Proteolytic degradation of the RGD-binding and non-RGD-binding conformers of human platelet integrin glycoprotein IIb/IIIa: clues for identification of regions involved in the receptor's activation

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The human integrin glycoprotein (GP)IIb/IIIa plays a central role in haemostasis as an inducible receptor for fibrinogen and other RGD-containing adhesive proteins at the platelet plasma membrane. Expression of the fibrinogen receptor on platelet activation involves conformational changes in the quaternary structure of GPIIb/IIIa. Little is known, however, about the nature of this conformational transition. Given that isolated GPIIb/IIIa contains a mixture of RGD-binding and non-RGD-binding heterodimers, we used limited proteolysis as a tool for investigating the structural differences between the two conformers. Comparison of their fragmentation patterns shows that, whereas in the non-RGD-binding form of GPIIb/IIIa the N-

terminal half of the heavy chain of GPIIb (GPIIbH) and the central region of GPIIIa are cleaved by endoproteinase Arg-C, these domains associate tightly with one another in the RGD-binding GPIIb/IIIa and are thus protected from proteolysis. In addition, the C-terminal half of GPIIb becomes more susceptible to degradation in the non-RGD-binding GPIIb/IIIa conformer. Our interpretation, in the context of available structural and functional data, is that a major relative reorientation of the GPIIbH and GPIIIa extracellular domains takes place along the subunit interface during the conformational transition of the platelet integrin.

INTRODUCTION

Human glycoprotein (GP)IIb/IIIa, a Ca^{2+} -dependent heterodimer (Kunicki et al., 1981), is the major integrin receptor on the platelet plasma membrane (Kieffer and Phillips, 1990). On resting platelets, GPIIb/IIIa (integrin $\alpha\text{IIb}\beta 3$) (Hynes, 1992) is maintained in an inactive conformation and serves as a low-affinity adhesion receptor for surface-coated fibrinogen (Savage et al., 1992). On platelet activation, signals within the cytoplasm alter the receptor function of GPIIb/IIIa (inside-out signalling) (O'Toole et al., 1991; Chen et al., 1992; Ginsberg et al., 1992), which undergoes a measurable conformational change (Parise et al., 1987) within its extracellular domains (Sims et al., 1991) and becomes a competent receptor for soluble fibrinogen and some other adhesive plasma proteins [reviewed by Plow and Ginsberg (1989) and Phillips et al. (1991)]. Although the C-terminal dodecapeptide of the human fibrinogen γ -chain ($\gamma^{400-411}$) seems to be the major site for interaction between activated GPIIb/IIIa and soluble fibrinogen (Kirschbaum et al., 1992; Weisel et al., 1992), GPIIb/IIIa binds to its other ligands (von Willebrand factor, fibronectin and vitronectin) via an RGD-sequence-specific mechanism (reviewed by Plow et al. 1992a).

On ligand binding, further structural alterations expose neoepitopes (termed ligand-induced binding sites) on both GPIIb (Frelinger et al., 1988) and GPIIIa (O'Toole et al., 1990; Kouns et al., 1990; Frelinger et al., 1990, 1991), and trigger the self-association of receptor-occupied GPIIb/IIIa complexes. Receptor clustering within the plane of the membrane (Isenberg et al., 1987; Kouns et al., 1991a) promotes a cascade of intracellular biochemical reactions which result in cytoskeletal rearrangement

(outside-in signalling), a common theme of many integrin receptors [reviewed by Damsky and Werb (1992) and Juliano and Haskill (1993)], and irreversible platelet aggregation (Plow et al., 1992b). The simultaneous binding of dimeric fibrinogen molecules to GPIIb/IIIa patches on adjacent platelets provides the necessary physical link for platelet aggregation and clot retraction to occur.

The human GPIIb/IIIa complex is the most thoroughly studied integrin receptor. Much of its molecular biology and major features of its primary structure have been elucidated over the last 5 years [reviewed by Calvete (1993)]. In addition, localization of functionally relevant monoclonal antibody epitopes, determination of the cross-linking sites of inhibitory peptide ligands, proteolytic dissection of the isolated integrin and analysis of natural and artificial GPIIb/IIIa mutants have recently provided a wealth of information regarding structure-function relationships of human GPIIb/IIIa [reviewed by Calvete (1993)]. However, the precise location and topography of the integrin ligand-binding pocket and the nature of the conformational alterations on the quaternary structure of GPIIb/IIIa exposing the fibrinogen-binding site and the ligand-induced neoepitopes have yet to be defined.

The present study was undertaken to compare the degradation patterns of the affinity-purified non-RGD-binding and RGD-binding isoforms of isolated detergent-solubilized GPIIb/IIIa. The rationale behind this approach is that differences in the susceptibility to limited proteolysis may aid in the demarcation of particular regions that are differently exposed on the protein surface of each of the GPIIb/IIIa conformers (see Wilson, 1991), i.e. domains the relative orientation of which is

Abbreviation used: GP, glycoprotein.

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altered during the non-binding $\rightarrow$ binding conformational-state transition.

MATERIALS AND METHODS

Peptides

Peptides KYGRGDS and GRGDS were synthesized using a semiautomated peptide synthesizer (Labortec SP 640) and the Fmoc solid-phase chemistry as described (Calvete et al., 1992a). Peptides were deprotected and released from the polymeric resin by treatment with 85% (v/v) trifluoroacetic acid/5% (v/v) thianisole for 6 h at room temperature with gentle stirring, followed by precipitation with diethyl ether and freeze-drying. The structure of each peptide was confirmed by liquid secondary-ion fast-atom-bombardment mass spectrometry using a Finnigan MAT 900 instrument. When necessary, peptides were purified by reversed-phase h.p.l.c. using a Lichrospher RP-100 (Merck) column (25 cm $\times$ 0.4 cm; 5 μ m particle size) eluted at 1 ml/min with a linear gradient of 0.1% (v/v) trifluoroacetic acid in water (A) and acetonitrile (B). U.v. detection was at 220 nm. Quantification of peptides was by amino acid analysis after hydrolysis with 6 M HCl for 24 h at 110 °C.

Preparation of the RGD-affinity matrix

NH₂-CH₂-CH₂-NH-CH₂-CH₂-agarose (10 g) containing 75 $\times$ 10⁻⁶ primary amine equivalents/ml (Hispanagar S.A., Burgos, Spain) was resuspended in 50 ml of 6% glutaraldehyde in 50 mM phosphate, pH 7.0; the pH was readjusted to 7.0, and the mixture was allowed to react with gentle stirring for 12–14 h at room temperature (Fernández-Lafuente et al., 1994). The derivatized matrix was washed exhaustively with 50 mM phosphate, pH 7.0, and resuspended in 50 ml of 0.1 M phosphate, pH 7.5, containing 100 mg of peptide KYGRGDS. The mixture was allowed to react for 2 h at room temperature with constant stirring, reduced with BH₄Na, washed with column buffer [10 mM Tris/HCl, 150 mM NaCl, 1 mM CaCl₂, 1% (w/v) *n*-octyl β -D-glucopyranoside (Sigma), pH 7.4], packed into a 15 ml column and equilibrated with the same buffer overnight.

Isolation of the RGD-binding GPIIb/IIIa fraction

GPIIb/IIIa was isolated from outdated human platelets as described (Rivas et al., 1991). Isolated GPIIb/IIIa (5 mg in 2 ml of column buffer) was applied to the RGD-affinity column, which was then washed with column buffer at a rate of 2 ml/h. The retained fraction was eluted with 1 mM GRGDS in column buffer at 0.1 ml/min. The column was then equilibrated with column buffer and the flow-through fraction rechromatographed. The retained and unretained fractions were pooled and concentrated by ultrafiltration (using an Amicon PM100 membrane) to about 2 mg/ml. Alternatively, the RGD-binding fraction was dialysed against column buffer to separate GPIIb/IIIa and GRGDS.

Proteolytic digestion and isolation of fragments

Some 2 mg of the non-RGD-binding and RGD-binding GPIIb/IIIa fractions (with or without 1 mM GRGDS peptide) in column buffer were digested with endoproteinase Arg-C (Boehringer-Mannheim) at an enzyme/GPIIb/IIIa ratio of 1:100 (w/w) at

37 °C for 1, 6 and 24 h. The digestion was stopped at the selected time by addition of a 25-fold molar excess of phenylmethanesulphonyl fluoride (Sigma). The digestion mixtures were cleared by centrifugation at 20000 *g* for 10 min, and the supernatants subjected to size-exclusion chromatography on a tandem of TSK SW 4000 and 3000 columns (0.75 cm $\times$ 60 cm each) equilibrated in column buffer. The isolated fractions were dialysed against MilliQ water and freeze-dried. For isolation of the peptides contained in each fraction, the GPIIb/IIIa fragments were dissociated with SDS [final concentration 1% (w/v)] at 60 °C for 5 min followed by size-exclusion chromatography as above but replacing 1% (w/v) *n*-octyl β -D-glucopyranoside with 0.1% SDS.

Characterization of proteolytic products

The isolated proteolytic fragments were subjected to SDS/PAGE, amino acid analysis and N-terminal sequencing as described previously (Calvete et al., 1992b). The N-terminal sequences were assigned to polypeptide stretches coded by the cDNA sequences of GPIIb (Poncz et al., 1987) and GPIIIa (Fitzgerald et al., 1987; Rosa et al., 1988). Immunochemical characterization was carried out by e.l.i.s.a. using the monoclonal antibodies P₃₇, M₁, M₂, M₃, M α ², M₄, M₅ and M₆, directed against continuous epitopes located on GPIIIa-(101–109), GPIIbL₂-(1–24), and GPIIbH-(747–755), -(849–857), -(837–843), -(143–151), -(550–558) and -(657–665) respectively (Calvete et al., 1991a,b).

RESULTS

Separation of the non-RGD-binding and the RGD-binding conformers of isolated GPIIb/IIIa

When isolated GPIIb/IIIa was chromatographed on an affinity matrix containing 10 mg of immobilized KYGRGD peptide/ml of gel, approx. 80% of the initial material was recovered in the flow-through eluate. This was not due to saturation of binding sites, as the unretained GPIIb/IIIa fraction failed to bind to a second affinity column. This GPIIb/IIIa pool was designated the 'non-RGD-binding' (RGD⁻ or R⁻) conformer, whereas the bound fraction, which could be eluted with 1 mM GRGDS, was termed the 'RGD-binding' conformer (RGD⁺ or R⁺). The RGD⁺ fraction retained its activity after exhaustive dialysis against column equilibration buffer.

Pattern of fragmentation of the RGD⁻ GPIIb/IIIa fraction with endoproteinase Arg-C

The time course of the digestion with endoproteinase Arg-C of the affinity chromatography column flow-through fraction was followed by SDS/PAGE (Figure 1a). Two major products with apparent molecular masses of 80 and 55 kDa were observed after 6 h digestion and were apparently stable for up to 24 h proteolysis. The upper band was identified by an anti-GPIIIa rabbit polyclonal serum, whereas the 55 kDa product reacted with rabbit polyclonal antibodies against both GPIIIa and GPIIb (results not shown). The 80 and 55 kDa products were separated by size-exclusion chromatography after dissociation with SDS. N-terminal sequence analysis showed that the 80 kDa fragment contained three disulphide-bonded GPIIIa peptides, ¹GPNIXT-TRGV..., ²¹⁷DAPEGGFDAI... and ³⁵³SKVELEVRDL..., whereas the 55 kDa product was made up of two disulphide-bridged fragments of GPIIbH, ¹LNLDPVQLTF... and ¹⁵³IYV-ENDF..., and two disulphide-bonded fragments of GPIIIa, ¹GPNIXTT... and ³⁵³SKVELEVRDL....

Figure 1 Digestion of the RGD-binding and non-RGD-binding conformers with endoproteinase Arg-C

(a) Non-reducing SDS/PAGE (10% gels) analysis of the time course of digestion of non-RGD-binding GPIIb/IIIa with endoproteinase Arg-C (100:1, w/w) at 37 °C for 0 h (lanes A and A'), 2 h (lane B), 6 h (lane C), 12 h (lane D) and 24 h (lane E). Lane F shows the digestion pattern of a sample identical with that in lane E but which contained 1 mM GRGDS in the digestion buffer. GPIIb/IIIa (50 µg) was loaded in each lane, except A' which contained 100 µg of GPIIb/IIIa. The gel was stained with Coomassie Blue. Molecular masses of the major proteolytic products (in kDa) are indicated. (b) Degradation pattern of the RGD-binding fraction of isolated GPIIb/IIIa analysed using the same experimental protocol as in (a). Digestion times were: lane A, 30 min; lane B, 1 h; lane C, 2 h; lane D, 6 h; lane E, 12 h; lane F, 24 h. Lane G, 24 h digestion products of a similar sample to that in lane F but previously dialysed to remove GRGDS.

When the 6 h digestion mixture was chromatographed under non-dissociating conditions, three major products with apparent molecular masses of 185, 85 and 30 kDa were recovered (Figure 2a). N-terminal sequence analysis of these intact size-exclusion chromatography products revealed the presence of the amino acid sequences found in the dissociated 80 and 55 kDa products and additional peptides (Table 1). This indicated that each fraction contained a single proteolytic product produced by non-covalent association of the 80 and 55 kDa products with other (small) peptides. The apparent size of the undissociated products, together with the distribution of the epitopes for a set of GPIIb- and GPIIIa-specific monoclonal antibodies among the digestion products (Table 2A), allowed us to determine the length of the constituent peptides (Table 1) as follows: the 185 kDa product contains the 80 kDa product of GPIIIa (made up of the polypeptide segments 1–150, 217–352 and 353–762 held together by disulphide bridges) non-covalently associated with the 55 kDa GPIIbH fragment (1–152-S-S-153–553), and with GPIIbH-(744–856) disulphide-bonded to GPIIbL₂-(1–86). The 85 kDa product is formed by association of the 55 kDa fragment of GPIIIa-(1–150 disulphide-bonded to 353–762) with GPIIbL₂-(30–86). The 30 kDa product contains small fragments of all

Figure 2 Isolation by size-exclusion chromatography of the proteolytic products of digestion of the non-RGD-binding and RGD-binding fractions of isolated GPIIb/IIIa with endoproteinase Arg-C

(a) Size-exclusion pattern of the products of RGD⁻ GPIIb/IIIa after 6 h digestion. Separation was performed on two TSK columns (4000 and 3000) arranged in tandem and equilibrated in column buffer (see the Materials and methods section). The apparent molecular mass of each chromatographic peak is shown. Characterization of the peptide composition of each TSK fraction is shown in Tables 1 and 3. The same results were obtained when a similar sample (6 h digestion) but containing 1 mM GRGDS in the digestion mixture was analysed. (b) Elution profile of the products obtained from the RGD-binding fraction of isolated GPIIb/IIIa after digestion with endoproteinase Arg-C for 1 h (—), 6 h (●) and 24 h (○). The apparent molecular masses are shown. Compositional characterizations of the isolated chromatographic fractions are given in Tables 1 and 3. The elution position of the standards used for size calibration are given at the top of the Figure: DB, Dextran Blue (2000 kDa); Fb, fibrinogen (340 kDa); IgG, immunoglobulin G (150 kDa); BSA (67 kDa); Ovo, chicken egg albumin (43 kDa); STI, soya-bean trypsin inhibitor (21 kDa); Trp, tryptophan, (0.2 kDa).

three chains of GPIIb/IIIa: GPIIIa-(217–261-S-S-262–352); GPIIbH-(744–856); and GPIIbL₂-(1–29).

Thus the 85 kDa and the 30 kDa products are late degradation products of the larger 185 kDa fragment, whereas the 55 kDa product of GPIIbH must have been degraded to small peptides. Identical results were obtained when the 24 h digestion products were analysed, or when 1 mM GRGDS peptide was included in the digestion mixture.

Proteolytic digestion of the RGD⁺ conformer of isolated GPIIb/IIIa with endoproteinase Arg-C

Figure 1(b) shows the time-course of endoproteinase Arg-C degradation of the RGD-binding form of GPIIb/IIIa in solution. Polyclonal monospecific anti-GPIIb and anti-GPIIIa sera were used to identify the degradation products (results not shown). Whereas GPIIb is cleaved very early to a 110 kDa product, which in turn is slowly degraded to a 50 kDa peptide, GPIIIa

Table 1 Products of digestion of the non-RGD-binding GPIIb/IIIa fraction

Degradation of RGD⁻ GPIIb/IIIa (isolated as in Figure 1a) was performed with endoproteinase Arg-C (100:1, w/w) for 24 h at 37 °C. The same results were obtained after 6 h Arg-C digestion.

Digestion products	N-terminal sequence	cDNA sequence (residue no.)
R ⁻ (185 kDa)		
GPIIIa	GPNIXTTRGV	1–150
	DAPEGGFDAI	217–352
	SKVELEVRDL	353–762
GPIIbH	LNLDPVQLTF	1–152
	IYVENDFSWD	153–553
	AEAQVELRGN	744–856
GPIIbL ₂	LQDPVLVSXD	1–86
R ⁻ (85 kDa)		
GPIIIa	GPNIXTTRGV	1–150
	SKVELEVRDL	353–762
GPIIbL ₂	AMVTVLAFW	30–86
R ⁻ (30 kDa)		
GPIIIa	DAPEGGFDAI	217–261
	LAGIVQPN DG	262–352
GPIIbH	AEAQVELRGN	744–856
GPIIbL ₂	LQDPVLVSXD	1–29

Table 2 Distribution of epitopes among the digestion products of the GPIIb/IIIa conformers

Binding of GPIIb- and GPIIIa-specific monoclonal antibodies to the endoproteinase Arg-C degradation products of (A) the non-RGD-binding (R⁻) and (B) the RGD-binding (R⁺) GPIIb/IIIa conformers isolated as in Figure 1. For specificity of each monoclonal antibody, see the Materials and methods section. Peptide compositions are given in Tables 1 and 3. + and – indicate the presence and absence respectively of the epitope.

Digestion product	Presence of epitope							
	P ₃₇	M ₁	M ₂	Mα ²	M ₃	M ₄	M ₅	M ₆
(A)								
R ⁻ (185 kDa)	+	+	–	+	+	+	–	–
R ⁻ (85 kDa)	+	–	–	–	–	–	–	–
R ⁻ (30 kDa)	–	+	–	+	+	–	–	–
(B)								
R ⁺ (220 kDa)	+	+	+	–	–	+	+	+
R ⁺ (180 kDa)	+	–	+	–	–	+	+	+
R ⁺ (135 kDa)	+	–	–	–	–	+	–	–

remains intact for up to 24 h digestion (Figure 1b). This was not due to the presence of 1 mM GRGDS peptide in the digestion mixture, as a sample without GRGDS (removed by exhaustive dialysis) showed the same fragmentation pattern. When the 1 h, 6 h and 24 h digestion products were separated by size-exclusion chromatography under non-denaturing conditions, a single major peak was obtained in each case, with an apparent molecular mass of 220, 180 or 135 kDa respectively (Figure 2b). N-terminal sequence analysis and immunochemical characterization of these products (Table 2B) revealed that all three contained undegraded GPIIIa non-covalently associated with progressively degraded fragments of GPIIb (Table 3). Thus the undissociated 220 kDa product contains intact GPIIIa complexed with GPIIbH-(1–520), disulphide-bonded to GPIIbH-(521–768), and GPIIbL₂-(1–86); the 180 kDa product is identical with the former

Table 3 Proteolytic degradation of the RGD-binding GPIIb/IIIa fraction

Fragments of the RGD-binding GPIIb/IIIa fraction (R⁺) were isolated by size-exclusion chromatography, as described in Figure 1(b), after digestion with endoproteinase Arg-C (100:1, w/w) at 37 °C for (A) 1 h, (B) 6 h and (C) 24 h. The same results were obtained in the presence or absence of 1 mM GRGDS.

Digestion products	N-terminal sequence	cDNA sequence (residue no.)
(A)		
R ⁺ (220 kDa)		
GPIIIa	GPNIXTTRGV	1–762
GPIIbH	LNLDPVQLTF	1–520
	VLLGSSQQAG	521–768
GPIIbL ₂	LQDPVLVSXG	1–86
(B)		
R ⁺ (180 kDa)		
GPIIIa	GPNIXTTRGV	1–762
GPIIbH	LNLDPVQLTF	1–520
	VLLGSSQQAG	521–768
(C)		
R ⁺ (135 kDa)		
GPIIIa	GPNIXTTRGV	1–762
GPIIbH	LNLDPVQLTF	1–449

except that it has lost the GPIIbL₂ fragment; and the 135 kDa product contains undegraded GPIIIa associated with the N-terminal fragment of GPIIbH encompassing residues 1–449 (Table 3).

DISCUSSION

Our results indicate that both the non-RGD-binding and the RGD-binding forms of GPIIb/IIIa are conformationally stable. This agrees with a previous report by Steiner et al. (1989). We have analysed the structure of both conformers of GPIIb/IIIa as a model for investigating the mechanism of the GPIIb/IIIa inactive-to-active conformational transition. To this end, the affinity-purified conformers were partially degraded under non-denaturing conditions with endoproteinase Arg-C, and the resulting products were isolated by size-exclusion chromatography and characterized by protein-chemistry and immunochemical methods. Our approach assumes that limited proteolysis may aid in the demarcation of structural domains and thus that differences in the fragmentation patterns of identically treated GPIIb/IIIa conformers may reflect changes in their quaternary structures. Indeed, electrophoretic analysis of the time-course degradation products of the non-RGD-binding of RGD-binding isoforms of GPIIb/IIIa (Figure 1) already indicated major differences. Particularly striking is the fact that GPIIIa remains uncleaved in the RGD⁺ GPIIb/IIIa form but is degraded to 80 kDa and 55 kDa products in RGD⁻ GPIIb/IIIa.

Structural and immunochemical analyses of size-exclusion-isolated degradation fragments of both GPIIb/IIIa conformers (Figure 2, Tables 1–3) clearly indicate that the major susceptible cleavage sites of RGD⁻ GPIIb/IIIa for endoproteinase Arg-C proteolysis are within the loop region (residues 100–400) of GPIIIa, between its N- and C-terminal disulphide-rich domains (Niewiarowski et al., 1989; Beer and Collier, 1989; Calvete et al., 1991c; Kouns et al., 1991b), and in the C-terminal half of both subunits of GPIIb. In addition, the N-terminal domain of GPIIbH is cleaved between Arg¹⁵³ and Ile¹⁵⁴, whereas this site is protected in RGD⁺ GPIIb/IIIa. On the other hand, the region of GPIIbH comprising residues 521–768 is more resistant to

Figure 3 Schematic model of the domain rearrangement involved in the GPIIb/IIIa receptor's activation conformational change

Relative distribution of some relevant epitopes on resting (non-RGD-binding) GPIIb/IIIa (a) P_{37} and M_5 are antigenic determinants recognized on resting platelets by the platelet-aggregation-inhibitory monoclonal antibodies P_{37} and M_5 ; P_{23} and M_6 are hidden epitopes which become surface exposed on platelet activation (b); R^{214} , arginine residue the substitution of which for tryptophan is related to the inability of thrombasthenic GPIIb/IIIa to undergo the conformational transition which exposes the macromolecular ligand-binding site, which is represented here by ●; 294–314, GPIIb peptide stretch which interacts with fibrinogen and blocks its binding to GPIIb/IIIa. The hatched surface represents the disulphide-rich core of GPIIIa. GPIIbH and GPIIbL, the heavy and the light chains of GPIIb respectively.

proteolysis in RGD⁺ GPIIb/IIIa than in the RGD⁻ form of GPIIb/IIIa.

The degradation pattern of the inactive conformer of GPIIb/IIIa is consistent with our recently reported model of the GPIIb/IIIa complex in solution, which is based on analysis of the proteolytic digestion of isolated GPIIb/IIIa with trypsin, chymotrypsin and endoproteinase Glu-C (Calvete et al., 1992b). According to this rudimentary domain connectivity map, interactions between GPIIbH-(486–553) and GPIIIa-(324–366), GPIIbH-(780–817) and GPIIIa-(217–298), and GPIIbL₂-(30–75) and GPIIIa-(403–421) may hold together the non-covalently associated peptides within fragments R⁻ (185 kDa), R⁻ (85 kDa) and R⁻ (30 kDa) (Table 1). This model for GPIIb/IIIa in octyl glucoside solution may also be applied to GPIIb/IIIa on the resting platelet membrane. Thus chymotryptic degradation of GPIIb/IIIa in washed resting platelets releases into the supernatant a 95 kDa product containing GPIIbH-(17–551), whereas the GPIIIa region 100–348 is degraded. A 66 kDa GPIIIa proteolysis-resistant product, corresponding to (1–100)-S-S-(349–C-terminal) (Niewiarowski et al., 1989), remains membrane bound and non-covalently associated with a 25 kDa product of GPIIbH (encompassing residues 558–747) (Calvete et al., 1991a). Altogether, these data indicate that the conformation of inactive GPIIb/IIIa in the platelet membrane is essentially preserved in solution.

On the other hand, the peptide composition of the late (24 h) digestion product of the RGD-binding active form of GPIIb/IIIa, R⁺ (135 kDa) (Table 3), may be the same as that reported for a high-affinity fibrinogen-binding complex made up of intact GPIIIa associated with an N-terminal 58 kDa fragment of GPIIb (Kouns et al., 1992). R⁺ (135 kDa) is also similar to a chymotryptic degradation product (containing the 55 and 85 kDa N-terminal fragments of GPIIb and GPIIIa respectively) generated

from immobilized GPIIb/IIIa on an RGD-Sepharose column (Lam, 1992). In addition to these studies, the characterization of the peptide composition of R⁺ (220 kDa) and R⁺ (180 kDa) reported here shows the time-course degradation pathway of GPIIb. The association of the N-terminal half of GPIIbH with GPIIIa in R⁺ (135 kDa) can be explained within the context of the model of GPIIb/IIIa in solution (Calvete et al., 1992b) by interaction of GPIIbH-(486–553) with GPIIIa-(324–366). However, the loss of the GPIIbL₂-(1–86) domain in R⁺ (180 kDa) and of the GPIIbH C-terminal peptides on degradation of R⁺ (180 kDa) into R⁺ (135 kDa) (Table 3), as well as the resistance of GPIIIa to proteolysis, clearly indicate that the connectivity map outlined for isolated GPIIb/IIIa (Calvete et al., 1992b) may not be valid to describe the quaternary structure of the RGD-binding conformer of GPIIb/IIIa. Indeed, comparison of the fragmentation patterns of inactive and active GPIIb/IIIa conformers (Tables 1 and 3) suggest that, on receptor induction, the N-terminal half of GPIIbH changes its relative orientation protecting itself and the GPIIIa-(100–348) region from proteolysis, whereas the C-terminal half of GPIIbH becomes more exposed (Figure 3).

The different conformations of the two GPIIb/IIIa forms may in part explain the different cross-linking patterns obtained for RGD and γ -chain peptides when the experiment was carried out with activated platelets (D'Souza et al., 1988, 1990) or with isolated GPIIb/IIIa (Calvete et al., 1992a). Thus D'Souza et al. (1988) have identified a region within GPIIIa (residues 109–171) to which the peptide KYGRGDS becomes chemically cross-linked in thrombin-stimulated platelets. A similar peptide (CGRGDS) was cross-linked to GPIIIa-(151–191), GPIIbH-(42–73) and GPIIbL₂-(30–75) within the isolated inactive GPIIb/IIIa complex (Calvete et al., 1992a). Furthermore, the fibrinogen γ -chain peptide K^[125I]YGGHHLGGAKQAGDV was covalently bound to thrombin-activated GPIIb/IIIa within GPIIbH-(294–314) (D'Souza et al., 1990), whereas CYHHLGGAKQAGDV was found cross-linked to GPIIIa-(6–37) and GPIIbH-(752–768) in isolated GPIIb/IIIa (Calvete et al., 1992a).

The RGD-binding site of non-activated GPIIb/IIIa is accessible to molecules which contain an RGD, RYD or KGD sequence at the tip of recognition loops protruding 1.4–1.7 nm from the protein core (Adler et al., 1991; Saudek et al., 1991; Tomiyama et al., 1992a,b). In contrast, soluble fibrinogen and other RGD-containing macromolecules such as fibronectin, vitronectin and von Willebrand factor bind to GPIIb/IIIa only after platelet activation (Bennett and Vialre, 1979; Marguerie et al., 1980; Plow and Ginsberg, 1989). Interestingly, Beer et al. (1992) have shown that the majority of RGD-binding sites on GPIIb/IIIa can be reached by peptides that extend out 1.1–3.2 nm from the surface of polyacrylonitrile beads, and that peptides in the lower length limit (approx. 1.1 nm) showed the greatest sensitivity to the platelet's activation state. These data suggest that the geometry of the binding pocket in non-activated GPIIb/IIIa may resemble a narrow tunnel, which must be opened through an activation-dependent conformational change to allow the entry of macromolecular ligands.

This hypothesis is substantiated by the results presented here and agrees with immunochemical studies showing that the epitope recognized by the platelet-aggregation-inhibitory monoclonal antibody M_6 [GPIIbH-(657–665); Calvete et al., 1991a] is hidden in GPIIb/IIIa on resting platelets but becomes exposed on thrombin activation. In addition, our results indicate that regions within GPIIIa-(100–348) also participate in the exposure of the GPIIb/IIIa active site.

The region 114–128 within GPIIIa seems to be involved in the conformational change(s) exposing the integrin's macromolecular

ligand-binding pocket, as it contains the platelet-activation-dependent epitopes recognized by monoclonal antibodies P₂₃⁷ [GPIIa-(114–122); Calvete et al., 1991b] and AC7 (GPIIIa 109–128; Andrieux et al., 1991). In addition, the point mutation Asp¹¹⁹ → Tyr produces a mutant α IIB β_3 integrin that lacks ligand recognition and shows perturbed interaction with bivalent cations (Loftus et al., 1990). The authors noticed that the linear spacing of oxygenated residues surrounding Asp¹¹⁹ (Ser¹²¹, Ser¹²³, Asp¹²⁶, Asp¹²⁷ and Ser¹³⁰) approximates that of the residues in the Ca²⁺-binding loop of EF hand proteins, and postulated that this clustering could provide co-ordination sites for bivalent cations essential for fibrinogen binding. Furthermore, cell lines expressing a mutant GPIIb/IIIa, in which the polypeptide GPIIIa-(129–133) was replaced by the corresponding amino acids from the integrin β_1 subunit (FNVKS), were hyperadherent and manifested spontaneous aggregation when fibrinogen was added to the medium (Bajt et al., 1992a). Although the basis for the capacity of this substitution to augment the ligand-binding function of this 'superintegrin' α IIB β_3 requires further structural characterization, it seems reasonable to assume that replacement of that GPIIIa region with its homologous region from an integrin that does not require activation to bind ligands, maintains the quaternary structure of GPIIb/IIIa in an activation-like conformation. Indeed, mutational studies on β_2 integrins indicate that the region homologous to that of GPIIIa (integrin β_3), where the activation-dependent epitopes are located is critical for association with the α -subunits and may be located at the subunit interface (Wardlaw et al., 1990; Arnaout et al., 1990; Back et al., 1992; Shuster et al., 1992).

GPIIa-(208–231) also contains an activation-dependent epitope (Piotrowicz and Kunicki, 1990), and the region around GPIIIa Arg²¹⁴ has been involved in the conformational change leading to a GPIIb/IIIa capable of binding fibrinogen. Thus mutation of this residue to Gln (Bajt et al., 1992b) or Trp (Lanza et al., 1992) abolishes fibrinogen binding in response to any platelet activation agonist. However, the Trp mutation does not impair the interaction of the mutated integrin with short synthetic RGD peptides in solution (Lanza et al., 1992), whereas the Gln substitution (Bajt et al., 1992b) results in reduced binding of the mutant integrin to immobilized RGD or γ -chain peptide. This may imply that the functional defect introduced by the Arg²¹⁴ → Trp substitution is related to the inability of the heterodimer to change its conformation and expose the macromolecular ligand-binding pocket: the mutation may change the character of subunit contacts, thus maintaining the quaternary structure of GPIIb/IIIa in a permanently non-activated-like conformation, i.e. the converse situation to that which appears to happen with the superintegrin described above.

The major conclusion is that all the GPIIb and GPIIIa regions that become exposed in the inactive-to-active-state conformational transition form part of the heterodimer subunit interface (Calvete et al., 1992b; Steiner et al., 1993). Interestingly, the different sequences of GPIIb and GPIIIa that have been reported to form part of the heterodimer ligand-binding pocket [GPIIIa-(114–128), GPIIIa-(204–219), GPIIbH-(294–314); GPIIbH 573, GPIIbH-(657–665), see Plow et al. (1992a), Tarantino and Nugent (1992) and Calvete (1993)] are also located at the subunit interface, except GPIIbH-(294–314). This evidence suggests that the integrin active site may be made up from regions of both subunits which, although distantly located within the linear primary structure, are brought into juxtaposition by the spatial folding of the polypeptide chains. This discontinuous receptor surface may be hidden within the resting heterodimer interface, and subtle motions between the subunits might be used in the regulation of the functional characteristics of the receptor. It is

tempting to speculate that GPIIbH-(294–314) is placed within the ligand-binding pocket as a consequence of the domain movement accompanying receptor activation. Our present results (schematically depicted in Figure 3) provide further clues for a better understanding of the GPIIb/IIIa activation-dependent conformational change.

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