the interchain bridge. When the unreduced CNBr products of cleavage of whole GPIIb were analysed by immunoelectroblotting, monoclonal antibodies M1 and M3 recognized a peptide of apparent molecular mass 20 kDa, whereas M4 recognized a peptide of 35 kDa (Fig. 6). When the reduced CNBr products were analysed, M4 still recognized the 35 kDa peptide, whereas M1 now recognized a peptide migrating with the electrophoretic front (Fig. 6). These experiments are further evidence against the 35 kDa peptide being the

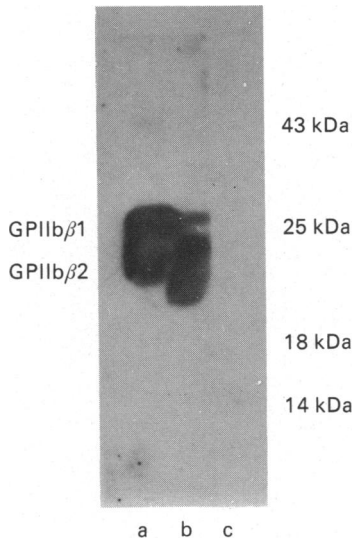


Fig. 7. Immunoelectroblotting analysis for the localization of the epitope for monoclonal antibody M1

For the chromatographic isolation of the CNBr products of cleavage of CM-GPIIbβ, see the Results section. SDS/polyacrylamide-gel electrophoresis in 12.5% polyacrylamide gels and immunoelectroblotting using M1 at 1:200 dilution and sample reduction with 1% 2-mercaptoethanol (2 min, 100 °C) were carried out as indicated in the Materials and methods section. Lane a, band 1 and band 2 of CM-GPIIbβ (Calvete & González-Rodríguez, 1986). Lane b, larger peptide fraction containing a mixture of three *N*-terminal peptide sequences: Pro-Val-Leu-Val-Ser, Ser-Ala-Pro-Cys and Val-Thr-Val-Leu-Ala. Lane c, smallest peptide fraction containing mainly a single peptide of *N*-terminal sequence Val-Thr-Val-Leu-Ala-Phe.

carrier of the half-cystine residue involved in the interchain disulphide bridge, and pointed once more to the 19 kDa CNBr product of cleavage of GPIIbα and therefore to the single cysteine residue in this fragment, which corresponds to the Cys-826 in the HEL GPIIb sequence.

Localization of the epitope for anti-GPIIbβ monoclonal antibody M1

When the products of 4 h cleavage of fully reduced and carboxymethylated GPIIbβ with CNBr were separated on a column (1 cm × 150 cm) of Sephadex G-75 in 0.1 M-phosphate/0.1% SDS buffer, pH 7.0, two fractions were obtained of apparent molecular masses 22 and 18 kDa; *N*-terminal sequence analysis showed clearly that the larger fraction is a mixture of three peptides whose most probable *N*-terminal sequences, after consideration of their apparent molecular masses, *N*-terminal sequence found here for GPIIbβ, and the cDNA predicted sequence for this subunit (Poncz *et al.*, 1987; Uzan *et al.*, 1988) were: Pro-Val-Leu-Val-Ser (residues 4–8); Ser-Ala-Pro-Cys (residues 11–14); Val-Thr-Val-Leu-Ala (residues 32–36). The smaller fraction contained mainly a single peptide whose *N*-terminal sequence (Val-Thr-Val-Leu-Ala-Phe) is the same as one of those found in the larger fraction and which corresponds to the peptide stretch beginning at residue 32 of GPIIbβ. When these two fractions were subjected to analysis by immunoelectroblotting, only the larger fraction was recognized by monoclonal antibody M1 (Fig. 7). Therefore all or part of the epitope for this antibody must be localized somewhere between Pro-4 and Met-24 (or Met-31) in the GPIIbβ sequence.

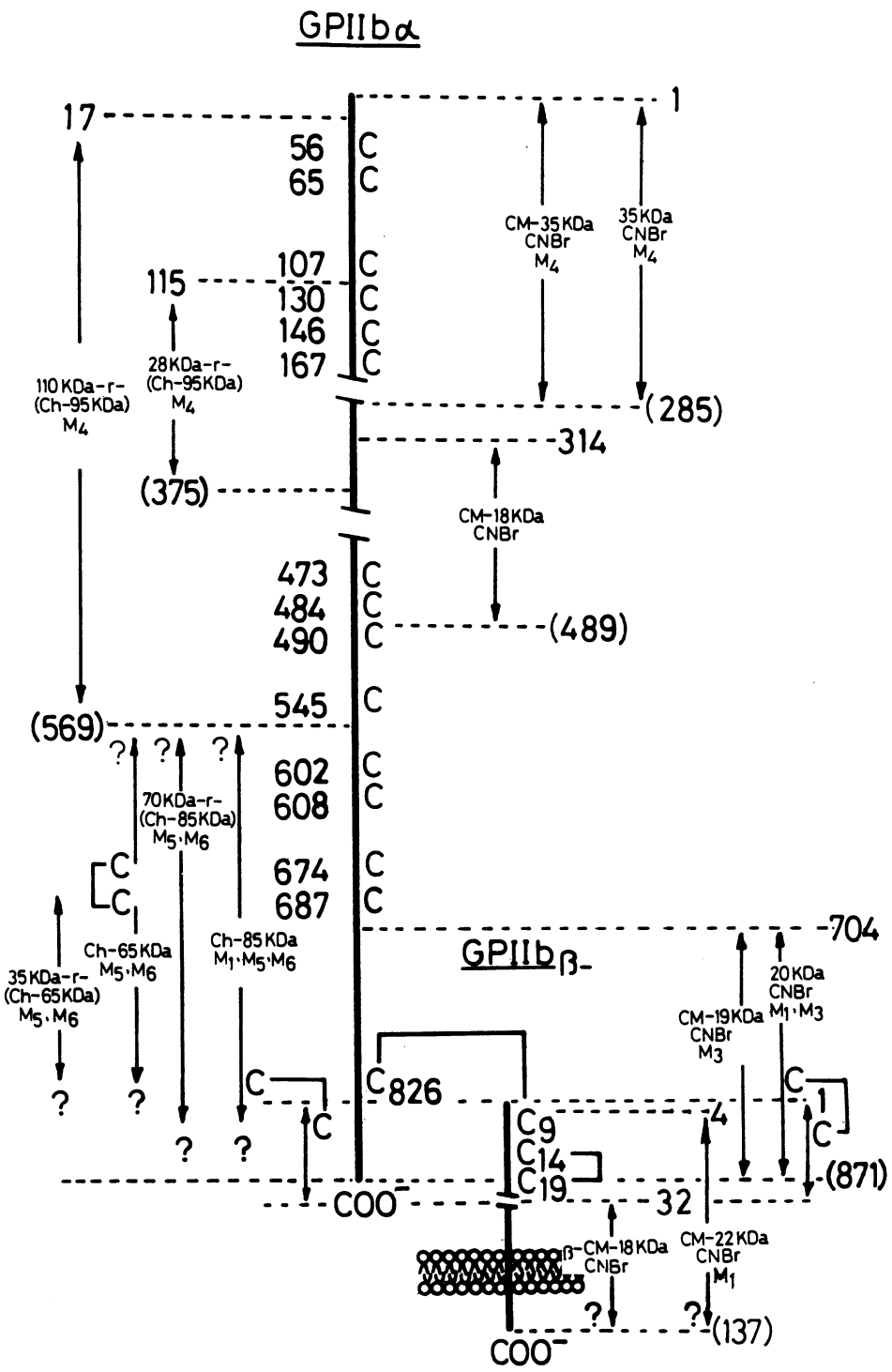
DISCUSSION

In Scheme 1, and on the basis of the amino acid sequence predicted from the cDNA sequence for HEL and megakaryocyte GPIIb (Poncz *et al.*, 1987; Uzan *et al.*, 1988) we outline the information obtained in the present work on the localization in platelet GPIIb of the interchain and intrachain disulphide bonds and of the epitopes for some monoclonal antibodies.

GPIIb interchain disulphide bond

First, we deal with disulphide-bond localization. Given that the cDNA sequence for HEL and megakaryocyte GPIIbα do not predict any transmembrane domain, it was reasonable to expect that the single disulphide bond joining the α- and β-subunits in GPIIb (Calvete & González-Rodríguez, 1986; footnote in Fitzgerald *et al.*, 1987) was extracellular. However, what it was not possible to predict was whether or not this disulphide bond was accessible to reduction from the extracellular medium, and whether or not GPIIbα would be liberated from the membrane after cleavage of the single interchain bond of GPIIb. Here we confirm our previous finding that, after its cleavage, the α-chain remains attached to the membrane and that this disulphide bond is accessible to extracellular reductants. These findings imply that, besides the interchain disulphide bridge, other non-covalent interactions of GPIIbα with other molecular components of the platelet plasma membrane (GPIIbβ, GPIIIa, lipids etc.) contribute to maintenance of the binding of this subunit to the membrane.

Several lines of evidence permit us to establish that the single interchain disulphide bridge in GPIIb is formed between GPIIbβ Cys-9 and GPIIbα Cys-826. Eosin–maleimide labelling after selective cleavage of the interchain disulphide, followed by isolation, full reduction and alkylation and *N*-terminal sequence analysis of the β-subunit, led us to conclude that Cys-9 of this subunit, the only eosin-labelled cysteine in the β-chain, is involved in the interchain disulphide bridge, whereas the two remaining cysteine residues (Cys-14 and Cys-19) form the single intrachain disulphide bond in GPIIbβ. The localization of the GPIIbα cysteine residue forming the interchain disulphide bridge was not as straightforward, because the eosin distribution (1.9 mol/mol of GPIIbα) between the CNBr cleavage fragments, mainly CM-35 kDa CNBr and CM-19 kDa CNBr (see Scheme 1), did not permit an unequivocal localization. So a set of CNBr and chymotryptic patterns of cleavage of GPIIb under different conditions had to be developed and the cleavage fragments recognized by means of monoclonal antibodies, amino acid composition and *N*-terminal sequence. In this way, fragment localization in the HEL GPIIb sequence was made by fragment *N*-terminal sequence analysis, and fragment *C*-terminal-end localization was tentatively assigned by the residue specificity of the cleavage procedure, the degree of agreement between the amino acid composition determined for the fragment and the theoretical composition of the assigned peptide stretch in HEL GPIIb sequence,



Scheme 1. Cleavage patterns and localization of disulphide bonds and epitopes for monoclonal antibodies (M1, M3, M4, M5 and M6) in human platelet GPIIb

Cleavage fragments are referred by their apparent molecular masses (kDa). The GPIIb linear amino acid sequence was taken to be identical with the cDNA-deduced amino acid sequence for HEL GPIIb (Poncz *et al.*, 1987). Abbreviations: CM- before CNBr fragments means cleavage done on the fully reduced and carboxymethylated subunit; CNBr fragments without CM-, cleavage done on unmodified GPIIb; Ch- before fragment, chymotryptic cleavage in whole platelet; r- after fragment, peptide obtained after reduction of the chymotryptic product in parentheses; terminal end positions of cleavage fragments which have not been determined by sequence analysis are in parentheses; C, cysteine.

and the fragment apparent molecular mass. This strategy permitted us to exclude CM-35 kDa CNBr fragment (residues 1–285) and to identify CM-19 kDa CNBr fragment (residues 704–871) as the carrier of the cysteine forming the interchain bridge. This peptide stretch contains a single cysteine residue, both in the HEL and in the megakaryocyte GPIIb sequence (Poncz *et al.*, 1987; Uzan *et al.*, 1988) and therefore we could conclude that Cys-826 is the cystine residue which forms the interchain disulphide bridge with Cys-9 in GPIIbβ.

Intrachain disulphide bonds in GPIIbα

At least four main peptide stretches non-disulphide-bonded among themselves can be outlined in GPIIbα (Scheme 1). The *N*-terminal stretch is represented by the 35 kDa CNBr peptide and contains the first six cysteine residues. A central region, containing four cysteine residues, stretches from Met-285 to the chymotryptic cleavage point of GPIIb in whole platelets; that is, the *C*-terminal end of Ch-95 kDa soluble peptide (tentatively assigned to Leu-569). A further stretch from Asn-570 up to Met-703 contains four cysteine residues. Finally, the *C*-terminal stretch, from Leu-704 up to the *C*-terminal end of GPIIbα, has within it Cys-826 only. Given the structural similarity of the two-chain α-subunits found so far among Arg-Gly-Asp adhesion receptors, such as platelet GPIIb–GPIIIa, vitronectin and fibronectin membrane receptors (Poncz *et al.*, 1987; Uzan *et al.*, 1988), the intrachain and interchain disulphide-bond pattern outlined here for platelet GPIIb will most probably be conserved in other cytoadhesins.

Monoclonal-antibody epitopes

The localization of monoclonal-antibody epitopes is outlined in Scheme 1. The M1 epitope is linear and it is localized somewhere between Pro-4 and Met-24 (or Met-31) of GPIIbβ, as found in the 20 kDa CNBr peptide from whole GPIIb and by CNBr cleavage of CM-GPIIbβ. The epitope for M3 is also linear and it must be localized somewhere between Cys-826 and the *C*-terminal end of GPIIbα, because this antibody recognizes both the 20 kDa CNBr and CM-19 kDa CNBr fragments, but not the Ch-85 kDa peptide, which contains the *N*-terminal part of the CM-19 kDa CNBr fragment, at least up to Cys-826. M4 also has a linear epitope which is located somewhere between residues 115 and 285 of GPIIbα, given the peptide recognition pattern of this antibody [35 kDa CNBr; CM-35 kDa CNBr; 110 kDa-r(Ch-95) kDa); and 28 kDa-r(Ch-95 kDa)]. The epitopes for M5 and M6 are located in the 35 kDa-r(Ch-65 kDa) fragment, somewhere between Cys-608 (or Cys-687) and Met-704. Recently Loftus *et al.* (1987) determined the epitope localization for PMI-1, a monoclonal antibody which inhibits platelet adhesion to collagen and whose epitope exposure is increased by the interaction of GPIIb–IIIa complex with Arg-Gly-Asp-containing ligands and suppressed by millimolar concentrations of Ca^{2+} or Mg^{2+}. This epitope is situated between residues 844 and 860 of GPIIb and must therefore be very close to the M3 epitope (residues 827–871). Lastly, comparison of the chymotryptic pattern of digestion of whole platelets obtained by Kieffer *et al.* (1984) with ours led us to conclude that their 76 and 60 kDa products should be equivalent to our Ch-85 kDa and CH-65 kDa membrane-bound fragments, and, therefore, that the Bak (Lek) alloantigen is most probably situated in the *C*-terminal half of GPIIbα in the same peptide that carries the M5 and M6 epitopes.

Finally, all the *N*-terminal amino acid sequences determined so far by us for GPIIbα, GPIIbβ and the cleavage fragments derived from them are identical with those determined previously (Charo *et al.*, 1986) or predicted by Poncz *et al.* (1987) and Loftus *et al.* (1987) for HEL GPIIb and by Uzan *et al.* (1988) for HEL and megakaryocyte GPIIb.

In the following paper (Calvete *et al.*, 1989) we deal with the complete localization of the seven intrachain disulphide bonds and the *N*-glycosylation points in GPIIbα. For the identification of the complete covalent structure of GPIIb, there are a number of features which remain to be determined. Among them: the localization of the *O*-glycosylation points, as well as the sugar sequences of the oligosaccharides; the molecular basis for the heterogeneity of GPIIb, which is already known from the existence of alloantigens localized in the α-subunit (Kieffer *et al.*, 1984), and from the heterogeneity of GPIIbβ (Calvete & González-Rodríguez, 1986; Loftus *et al.*, 1988); and the extent of identity among platelet GPIIb, megakaryocyte and HEL GPIIb, and the α-subunit of other cytoadhesins (Ginsberg *et al.*, 1988).

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REFERENCES

Bray, P. F., Rosa, J-P., Lingappe, V. R., Kan, Y. W., McEver, R. P. & Shuman, M. A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1480–1484

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

Calvete, J. J., Alvárez, M. V. & González-Rodríguez, J. (1986) in Monoclonal Antibodies and Human Blood Platelets (McGregor, J. L., ed.), pp. 179–190, Elsevier, Amsterdam

Calvete, J. J., Rivas, G., Maruri, M., Alvárez, 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

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

Clemetson, K. J. & McGregor, J. L. (1987) in Platelets in Biology and Pathology (MacIntyre, D. E. & Gordon, J. L., eds.), vol. 3, pp. 1–36, Elsevier, Amsterdam

Duperray, A., Berthier, R., Chagnon, E., Ryckewaert, J. J., Ginsberg, H. H., Plow, E. F. & Marguerie, G. A. (1987) J. Cell Biol. **104**, 1665–1673

Edman, P. & Henschen, A. (1975) in Protein Sequencing Determination (Needleman, S. B., ed.), pp. 232–279, Springer, Berlin

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

Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77

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

Ginsberg, M. H., Loftus, J. C. & Plow, E. F. (1988) Thromb. Haemostasis **59**, 1–6

Gross, E. & Witkop, B. (1962) J. Biol. Chem. **237**, 1856–1860

Henschen, A. (1986) in Advanced Methods in Protein Microsequence Analysis (Wittman-Liebold, B., Salnikow, J. & Erdmann, V. A., eds.), pp. 244–255, Springer, Berlin

Hew, C.-L., Wang, N. C., Yan, S., Cai, H., Sclater, A. & Fletscher, G. L. (1986) Eur. J. Biochem. **160**, 267–272
Hunkapiller, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) Methods Enzymol. **91**, 227–236
Jennings, L. K. & Phillips, D. R. (1982) J. Biol. Chem. **257**, 10458–10466
Kieffer, N., Boizard, B., Didry, D., Wautier, J.-L. & Nurden, A. T. (1984) Blood **64**, 1212–1219
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Leung, L. L. K., Kinoshita, T. & Nachman, R. L. (1981) J. Biol. Chem. **256**, 1996–1997
Loftus, J. C., Plow, E. F., Frelinger III, A. L., D'Souza, S. E., Dixon, D., Lacy, J., Sorge, J. & Ginsberg, M. H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7114–7118
Loftus, J. C., Plow, E. F, Jennings, L. K. & Ginsberg, M. H. (1988) J. Biol. Chem. **263**, 11025–11028
Marguerie, G. A., Ginsberg, M. H. & Plow, E. F. (1987) in Platelets in Biology and Pathology (MacIntyre, D. E. & Gordon, J. L., eds.), vol. 3, pp. 95–125, Elsevier, Amsterdam
Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) Anal. Biochem. **87**, 206–210
McEver, R. P., Baenziger, J. U. & Majerus, P. W. (1982) Blood **59**, 80–85
Melero, J. A. & González-Rodríguez, J. (1984) Eur. J. Biochem. **141**, 421–427
Nurden, A. T. & Caen, J. P. (1974) Br. J. Haematol. **28**, 253–260
Phillips, D. R., Jenkins, C. S. P., Lüscher, E. F., Larrieu, M. J. (1975) Nature (London) **257**, 599–600
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
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Usobiaga, P., Calvete, J. J., Saíz, J. L., Eirín, M. T. & González-Rodríguez, J. (1987) Eur. Biophys. J. **14**, 211–218
Uzan, G., Frachet, P., Lajmanovich, A., Prandini, M.-H., Dernarier, E., Duperray, A., Loftus, J., Ginsberg, M., Plow, E. & Marguerie, G. (1988) Eur. J. Biochem. **171**, 87–93
Zahler, W. L. & Cleland, W. W. (1968) J. Biol. Chem. **243**, 716–719

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