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Assignment of disulphide bonds in human platelet GPIIIa

A disulphide pattern for the β-subunits of the integrin family

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Integrins are cell-surface heterodimers formed by the association of one α- and one β-subunit. Glycoprotein IIIa (GPIIIa or β3 subunit) is the common β-subunit of the β3 subfamily of integrins, which, when associated with glycoprotein IIb (GPIIb), constitutes the receptor for fibrinogen and other adhesive proteins at the platelet surface (the GPIIb–IIIa complex) and, when associated with the α_v subunit, constitutes the vitronectin receptor present in several cell types. Protein chemical analysis of GPIIIa allows us to define the following structural domains: the cysteine-rich and proteinase-resistant *N*-terminal domain (GPIIIa 1–62); the adhesive-protein-binding domain (GPIIIa 101–422); the cysteine-rich and proteinase-resistant core (GPIIIa 423–622); and the *C*-terminal domain comprising an extracellular subdomain (GPIIIa 623–692), a transmembrane subdomain (GPIIIa 693–721), and a cytoplasmic subdomain (GPIIIa 722–762). We also assign unambiguously the disulphide bonds within the *N*-terminal, the fibrinogen-binding and the *C*-terminal domains, and the two long-range disulphide bonds which join the *N*-terminus to the proteinase-resistant core (Cys^5–Cys^{435}) and the fibrinogen-binding domain to the extracellular side of the *C*-terminal domain (Cys^{406}–Cys^{655}). In addition, we propose three alternative models for the arrangement of the disulphide bonds within the core and of the disulphide bonds joining the core to the extracellular side of the *C*-terminal domain, consistent with our experimental findings, favouring temporarily that which imposes less steric hindrance for the formation of these disulphide bonds.

On the basis of this information and on the highly conserved overall structure observed in the β-subunits of the integrin family known so far, except in β4, we propose to extend the cysteine-pairing pattern and the structural domains outlined here for GPIIIa to all the β-subunits of the integrin family.

INTRODUCTION

The term 'integrin' has recently been coined for the members of a family of cell-adhesion receptors functionally implicated in cell–substratum and cell–cell interactions and, therefore, in biological processes so diverse as haemostasis, embryogenesis, immunological recognition, wound healing, and tumour metastasis (Hynes, 1987). Individual integrins, expressed at the cell surface and consisting of heterodimers of non-covalently associated α- and β-subunits, have been divided into subfamilies based on the presence of distinct, but sequence-related, β-subunits (β1, β2, β3, β4 and β5) (Hynes, 1987; Rouslahti & Pierchbacher, 1987; Kajiji *et al.*, 1989; Ramaswamy & Hemler, 1990). Each β-subunit can associate with one of a number of α-subunits, enlarging the diversity of the integrin family further. Two members comprise, so far, the β3 subfamily (Fitzgerald *et al.*, 1987), the platelet GPIIb–IIIa complex and the vitronectin receptor, expressed, among others, by endothelial and melanoma cells (Pytela *et al.*, 1985; Cheresh, 1987; Cheresh & Spiro, 1987).

The GPIIb–IIIa complex, a Ca^{2+}-dependent heterodimer formed by glycoproteins IIb (GPIIb) and IIIa (GPIIIa or β3), is the major integrin at the platelet surface, which, after platelet activation, serves as the main fibrinogen receptor as well as the receptor for other adhesive proteins and, therefore, plays a primary role in platelet adhesion and aggregation (Bennett *et al.*, 1982; Nachman & Leung, 1982; Ruggeri *et al.*, 1983; Plow *et al.*, 1985; Thiagarajan & Kelly, 1988). In the last few years, the cDNA-derived amino acid sequence has been determined for most of the α- and β-subunits of the integrin family, among them GPIIb and GPIIIa (Fitzgerald *et al.*, 1987; Poncz *et al.*, 1987; Rosa *et al.*, 1988; Heidenreich *et al.*, 1990; Zimrin *et al.*, 1990). GPIIb (136 kDa) is a platelet-specific, bitopic, disulphide-bonded two-chain α-subunit whose covalent structure is mainly known (Calvete *et al.*, 1989; Calvete et al., 1990*a*,*b*), and whose topography is beginning to be revealed (Lam *et al.*, 1989; Calvete *et al.*, 1991*a*). GPIIIa (92 kDa) is a bitopic single-chain β-subunit, highly cross-linked by 28 disulphide bonds, whose covalent structure is mostly unknown and whose topography at the *N*-terminal region is becoming known (Calvete *et al.*, 1988; D'Souza *et al.*, 1988; Beer & Coller, 1989; Niewiarowski *et al.*, 1989; Pasqualini *et al.*, 1989; Calvete *et al.*, 1991*b*). Taking all this information together we have been able to outline four main structural domains in GPIIIa: the *N*-terminal cysteine-rich domain; the fibrinogen-binding domain, comprising at least the GPIIIa 100–348 sequence stretch; the cysteine-rich proteinase-resistant core, which is bound to the *N*-terminal domain by a single disulphide bond; and the *C*-terminal domain, comprising at least the transmembrane and the short cytoplasmic subdomains.

In the present work, and using protein chemical methods, we have been able to define more precisely the limits of the four structural domains referred to above and to assign unambiguously the disulphide bonds within the *N*-terminal, the fibrinogen-binding and the *C*-terminal domains, and the two long-range disulphide bonds which join the *N*-terminal domain to the *N*-terminal side of the proteinase-resistant core, and the *C*-terminal side of the fibrinogen-binding domain to the extracellular side of the *C*-terminal domain of GPIIIa respectively. In addition, we propose a cysteine-pairing pattern for the proteinase-resistant core and for the disulphide bonds joining the

Abbreviations used: GPIIb and GPIIIa, platelet glycoproteins IIb and IIIa; CM-GPIIIa, fully reduced and carboxymethylated GPIIIa; Tos-Phe-CH_2Cl-trypsin, tosylphenylalanylchloromethane('TPCK')-treated trypsin; TFA, trifluoroacetic acid.

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core to the extracellular side of the *C*-terminal domain of GPIIIa. On the basis of this information we outline a disulphide-bond pattern that may be extensible for all the β-subunits of the integrin family.

MATERIALS AND METHODS

Materials

Chymotrypsin and tosylphenylalanylchloromethane-treated trypsin (Tos-Phe-CH_2Cl-trypsin) and thermolysin were from Sigma (St. Louis, MO, U.S.A.), endoprotease Lys-C and endoprotease Glu-C (*Staphylococcus aureus* V8) were from Boehringer-Mannheim (Mannheim, Germany) and Miles (Elkhart, IN, U.S.A.) respectively, and eosin-5-maleimide and eosin-5-iodoacetamide were from Molecular Probes (Eugene, OR, U.S.A.). 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 GPIIIa and the fully reduced and carboxymethylated GPIIIa (CM-GPIIIa), were as described previously (Eirin *et al.*, 1986).

Analytical methods

Protein assay (Markwell *et al.*, 1978), amino acid and amino sugar analyses, SDS/PAGE (Laemmli, 1970), peptide blotting from SDS/polyacrylamide gels into polyvinylidene difluoride membranes (Matsudaira, 1987) were carried out as described previously (Calvete *et al.*, 1991*a*). *N*-Terminal sequence analyses were effected either in a prototype automated spinning-cup sequencer (Edman & Henschen, 1975) or in an Applied Biosystems 470 gas-phase sequencer, and the phenylthiohydantoin derivatives of the amino acids were analysed by reverse-phase h.p.l.c. (Henschen, 1986).

Early digestion of GPIIIa with trypsin

GPIIIa (2 mg/ml) in 50 mM-NH_4HCO_3/0.1 % (v/v) *N*-ethylmorpholine, pH 8.0 (NH_4HCO_3 buffer) was treated with Tos-Phe-CH_2Cl-trypsin at an enzyme/glycoprotein ratio of 1:250 (w/w) for 45 min at 37 °C. The tryptic products were fractionated on a Sephacryl S-200 column (85 cm × 1.6 cm) using 0.1 M-sodium phosphate/1 mM-EDTA, pH 6.8, containing 0.1 % SDS and 0.025 % NaN_3 (phosphate buffer) as elution buffer. The major tryptic fraction (70 kDa), after extensive dialysis against Milli Q water, was freeze-dried and further solubilized (5 mg/ml) in 50 mM-Tris/HCl/1 mM-EDTA/0.1 % SDS, pH 8.0 (Tris buffer) and reduced with dithioerythritol (0.1 mM final concn.) for 1 h at room temperature. Afterwards the thiol groups formed were alkylated with a 1.5-fold molar excess of eosin-5-maleimide over reducing agent for 15 min, at room temperature and in the dark. The alkylated sample was diluted with Milli Q water to bring the SDS concentration below its critical micellar concentration (Usobiaga *et al.*, 1987), and the unbound dye was separated by passage through a column (50 cm × 1.6 cm) of Sephadex G-50 equilibrated in 20 mM-phosphate/0.025 % SDS, pH 6.8. The eosin-labelled peptides (50 kDa and 17 kDa) were isolated on Sephacryl S-200 as described above.

The 50 kDa fragment was subsequently fully reduced with a 150-fold molar excess of dithioerythritol over its cysteine content, and the new thiol groups formed alkylated with 4-vinylpyridine. After extensive dialysis against Milli Q water, the sample was freeze-dried. Part of the sample (10 mg/ml) was cleaved with CNBr (100 mg/ml) in 70 % (v/v) formic acid, under N_2 and in the dark. After 4 h at room temperature the mixture was diluted with Milli Q water and freeze-dried. The rest of the sample was further digested with Tos-Phe-CH_2Cl-trypsin at an enzyme/peptide ratio of 1:25 (w/w) in NH_4HCO_3 buffer for 16 h at 37 °C, and the tryptic products were isolated by reverse-phase h.p.l.c. on a C4 (pore size 30 nm, particle size 10 μm) Vydac column (25 cm × 0.6 cm) equilibrated in a mixture of 0.1 % trifluoroacetic acid in water (A) and 0.1 % trifluoroacetic acid (TFA) 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.

The 17 kDa fragment from the Sephacryl 200 column was further digested with Tos-Phe-CH_2Cl-trypsin at an enzyme/substrate ratio of 1:25 (w/w) for 16 h at 37 °C, and the tryptic products isolated by reverse-phase h.p.l.c. on a C_{18} (pore size 30 nm, particle size 10 μm) Vydac column (25 cm × 0.46 cm) equilibrated in a TFA/water/acetonitrile mixture (80 % A/20 % B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 50 % B in 80 min. Fractions T6 and T7 from this column were successively freeze-dried, solubilized in 10 mM-triethylamine, pH 9.0, reduced with 1 % (v/v) 2-mercaptoethanol at 100 °C for 2 min, alkylated with a 5-fold molar excess of 4-vinylpyridine over 2-mercaptoethanol, freeze-dried, solubilized in NH_4HCO_3 buffer and digested with Tos-Phe-CH_2Cl-trypsin at an enzyme/peptide ratio of 1:50 (w/w) for 16 h at 37 °C. The tryptic products were isolated on a C_{18} Vydac column equilibrated in a TFA/water/acetonitrite mixture (80 % A/10 % B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 25 % of B in 25 min, and then up to 50 % of B in 75 min.

Late digestion of GPIIIa with trypsin

GPIIIa (5 mg/ml) in NH_4HCO_3 buffer was treated with Tos-Phe-CH_2Cl-trypsin at an enzyme/glycoprotein ratio of 1:50 (w/w) for 16 h at 37 °C. The late tryptic products were isolated either on a Sephacryl S-200 column, as described above, or on a C_{18} Vydac column equilibrated in a TFA/water/acetonitrile mixture (90 % A/10 % B) eluted at 1 ml/min, first isocratically for 10 min, followed by a linear gradient up to 40 % B in 110 min, and then up to 70 % B in 30 min.

The main fraction (35 kDa tryptic fragment) obtained on the Sephacryl S-200 column was extensively dialysed against Milli Q water and freeze-dried. Part of this fraction (0.5 mg) was solubilized in 10 mM-triethylamine, pH 9.0, reduced with 1 % 2-mercaptoethanol for 2 min at 100 °C, alkylated with a 5-fold molar excess of 4-vinylpyridine over reductant, and either fractionated on a TSK-2000 SW (600 mm × 7.5 mm) column equilibrated and eluted with phosphate buffer, or on a C_{18} Vydac column equilibrated in TFA/water/acetonitrile mixture (90 % A/10 % B), and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 50 % B in 85 min. Alternatively, the main fraction (35 kDa tryptic fragment) obtained on the Sephacryl S-200 column, once dialysed and freeze-dried, was digested with Tos-Phe-CH_2Cl-trypsin in NH_4HCO_3 buffer at an enzyme/peptide ratio of 1:25 (w/w) for 16 h at 37 °C. The tryptic products were fractionated on a Sephacryl S-200 column as described above. The main fraction (30 kDa fragment) was fully reduced, alkylated, and fractioned either on a TSK-2000 SW column or on a C_{18} Vydac column, as described above for the preparation and isolation of the full reduction and alkylation products of the 35 kDa tryptic fragment.

Chymotryptic digestion of GPIIIa

GPIIIa (5 mg/ml) in 0.1 M-Tris/HCl, pH 7.6, was digested with chymotrypsin at an enzyme/glycoprotein ratio of 1:10 (w/w) for 24 h at 37 °C, and the chymotryptic products fractionated on a Sephacryl S-200 column as described above. The main fraction (35 kDa chymotryptic fragment) was further fully reduced, alkylated and fractionated, either on a TSK-2000 SW column or on a C_{18} Vydac column, as described above

for the full reduction and alkylation products of the 35 kDa tryptic fragment.

Sequential digestion of GPIIIa with endoprotease Glu-C (*Staph. aureus* V8 proteinase) and trypsin

GPIIIa (5 mg/ml) in 50 mM-phosphate buffer, pH 8.0, was digested with V8 proteinase at an enzyme/glycoprotein ratio of 1:50 (w/w) for 18 h at 37 °C, and the digestion products fractionated on a Sephacryl S-200 column, as described above. The main fraction (35 kDa V8-proteinase-resistant fragment) was extensively dialysed against Milli Q water and freeze-dried. Part of this fraction was reduced, alkylated and fractionated as described above for the 35 kDa tryptic product of GPIIIa. Alternatively, the 35 kDa V8-proteinase-resistant fragment was solubilized in NH_4HCO_3 buffer and treated with Tos-Phe-CH_2Cl-trypsin at an enzyme/peptide ratio of 1:50 (w/w) for 20 h at 37 °C and the tryptic products isolated on a C_{18} Vydac column equilibrated in a TFA/water/acetonitrile (90% A/10% B) mixture and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 50% of B in 85 min.

Tryptic digestion of partially reduced GPIIIa

GPIIIa (5 mg/ml) in 50 mM-NH_4HCO_3/0.1% SDS, pH 8.0, was reduced with dithioerythritol at 1 mM final concentration, for 1 h at room temperature. The thiol groups formed were alkylated with a 1.5-fold molar excess of eosin 5-maleimide over reductant, for 15 min in the dark. The labelled glycoprotein, once diluted with Milli Q water, was freed from unbound eosin by passage through a Sephadex G-50 column (see above). A portion of the labelled glycoprotein (5 mg/ml) was dissolved in the initial buffer, and fully reduced with 1% 2-mercaptoethanol for 2 min at 100 °C and alkylated with 4-vinylpyridine and, after extensive dialysis against Milli Q water, freeze-dried, dissolved in NH_4HCO_3 buffer and digested with Tos-Phe-CH_2Cl-trypsin at an enzyme/glycoprotein ratio of 1:25 (w/w) for 16 h at 37 °C. The tryptic products were isolated on a C_{18} (pore size 30 nm, particle size 5 μm) Lichrospher column equilibrated in a TFA/water/acetonitrile mixture (90% A/10% B) and eluted at 1 ml/min, first isocratically for 10 min, followed by a linear gradient up to 25% of B in 25 min, and then up to 50% in 70 min. The rest of the labelled glycoprotein was directly dissolved in NH_4HCO_3 buffer and digested with Tos-Phe-CH_2Cl-trypsin in the same way, and the tryptic products were isolated on a C_4 Vydac column equilibrated in a TFA/water/acetonitrile mixture (90% A/10% B) and eluted at 1 ml/min, first isocratically for 5 min, followed a linear gradient up to 40% of B in 90 min.

RESULTS AND DISCUSSION

Localization in the GPIIIa cysteine proteinase-resistant core of the peptide carrying the cysteine residue, which forms the disulphide bond which joins the core to the *N*-terminal domain

In previous work (Calvete *et al.*, 1988, 1991*b*) we had found that the *N*-terminal cysteine-rich domain of GPIIIa, comprising the first 100 residues and, therefore, containing seven cysteine residues (the 14 kDa reduction product of chymotryptic digestion of GPIIIa in whole platelets), must be bound by a single disulphide bond to a cysteine residue upstream of Gly-349.

When pure GPIIIa was digested with Tos-Phe-CH_2Cl-trypsin, as indicated in the Materials and methods section, a fragment of apparent molecular mass 70 kDa was obtained which, upon reduction, splits into two products of 50 and 17 kDa (Fig. 1*a*). The 70 kDa fragment was isolated by size-exclusion chromatography on a Sephacryl S-200 column (Fig. 1*b*) and its *N*-terminal amino acid sequence determined. Two sequences were found, namely GPNIXTTRGV and DLPEELSLSF. The first corresponds to the *N*-terminal sequence of intact GPIIIa and therefore to the 17 kDa fragment, as determined previously (Calvete *et al.*, 1988), and the second corresponds to the peptide stretch beginning at Asp[361] and therefore to the 50 kDa fragment. When the 70 kDa fragment was partially reduced with 0.1 mM-dithioerythritol, alkylated with a 1.5 molar excess of eosin-5′-maleimide over dithioerythritol, and freed from unbound eosin by gel filtration (see the Materials and methods section), a labelling ratio of 2.75 mol of eosin/mol of fragment was determined. The reduced

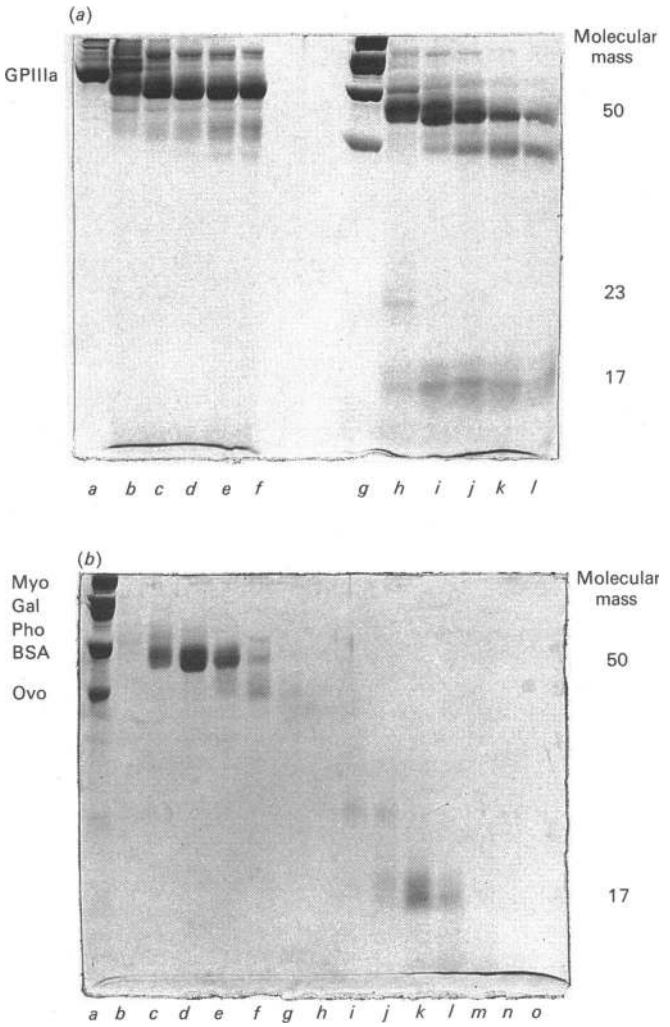

Fig. 1. Electrophoretic analysis of the early products of tryptic digestion of pure GPIIIa and their isolation by size-exclusion chromatography

GPIIIa in NH_4HCO_3 buffer was digested with Tos-Phe-CH_2Cl-trypsin at an enzyme/glycoprotein ratio of 1:250 (w/w) at 37 °C for various periods of time (see the Materials and methods section). Gels containing 15% polyacrylamide were used and stained with Coomassie Blue. (*a*) Lanes *a*, *b*, *c*, *d*, *e*, and *f*, unreduced tryptic products of GPIIIa after 0, 10, 30, 60, 120, and 180 min of digestion respectively. Lanes *h*, *i*, *j*, *k* and *l*, reduced tryptic products of GPIIIa after 10, 30, 60, 120 and 180 min of digestion respectively. Lane *g*, reduced molecular-mass markers: Myo, myosin (205 kDa); Gal, β-galactosidase (116 kDa); Pho, phosphorylase *b* (97 kDa); BSA (66 kDa); Ovo, ovalbumin (45 kDa). Sample reduction was carried out with 1% 2-mercaptoethanol for 1 min at 100 °C. (*b*) Electrophoretic analysis of the 50 kDa and 17 kDa tryptic products of 45 min digestion of GPIIIa isolated on a Sephacryl S-200 column after partial reduction with dithioerythritol and alkylation with eosin-5-maleimide (see the Materials and methods section). Lane *a*, molecular-mass markers as in Fig. (*a*). Lanes *b*–*n*, fractions along the elution profile of the Sephacryl S-200 column.

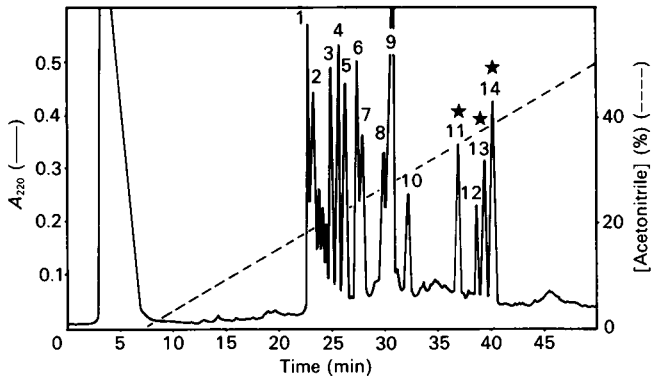

Fig. 2. Isolation by reverse-phase h.p.l.c. of the tryptic (R-) products of the fully reduced, vinylpyridine-alkylated, eosin-labelled, 50 kDa tryptic product of early digestion of GPIIIa

The fully reduced and alkylated, eosin-labelled 50 kDa tryptic product of GPIIIa, isolated as shown in Fig. 1(*b*), was digested with Tos-Phe-CH_2Cl-trypsin (see the Materials and methods section), and the tryptic products were separated on a C_4 (30 nm particle size, 5 μm pore size) Vydac column (25 cm × 0.46 cm) equilibrated in 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) (100% A/0% B), first isocratically for 5 min, followed by a linear gradient up to 70% B in 70 min. The *N*-terminal sequences of the separated fractions are given in Table 1. The stars indicate those fractions containing eosin-labelled peptides.

Table 1. *N*-Terminal sequence of the tryptic fragments of the eosin-labelled 50 kDa tryptic product of GPIIIa

The Table shows the *N*-terminal sequences of the tryptic fragments (R1–R14) of the fully reduced, 4-vinylpyridine-alkylated, eosin-labelled 50 kDa tryptic product of GPIIIa, isolated as shown in Fig. 2, and numbered according to their order of elution from the C_4 reverse-phase column. On the right-hand side of the Table is the numbering in the cDNA sequence of the terminal residues of each peptide ('cDNA sequence'), assigned after amino- acid- and *N*-terminal-sequence analysis.

Fragment	Sequence	cDNA sequence
	E G Q P V	490–498
R1		
	G C P Q E	405–410
R2	D A V X C T Y	651–658
R3	C N N G X G T	448–461
R4	F Q Y Y E	667–676
R5	C P T C P D A C T	601–612
R6	Y C E C D D F	520–530
R7	C E C G S C V	581–600
R8	G E C L C G Q	499–515
R9	C G P G W L G	462–489
R10	S F T I K P V	413–422
R11	S X M G L	385–390
R12	S I L Y V V E	677–689
R13	D S L I V Q V	423–447
R14	D L P E E L S L	361–384

and alkylated products were isolated on Sephacryl S-200, and the labelling ratios determined were 1.3 and 1.8 mol of eosin/mol of 50 and 17 kDa products respectively. The eosin-labelled 50 kDa product was fully reduced with dithioerythritol and further alkylated with 4-vinylpyridine; the fully alkylated product was cleaved with CNBr, analysed by SDS/PAGE electrophoresis and viewed under u.v. light. A main fluorescent band of 24 kDa was observed, whose *N*-terminal amino acid sequence, determined after being transferred to a polyvinylidene difluoride membrane, was ^{388}GLKIG, which suggested that the cysteine residue we were looking for was between Met^{387} and Met^{535}.

On the other hand, when the fully reduced and vinylpyridine-alkylated eosin-labelled 50 kDa product was further digested with Tos-Phe-CH_2Cl-trypsin and the tryptic products isolated by reverse-phase h.p.l.c. (Fig. 2) and identified by amino acid and *N*-terminal sequence analyses (Table 1), all the theoretically expected peptides containing cysteine, between Met^{387} and Met^{535}, were recovered. Only one among them, R 13, identified with the peptide stretch 423–447, contained eosin; therefore any of the three cysteine residues in this sequence must be bound to any of the seven cysteine residues in the *N*-terminal domain.

To find out which of those three cysteine residues is labelled with eosin, and therefore is involved in the long-range disulphide bond, 17 Edman degradation cycles were performed to fraction R 13; the following *N*-terminal sequence was obtained:

GPIIIa 423 D S L I V Q V T F D C D X A C Q A
| |
Pyr Pyr

which showed unambiguously *S*-β-4-pyridylethylcysteine residues (C-Pyr) in positions 433 and 437, and a gap in position 435; thus Cys^{435} is the cysteine residue that we were looking for. It will be shown below that the other two eosin-containing fractions, R 11 (385–390) and R 14 (361–384), are disulphide-bonded between themselves.

Assignment of the Cys^5–Cys^{435} disulphide bond

The eosin-labelled 17 kDa product, isolated by size-exclusion chromatography after partial reduction of the 70 kDa tryptic fragment of GPIIIa (see above), was further digested with Tos-Phe-CH_2Cl trypsin, at an enzyme/substrate ratio of 1:25 (w/w), for 16 h at 37 °C (see the Materials and methods section), and the tryptic products were isolated by reverse-phase h.p.l.c. (Fig. 3). Among the isolated fractions, only two contained eosin, namely T6 and T7. Amino acid composition, eosin labelling and *N*-terminal-sequence analyses showed that fractions T6 and T7

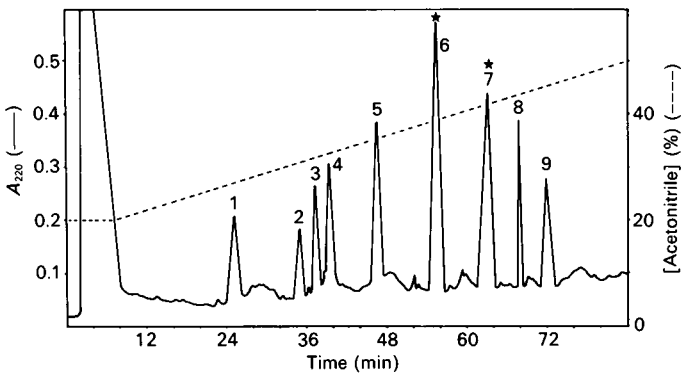

Fig. 3. Isolation, by reverse-phase h.p.l.c., of the tryptic products (T-) of the eosin-labelled 17 kDa tryptic product of early digestion of GPIIIa

The eosin-labelled 17 kDa tryptic product of early digestion of GPIIIa, isolated as shown in Fig. 1(*b*), was digested with Tos-Phe-CH_2Cl-trypsin (see the Materials and methods section), and the tryptic products (0.4 mg) were separated on a C_{18} (30 nm particle size, 10 μm pore size) Vydac column (25 cm × 0.46 cm) equilibrated in 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) (80% A/20% B) and eluted at 1 ml/min, first isocratically for 5 min, followed by a linear gradient up to 50% B in 80 min. The stars indicate those fractions containing eosin-labelled peptides.

contained the same peptide (the GIIIa 1–62 sequence) and differed in the relative peptide content (1 and 1.5) and in the eosin-labelling ratio (1.1 and 1.8 mol eosin/mol of peptide respectively), which is in good agreement with the 1.8 labelling ratio of the 17 kDa peptide found above. The high proteolytic resistance of the GPIIIa 1-62 sequence, even after partial reduction and alkylation, is remarkable.

When fractions T6 and T7 were fully reduced with dithioerythritol, alkylated with vinylpyridine and digested further with Tos-Phe-CH_2Cl-trypsin, at an enzyme/substrate ratio of 1:50 (w/w), for 16 h at 37 °C (see the Materials and methods section), the tryptic products were isolated by reverse-phase HPLC on a C_{18} column. A single eosin-labelled tryptic product was isolated from T6, the *N*-terminal sequence and amino acid analyses of which allowed us to identify it as:

GPIIIa 1–8 GPNIXTT(R)

which indicates that the first cysteine residue in the amino acid sequence of GPIIIa, Cys^5, is the cysteine we were looking for, and therefore the one which, together with Cys^{435}, disulphide-bonds the *N*-terminal domain of GPIIIa to the *N*-terminal side of the proteinase-resistant core.

Cysteine pairing at the *N*-terminal domain of GPIIIa

Three eosin-labelled tryptic products were isolated from fraction T7, whose *N*-terminal sequence and amino acid analyses correspond, we believe, to:

GPIIIa 1–8 GP NI XTT (R)
GPIIIa47–62 DNXA P E...
GPIIIa 9–37 GVS S CQQ X LAV...
Pyr

which indicates that Cys^{49} and Cys^{16} are eosin-labelled, whereas Cys^{13} and Cys^{38} are not. Therefore Cys^{49} could be bound to either of the three remaining cysteine residues in the GPIIIa 1–62 sequence: Cys^{16}, Cys^{23} and Cys^{26}.

To be able to assign the actual pairing of these residues, GPIIIa was digested with chymotrypsin at an enzyme/substrate ratio of 1:10, for 24 h at 37 °C (see the Materials and methods section), and the chymotryptic products analysed by SDS/PAGE. A product of apparent molecular mass 35 kDa (30 kDa under reduced conditions) is obtained that, after isolation on a Sephacryl S-200 column, was found to contain five different *N*-terminal sequences. When the fully reduced and alkylated 35 kDa chymotryptic product was fractionated by size-exclusion chromatography on a TSK-2000 column, a main fraction was obtained with the *N*-terminal sequence KDSLIVQVTFD... that, after amino acid analysis, was identified with the GPIIIa 422–625 sequence stretch. When the reduced and alkylated 35 kDa chymotryptic product was alternatively fractionated by reverse-phase h.p.l.c. on a C_{18} column, four fractions were isolated with the following *N*-terminal sequences:

GPIIIa	1	GP NI XTTRGV...
GPIIIa	46	KDNXAPESI E
GPIIIa	626	HDENTXN...
GPIIIa	681	VVEEPEXP...

which, after amino acid analysis, were identified with the GPIIIa sequences 1–25, 46–64, 626–634 and 681–694. This indicates that these four peptides are disulphide-bonded to the 422–625 sequence stretch, which is the proteinase-resistant core of GPIIIa. This means that Cys^{26} and Cys^{38} are disulphide-bonded between themselves, and, therefore, that the disulphide-bond assignment at the *N*-terminal cysteine-rich domain of GPIIIa is as follows: Cys^5–Cys^{435} (eosin-labelled); Cys^{13}–Cys^{23}; Cys^{16}–Cys^{49} (eosin-labelled); and Cys^{26}-Cys^{38} (Fig. 4). A similar disulphide-bond arrangement has recently been found at the III-T2 fragment of the Von Willebrand factor (Marti *et al.*, 1987).

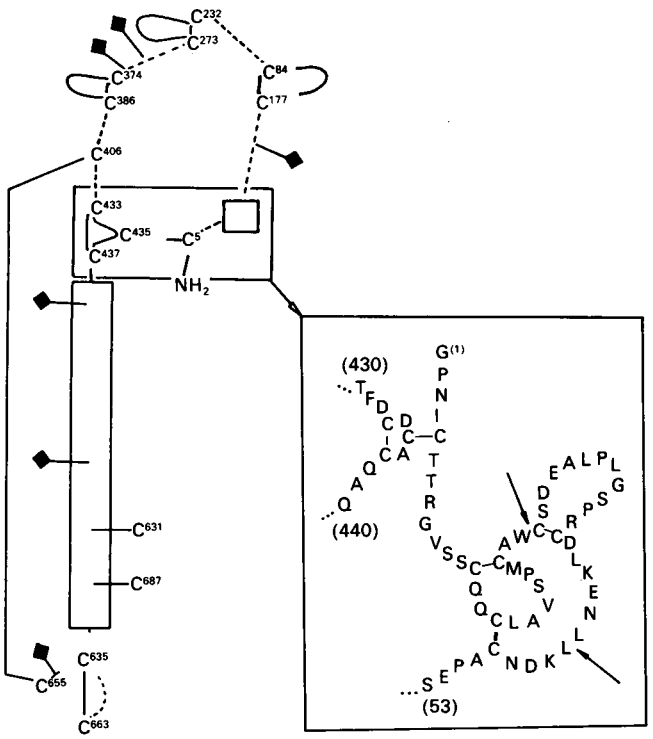

Fig. 4. Summary of the unambiguously assigned disulphide bonds in human platelet GPIIIa

It is shown the cysteine-pairing within the *N*-terminal domain (enlarged and detailed on the right-hand side), the putative fibrinogen-binding domain and the *C*-terminal domain, as well as the two long-range disulphide bridges joining the *N*-terminal domain to the proteinase-resistant core (represented as a rectangle) and the putative fibrinogen-binding domain to the *C*-terminal domain respectively. ◆–, *N*-glycosylation points; →, chymotryptic cleavage points within the *N*-terminal domain.

Cysteine pairing at the fibrinogen-binding domain and at the extracellular side of the *C*-terminal domain of GPIIIa

To assign the disulphide bonds in these domains, GPIIIa was digested with Tos-Phe-CH_2Cl-trypsin, at an enzyme/substrate ratio of 1:50, for 16 h at 37 °C and pH 8.0. Under these conditions a large fragment of apparent molecular mass 35 kDa was obtained (see below) and a set of short tryptic peptides, which were analysed by reverse-phase h.p.l.c. on a C_{18} column (Fig. 5). Amino acid and *N*-terminal sequence analyses of the fraction obtained (TR1–TR21) allowed us to identify the isolated peptides in each of them (Table 2). Fractions containing cysteine-carrying peptide pairs allowed us to assign the following disulphide bonds (Fig. 4): Cys^{177}–Cys^{184} (TR18); Cys^{232}–Cys^{273} (TR19); Cys^{374}–Cys^{386} (TR21); Cys^{406}–Cys^{655} (TR17); and Cys^{635}–Cys^{663} (TR12). The first three disulphides establish the nearest-neighbour pattern of cysteine pairing in the fibrinogen-binding domain. The fourth disulphide bond, Cys^{406}–Cys^{655}, is the other long-range disulphide in GPIIIa, which joins the *C*-terminal side of the fibrinogen-binding domain to the extracellular side of the *C*-terminal domain of GPIIIa. And the fifth, Cys^{635}–Cys^{663}, is a disulphide bond within the extracellular side of the *C*-terminal domain of GPIIIa.

The 35 kDa tryptic fragment of GPIIIa (see above) was isolated by size-exclusion chromatography on a Sephacryl S-200 column

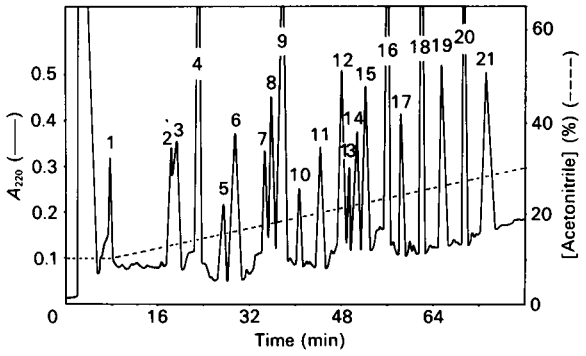

Fig. 5. Isolation by reverse-phase h.p.l.c. of the tryptic products of the late digestion of GPIIIa

GPIIIa in NH_4HCO_3 buffer was treated with Tos-Phe-CH_2Cl-trypsin for 16 h at 37 °C (see the Materials and methods section) and the late tryptic products isolated on a C_{18} Vydac column equilibrated in a mixture of 0.1 % TFA in water (A) and 0.1 % TFA in acetonitrile (B) (90 % A/10 % B), eluted at 1 ml/min, first isocratically for 10 min, followed by a linear gradient up to 40 % B in 110 min, and then up to 70 % B in 30 min. The *N*-terminal sequences of the separated fractions are given in Table 2.

Table 2. *N*-Terminal sequences of the fractions of the late tryptic digestion of GPIIIa, isolated by reverse-phase h.p.l.c.

The Table shows the *N*-terminal sequences of the tryptic fragments (TR1–TR21) of the late tryptic digestion of GPIIIa, prepared and isolated as shown in Fig. 5, and numbered according to their order of elution from the C_{18} reverse-phase column. On the right-hand side of the Table is the numbering in the cDNA sequence of the terminal residues of each peptide ('cDNA sequence'), assigned after *N*-terminal-sequence and amino-acid analysis.

Fragment	Sequence	cDNA sequence
TR1	F N E E V K	203–208
	D E I S V K	637–643
	L A T Q M R	138–143
TR2	V E L E V R	355–360
	T H I A L D G R	254–261
TR3	V L E D R P L	63–72
	F Q Y Y E D S	667–676
TR4	I G W R	236–239
TR5	L R P D D S K	92–98
TR6	X F S I Q V R	99–105
TR7	I G D T V S F	391–402
TR8	H V L T L T D Q V	192–202
TR9	W D T A N N	739–748
TR10	E A T S T F T	749–760
TR11	Q V E D Y P V D I Y	106–125
TR12	Y	634–636
	N E D D X V V	659–666
TR13	S F T I K P V G F	413–422
TR14	D D L W S I Q N L	126–137
TR15	I G D T V S F	391–402
TR16	N D A S H L L	240–253
TR17	D A V X X T Y	651–658
	G X P Q E	405–410
TR18	I G F G A F V D K P	151–181
	T T X L P M F G Y	182–191
TR19	L A G I V Q P N	262–298
	D A P E G G F D	217–235
TR20	N I N L I F A V	303–350
TR21	D L P E E L S L	361–384
	S X M G L	385–390

and after amino-acid- and *N*-terminal-sequence analyses, it was found to contain four *N*-terminal sequences. When the fully reduced and alkylated 35 kDa tryptic fragment was fractionated by size-exclusion chromatography on a TSK-2000 column, a major peptide was obtained that, after amino-acid- and *N*-terminal-sequence (DSLIVQVTFDXDXA...) analyses was identified with the GPIIIa 423–622 sequence stretch, that is, the proteinase-resistant core. When the same reduced and alkylated 35 kDa fragment was fractionated by reverse-phase h.p.l.c. on a C_{18} column, three tryptic peptides were obtained that, after amino-acid- and *N*-terminal-sequence analyses, were identified with the GPIIIa 1–62 (GPNIXTTRGVSSXQ...), 623–633 [GALHDENTXN(R)], and 677–689 [SILYVVEEPEXP(K)] sequences. This means that these three peptide stretches are disulphide-bonded to the 423–622 stretch in GPIIIa, and thus confirming the Cys^5–Cys^{435} long-range disulphide bond and showing that the extracellular side of the *C*-terminal domain is twice disulphide-bonded to the proteinase-resistant core by means of Cys^{631} and Cys^{687}. These results are very similar to those described above after chymotrypsin digestion and to those found after thermolysin or V8-proteinase digestion of GPIIIa.

A second tryptic digestion of the isolated 35 kDa tryptic fragment [trypsin/fragment ratio 1:25 (w/w) for 16 h at 37 °C] breaks it down to a 30 kDa fragment, which was isolated on a Sephacryl S-200 column and found to contain three *N*-terminal sequences. The fully reduced and alkylated 30 kDa fragment was further fractionated on a TSK-2000 column and a major peptide isolated that, after amino-acid- and *N*-terminal-sequence analyses, was identified with the GPIIIa 423–600 peptide stretch (DSLIVQVT...) and, therefore, with the proteinase-resistant core. When the fully reduced and alkylated 30 kDa fragment was alternatively fractionated by reverse-phase h.p.l.c. on a C_{18} column, two peptides were isolated that, after amino-acid- and *N*-terminal-sequences analyses, were identified with GPIIIa 1–8 [GPNIXTT(R)] and 623–633 (GALHDEN...) peptide stretches. This means that these two peptides are disulphide-bonded to the proteinase-resistant core, confirming, once more, the Cys^5–Cys^{435} long-range disulphide bond and the cross-linking of the extracellular side of the *C*-terminal domain to the core by means of Cys^{631}. Moreover, given that the GPIIIa 677–689 peptide has no trypsin cleavage point within it, its tryptic liberation from the core, during the tryptic digestion of the 35 kDa tryptic fragment, has to take place by cleavage of a peptide within the 423–622 sequence, which has an uneven number of cysteine residues and is flanked by two trypsin cleavage points. This peptide has to be the GPIIIa 601–622 stretch, which has five cysteine residues and is cleaved from the *C*-terminus of the 426–622 core at the time of the 677–689-peptide-sequence release. Therefore, one of the cysteine residues (at position 601, 604, 608, 614 or 617) in the 601–622 stretch has to be disulphide-bonded to Cys^{687} within the GPIIIa 677–689 stretch, and the rest of them must form two disulphide bonds among themselves.

Disulphide-bond assignment in the proteinase-resistant core of GPIIIa

Among several attempts to cleave the proteinase-resistant core of GPIIIa to reach full assignment of the disulphide bonds within it, two were partially successful, and were based either on sequential digestion of the core with different proteinases or on partial reduction and alkylation of GPIIIa followed by tryptic digestion. When GPIIIa was digested with V8 proteinase [V8/GPIIIa ratio 1:50 (w/w), for 18 h at 37 °C and at pH 8.0] and the core fragment isolated on a Sephacryl S-200 column, four GPIIIa sequences were found in addition to a 20 % contribution from the *N*-terminal sequence of the V8 proteinase itself. After full reduction and alkylation of the V8-resistant core, and alternative

fractionation by size-exclusion chromatography or by reverse-phase h.p.l.c., and amino-acid- and *N*-terminal-sequence analyses of the isolated products (as described above for the 35 kDa trypsin-resistant core), four peptides were identified:

GPIIIa 1–55 GP NI XTTR GV...
GPIIIa 401–409 A K V R GXP Q(E)
GPIIIa 424–683 S L I V QVTF D...
GPIIIa 684–692 E P E XP KGP (D)

The isolated V8-resistant core was further dialysed against Milli Q Water, freeze-dried, dissolved in ammonium bicarbonate buffer, and digested with TPCK-trypsin at an enzyme/substrate ratio 1:50 (w/w) for 20 h at 37 °C. The tryptic products were isolated by reverse-phase h.p.l.c., and, besides the digestion products of V8 protease and trypsin, two GPIIIa tryptic products were identified by amino acid and N-terminal sequence analyses:

GPIIIa 1–8 GP NI X T T(R)
GPIIIa 424–447 S L I VQ V T...and
GPIIIa 462–489 X GP GW L GS Q...

The first product, with two *N*-terminal sequences, as well as confirming once more the Cys^{5}–Cys^{435} disulphide bond, reveals also the Cys^{433}–Cys^{437} disulphide bond. The second product shows that the four cysteine residues within the 462–489 peptide stretch (Cys^{462}, Cys^{471}, Cys^{473} and Cys^{486}) must form two disulphide bonds among themselves.

The second attempt to cleave the proteinase-resistant core consisted, first, in a partial reduction of GPIIIa (5 mg/ml) in 50 mM-NH_4HCO_3/1 mM-dithioerythritol/0.1 % SDS, pH 8.0, for 1 h at room temperature, followed by alkylation with a 1.5 molar excess of eosin-5′-maleimide (iodoacetamide) over dithioerythritol, gel filtration on Sephadex G-50 to separate labelled GPIIIa from free dye, and dialysis against the original buffer. Then part of the sample was fully reduced with 2-mercaptoethanol, alkylated with 4-vinylpyridine, dialysed against the original buffer, and digested again with Tos-Phe-CH_2Cl-trypsin, at a trypsin/GPIIIa ratio of 1:25 (w/w) for 16 h at 37 °C. Reverse-phase h.p.l.c. peptide mapping, amino-acid- and *N*-terminal-sequence analyses, and eosin/peptide molar-ratio determination of the tryptic peptides showed that GPIIIa cysteine residues (5; 232; 273; 374; 386; 435; one among 501, 503, 506, 508; 521; one among 601, 604, 608; 614; 617; and 687) are at least partially labelled with eosin. Besides confirming previous findings, these results suggest that Cys^{521} is disulphide-bonded to any of the cysteine residues within the 501–508 stretch, because the rest of the labelled cysteine residues are either already assigned to established disulphide bonds (Cys^{5}–Cys^{435}; Cys^{232}–Cys^{273}; Cys^{374}–Cys^{386}) or have been found forming groups of disulphide pairs among themselves (Cys^{601}, Cys^{604}, Cys^{608}, Cys^{614}, Cys^{617} and Cys^{687}).

The rest of the sample of partially reduced, eosin-labelled GPIIIa was directly digested with Tos-Phe-CH_2Cl-trypsin, at a trypsin/GPIIIa ratio of 1:25 (w/w) for 16 h at 37 °C. The tryptic products were isolated by reverse-phase h.p.l.c., analysed for amino acid composition, *N*-terminal sequence and eosin/peptide molar ratio, and the following peptides were identified:

GPIIIa 520–530 Y C E X D D F S X V(R)
|
eosin

which, given that Cys^{521} is eosin-labelled (see above), indicates that Cys^{523} and Cys^{528} are most probably disulphide-bonded between themselves, as was subsequently ascertained by full reduction, alkylation with vinylpyridine and *N*-terminal-sequence analysis of this peptide:

GPIIIa 581–600 X E X G(S) X V X I Q...
623–633 GAL(H)D E NT...

which indicates that Cys^{631}, together with cysteine residues 581, 583, 586, 588 and 598, form three disulphide bonds among themselves; and:

GPIIIa 1–8 GP NI XTT(R)
423–461 DS L I VQV T FD...
490–498 E GQ P V...

which indicates that Cys^{495}, together with Cys^{448}, Cys^{457} and Cys^{460}, form two disulphide bonds among themselves, if we take into consideration previous findings that allowed to assign the Cys^{5}-Cys^{435} and Cys^{433}–Cys^{437} disulphide bonds (see above).

Summarizing the experimental data on the proteinase-resistant core, we have assigned two disulphide bonds, Cys^{433}–Cys^{437} and Cys^{523}–Cys^{528}. In the first cysteine-rich repeat of GPIIIa we have established that cysteine residues 448, 457, 460 and 495 form two disulphide bonds among themselves and that cysteine residues 462, 471, 473 and 486 are also paired among themselves. We have also shown that Cys^{521} is disulphide-bonded to any of the cysteine residues 501, 503, 506 or 508, in the second cysteine-rich repeat, and we have in addition established that, in the fourth cysteine-rich repeat, cysteine residues 581, 583, 586, 588, 598 and 631 form three disulphide bonds among themselves, and that cysteine residues 601, 604, 608, 614, 617 and 687 are also paired among themselves. In Fig. 6 the sequences of the four cysteine-

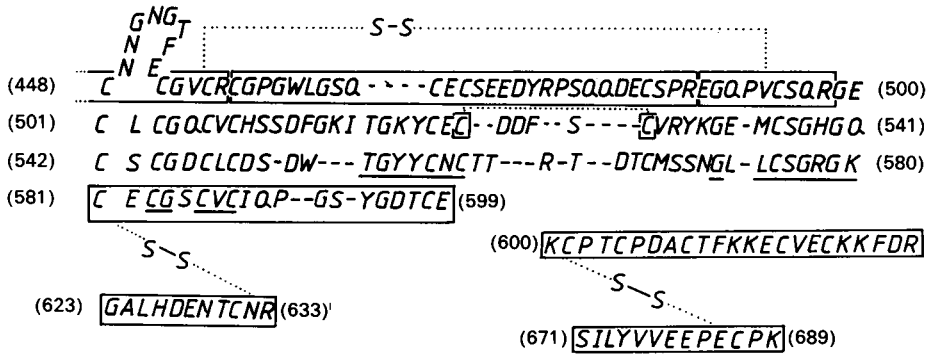

Fig. 6. Amino-acid-sequence alignment of the four cysteine-rich repeats within the proteinase-resistant core of GPIIIa

The cysteine-rich repeat sequences are aligned with their cysteine residues in register, and the highly conserved sequence stretches are underlined. Assigned disulphide bonds or groups of cysteine residues paired among themselves are boxed.

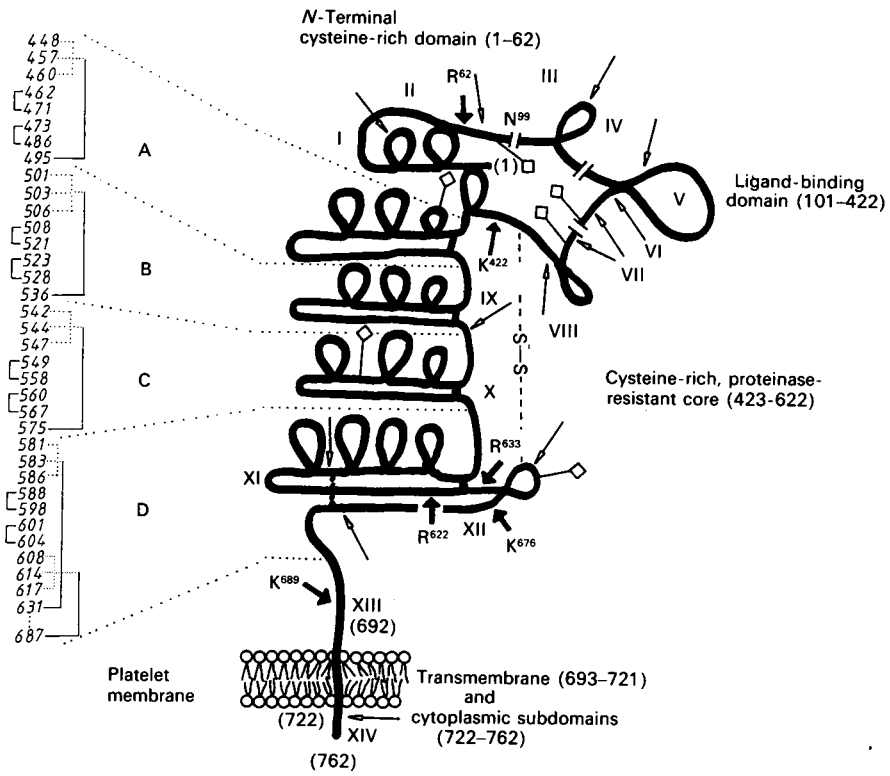

Fig. 7. Outline of the disulphide-bond pattern and main structural domains of GPIIIa

On the left-hand side is the list of the disulphide bonds assigned and the three cysteine-pairing possibilities of the first three cysteine residues in each repeat and of Cys^{687} and the last three cysteine residues of the fourth repeat, within the proteinase-resistant core, assuming that all the cysteine residues within each repeat are paired among themselves and that the pairing pattern is the same in each repeat. The assignment of the rest of the disulphides is as in Fig. 5. Symbols: ◇–, glycosylation points; →, arginine and lysine tryptic cleavage points; ⊸, exon delimitations taken from Heidenreich *et al.* (1990).

rich repeats of the proteinase-resistant core are shown, aligned with their eight cysteine residues in register, together with the experimental results found here. It can be observed that the first cysteine-rich repeat has an extra amino-acid-sequence stretch in between the first two cysteine residues (Cys^{448} and Cys^{457}) and that the first four cysteine residues of the fourth repeat are in register, whereas the last four are not. If we combine the information gathered in Fig. 6 with the assumption that all the cysteine residues within each repeat are paired among themselves, and that the pairing pattern is the same in each repeat, then the following conclusions can be drawn. The fourth cysteine residue in each repeat should be bound to the fifth, and the sixth to the seventh, being the first three cysteine residues together with the eighth paired among themselves. There are three cysteine-pairing possibilities: 1st–2nd, 3rd–8th; 1st–3rd, 2nd–8th; and 1st–8th, 2nd–3rd. In Fig. 7 we outline the disulphide-bond pattern for GPIIIa, favouring the last cysteine-pairing possibility for steric reasons, and at the side we list the three alternatives. Further experimental evidence is required to ascertain the definitive disulphide-bond pattern within each cysteine-rich repeat and the pairing of cysteine residues 608, 614, 617 and 687.

Disulphide-bond pattern for the β-subunits of the integrin family

In all known β-subunits of the intregrin family, except β4, from man down to *Drosophila*, there are certain structural and functional features which are becoming apparent and which are highly maintained overall: the size, about 760 amino acids; the single transmembrane domain; the short cytoplasmic domain of about 40–50 amino acids; the position and number of the 56 cysteine residues; the four cysteine-rich repeats; the highly similar amino acid sequences within the 100–350-peptide stretch; the location of the adhesive-protein-binding domain in the 100–350-peptide stretch, which most probably is not involved in the interaction with the corresponding subunit to form the heterodimer, the integrin; the presence of a putative tyrosine phosphorylation site in the cytoplasmic domain. So far, only one exception to this general pattern is known: the β4 subunit of a recently found integrin (Kajiji *et al.*, 1989) whose amino acid sequence has just been derived from its cDNA (Suzuki *et al.*, 1990; & Hogervost *et al.*, 1990). This β4 subunit has a molecular mass more than double that of the other β-subunits, four cysteine-rich repeats, but only 48 cysteine residues in the extracellular domain, and has a cytoplasmic domain more than twenty times the size of the cytoplasmic domain of the other β-subunits.

On the basis of this highly conserved overall structure, we propose to extend the cysteine-pairing pattern outlined here for GPIIIa (β3) (Fig. 7) to all the β-subunits of the integrin family, as well as to define in them the following domains: the *N*-terminal, cysteine-rich and proteinase-resistant domain (GPIIIa 1–62); the adhesive-protein-binding domain (GPIIIa 101–422); the cysteine-rich proteinase-resistant core (GPIIIa 423–622); and the *C*-terminal domain, comprising an extracellular side subdomain (GPIIIa 623–692), a transmembrane subdomain (GPIIIa 693–721), and cytoplasmic subdomain (GPIIIa 722–762). Finally, there is no apparent correlation between the exon boundaries in the GPIIIa gene (Heidenreich *et al.*, 1990) and the domain organization outlined here (Fig. 7).

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