Evidence that the central region of glycoprotein IIIa participates in integrin receptor function

Priya RAMSAMOOJ,* Mark O. LIVELY and Roy R. HANTGAN†

Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, 300 South Hawthorne Road, Winston-Salem, NC 27103, U.S.A.

We have obtained evidence that the ligand-recognition region of the integrin β -subunit, platelet glycoprotein IIIa (GPIIIa), is discontinuous. Receptor function can be localized to residues near the *N*-terminus and to the central region of the polypeptide chain. The epitope recognized by our monoclonal antibody, CS-1, which substantially inhibits fibrin(ogen) binding to ADP- and thrombin-stimulated platelets [Ramsamooj, Doellgast & Hantgan (1990) *Thromb. Res.* **58**, 577–592], is contained within residues 349–422 of GPIIIa. This sequence is adjacent to a proteinase-resistant domain of GPIIIa which is linked by disulphide bond(s) to an *N*-terminal segment near to the putative Arg-Gly-Asp recognition site [D'Souza, Ginsberg, Burke, Lam & Plow (1988) *Science* **242**, 91–93]. Limited trypsin digestion of purified platelet GPIIIa yielded a mixture of two-chain molecules comprised of an *N*-terminal fragment disulphide-bonded to one of four fragments, which began at residues 299, 303, 353 or 423. Tryptic cleavage of the 300–422 segment correlated with loss of immunoreactivity with anti-GPIIIa monoclonal antibody, CS-1. Chymotrypsin cleavage of GPIIIa resulted in an *N*-terminal 19 kDa fragment joined by at least one intrachain cystine residue to a 46 kDa polypeptide beginning at residue 349. Partial reduction with dithiothreitol released the larger chymotryptic fragment with its epitope for CS-1 intact. These results have enabled us to localize the epitope recognized by our inhibitory monoclonal antibody, CS-1, to residues 349–422 of GPIIIa. Our data are consistent with a structure in which both the *N*-terminal and central regions of GPIIIa, which may be in close proximity in the functional GPIIb–IIIa complex, participate in ligand binding.

INTRODUCTION

The non-covalent heterodimeric complex formed by platelet glycoproteins IIb and IIIa (GPIIb and GPIIIa) is the prototypical member of the integrin family of cell-adhesion receptors, which are characterized by considerable sequence similarity of their respective α - and β -subunits (recently reviewed by Phillips *et al.* [1]). GPIIIa, which has been identified as the β -subunit of the integrin subclass termed 'cytoadhesins' [2], is a single polypeptide chain of 762 residues. Important features of the GPIIIa primary structure [3,4] include an *N*-terminal segment of approx. 200 residues, which contains a putative Arg-Gly-Asp recognition site [5–7], followed by a proteinase-susceptible region [8], and a large extracellular domain containing 45 cysteine residues, 31 of which are arranged in a set of four tandem repeats [1,3,4]. This domain is followed by a short stretch of hydrophobic residues tentatively identified as a membrane-spanning region and a short intracellular sequence extending to the *C*-terminus [1,3,4].

Although biochemical [5–7] and immunological [9] data have clearly demonstrated an important role for the *N*-terminal region of platelet GPIIIa in ligand binding and platelet aggregation, the presence of disulphide bond(s) between the *N*-terminal and cysteine-rich domains of the molecule [8–10] indicates that regions of GPIIIa distant in linear sequence are in close proximity in the folded structure and could contribute to receptor function. Here we show that our anti-GPIIIa monoclonal antibody, CS-1, which inhibits platelet–fibrin(ogen) interactions [11], recognizes an epitope contained within residues 349–422 of GPIIIa. As this sequence is present on a central section of GPIIIa distinct from the Arg-Gly-Asp-recognition site on the *N*-terminus, we propose

that the central region of GPIIIa also participates in integrin-receptor function.

MATERIALS AND METHODS

Purification of GPIIb–IIIa and development of monoclonal antibody CS-1

The GPIIb–IIIa complex was purified by a combination of lentil-lectin affinity chromatography [12] and Sepharose-4B gel-filtration chromatography of solubilized platelet membrane proteins [13] as previously described [11].

Also previously described was the development of CS-1 by standard hybridoma technology [14] with purified GPIIb–IIIa as the antigen; immunoblotting demonstrated that CS-1 is specific for non-reduced GPIIIa, but does not recognize the completely reduced polypeptide. Radiolabelled-ligand binding studies showed that CS-1 recognizes approx. 55000 sites/platelet and binds with a dissociation constant in the nanomolar range, independent of the state of platelet activation. We have also shown that CS-1 causes a 2.5–3-fold reduction in binding of fibrinogen and soluble fibrin polymers to ADP- and thrombin-stimulated platelets with IC_{50} values in the range 9–10 nM, and that CS-1 inhibits aggregation of ADP- and thrombin-stimulated platelets by 2- and 4-fold respectively [11].

Isolation of GPIIIa by high-performance size-exclusion chromatography (h.p.s.e.c.)

Purified GPIIb–IIIa was separated into GPIIb and GPIIIa according to the h.p.s.e.c. method of Eirin *et al.* [15]. Purified

Abbreviations used: GPIIIa, platelet glycoprotein IIIa; GPIIb–IIIa, platelet glycoprotein IIb–IIIa complex; TX-100R, Triton X-100 reduced; h.p.s.e.c., high-performance size-exclusion chromatography; HRP-CS-1, horseradish peroxidase-conjugated monoclonal antibody CS-1; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin.

* Present address: Georgetown University Medical Center, Department of Radiation Medicine, Vincent T. Lombardi Cancer Research Center, 3800 Reservoir Road, N.W., Washington, DC 20007-2197, U.S.A.

† To whom correspondence should be sent.

GPIIb–IIIa (250 μ g) was applied to an h.p.s.e.c. system consisting of a TSK 4000 and a TSK 3000 column (Beckman Instruments, San Ramon, CA, U.S.A.) (each 7.5 mm $\times$ 30 cm) coupled in series. The mobile phase contained 0.1 % SDS, 0.1 M-phosphate, pH 6.8, 1 mM-EDTA and 0.025 % NaN_3 . The resultant peaks were analysed by SDS/PAGE and immunoblotting to confirm separation of GPIIb and GPIIIa and maintenance of GPIIIa immunoreactivity (results not shown). The fractions containing a majority of GPIIIa (approx. 70 % GPIIIa as judged by densitometric scans of stained SDS/PAGE gels) were pooled and the concentration determined by A_{280} using an absorption coefficient of $1 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ [16].

SDS/PAGE and immunoblotting

SDS/PAGE was performed on 0.75 mm-thick slab gels of 10 or 12.5 % polyacrylamide (Bio-Rad, 5.8 cm $\times$ 8.3 cm) according to the method of Laemmli [17]. Approx. 10–20 μ g of protein/lane were applied (based on a molar absorption coefficient (280 nm) of $1.18 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ for the complex [11] or $1 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ for GPIIIa [16]). The gels were run at constant voltage (200 V) for 45 min. Molecular-mass standards were prestained. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 [0.1 % in methanol/acetic acid/water (5:1:4, by vol.)] and destained with methanol/acetic acid/water (5:1:4, by vol.) (1–2 h) and methanol/acetic acid/water (5:7:88, by vol.) (overnight), if necessary.

Alternatively, gels were transferred electrophoretically to nitrocellulose (70 V for 10–20 min) with a buffer containing 192 mM-glycine, 25 mM-Tris/HCl, pH 8.3, 20 % (v/v) methanol and 0.1 % SDS [18]. The efficiency of transfer was assessed by revealing the prestained standards in the nitrocellulose. After the electrophoretic transfer, the nitrocellulose was placed in blocking buffer [10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/3 % casein] overnight. The nitrocellulose was then probed with HRP-CS-1 (200 ng/ml) in blocking buffer for 2 h. Development of the blot required incubation in a Tris/saline buffer (10 mM-Tris/HCl, pH 7.4) containing 3.4 μ M-4-chloro-1-naphthol and 0.02 % H_2O_2 until bands appeared, typically 5–15 min. Development was stopped by rinsing in distilled water.

Limited proteolysis of GPIIIa

Purified GPIIIa (obtained by means of h.p.s.e.c.) was subjected to limited proteolysis with trypsin, α -chymotrypsin or staphylococcal V8 proteinase or combinations of these proteinases. All enzymes were present in the digestion mixture at a 1:100 (w/w) enzyme/protein ratio. A 3 h digestion period with trypsin at 22 °C was adequate to obtain limited proteolysis of GPIIIa; prolonged digestion for 24 h did not result in further cleavage, as judged by SDS/PAGE. The peptides resulting from proteolysis with these enzymes, alone and in various combinations, were analysed by SDS/PAGE and immunoblotting.

Electroelution

Fragments of GPIIIa resulting from limited proteolysis were purified by electroelution using a CBS Scientific electroelution apparatus as described by Hunkapillar *et al.* [19]. The samples were collected, and approx. 5 % of the total sample was analysed by SDS/PAGE to ensure that the correct polypeptide was electroeluted and to obtain an estimate of its purity.

Electrophoretic transfer to poly(vinylidene difluoride) (PVDF)

As an alternative to electroelution, peptides for *N*-terminal amino acid sequence analysis were transferred from 10 % (w/v) polyacrylamide gels to PVDF (Immobilon-P, Millipore) membranes by a minor modification of the procedure of Matsudaira [20]. Gels were equilibrated in transfer buffer [192 mM-

glycine/25 mM-Tris/HCl (pH 8.3)/0.1 % SDS/20 % (v/v) methanol] and the PVDF membrane equilibrated in 100 % methanol. The proteins were transferred using a Bio-Rad transblot apparatus and power supply (Bio-Rad 250/2.5 power supply, 70 V for 10 min). Efficiency of transfer was assessed by revealing the prestained standards in the membrane. The PVDF membrane was then stained for 5 min (0.1 % Coomassie Brilliant Blue R-250/50 % methanol), destained (methanol/acetic acid/water, 5:1:4, by vol.) for 10 min, rinsed with Milli-Q water and dried between two sheets of filter paper. The bands of interest were then excised and subjected to *N*-terminal amino acid sequence analysis.

N-Terminal amino acid analysis

Proteins obtained by electroelution were precipitated with ethanol (9 vol. of ethanol, –20 °C, for at least 1 h). After centrifugation [Servall RC-2 centrifuge, HB-4 rotor, 8000 rev./min (10 550 *g*) for 20 min], aspiration of the excess ethanol and dissolution of the pellet in 50 % acetic acid, the samples were applied to an Applied Biosystems model-475A protein sequencer equipped with an on-line phenylthiohydantoin (PTH) amino acid derivative analyser.

Partial reduction

The purified, 49 kDa α -chymotryptic fragment (the smallest fragment recognized by CS-1) was subjected to partial reduction with dithiothreitol to separate the constituent polypeptides of the fragment. This was achieved by a modification of the selective reduction technique of Calvete *et al.* [9]. The concentration of the electroeluted fragment was estimated by microbiuret assay [21]. The fragment was precipitated with ethanol (9 vol. of ethanol, –20 °C, overnight), centrifuged, and the pellet resuspended in 50 mM-Tris/HCl (pH 8.0)/0.1 % SDS/1 mM-EDTA. Aliquots of the protein were incubated with dithiothreitol at a concentration 0.25-, 0.5-, 1- and 2-fold the estimated half-cystine content of the fragment for 1 h at room temperature. Iodoacetamide at a 1.5-fold excess over dithiothreitol was then added and the mixture further incubated for 30 min. The sample was then analysed by SDS/PAGE, using 12.5 %-(w/v)-polyacrylamide gels. One of the gels was stained with Coomassie Brilliant Blue; the other was electrophoretically transferred to nitrocellulose and probed for binding with HRP-CS-1 as described above.

Primary structure comparisons and predictive algorithms

Programs FASTA [22] and PEPTIDESTRUCTURE (in particular, the surface probability algorithm of Emini *et al.* and Janin *et al.* [23,24] and the hydrophobicity algorithm of Kyte & Doolittle [25] of the GCG Sequence Analysis Software Package [26]) were used for analyses of the primary structure of GPIIIa.

RESULTS AND DISCUSSION

Limited proteolysis with trypsin, α -chymotrypsin and staphylococcal V8 proteinase

Trypsin, α -chymotrypsin and staphylococcal V8 proteinase, alone or in combination, were all found to make limited cleavages in purified GPIIIa, as evidenced by SDS/PAGE (Fig. 1a). An identical gel was electrophoretically transferred to nitrocellulose and probed with HRP-CS-1 (Fig. 2b). The results indicated that a peptide of approx. 49 kDa was the smallest immunoreactive fragment obtained, independently of the combination of proteinases used.

Limited proteolysis with trypsin

To examine this 49 kDa polypeptide in greater detail, we focused on the immunoreactive tryptic fragment. Initial experi-

Fig. 1. SDS/PAGE analysis of the products obtained by limited proteolysis of purified GPIIIa

Two 10%-(w/v)-polyacrylamide gels were run containing 15 µg/lane of a digestion mixture of GPIIIa and various proteinases, each at a 1:100 ratio (w/w) enzyme/protein for 3 h at 22 °C: trypsin (lane 2); trypsin and α-chymotrypsin (lane 3); α-chymotrypsin (lane 4); α-chymotrypsin and staphylococcal V8 proteinase (lane 5); staphylococcal V8 proteinase (lane 6); staphylococcal V8 proteinase and trypsin (lane 7). Lanes 1 and 8 contained molecular-mass standards. The gel in (a) was developed with Coomassie Brilliant Blue R-250. The other (b) was electrophoretically transferred to nitrocellulose, probed with HRP.CS-1 and developed as described in the Materials and methods section. The results indicated that the fragment with an apparent molecular mass of 49 kDa was the smallest immunoreactive fragment obtained, independently of the combination of proteinases used.

ments were performed to examine the time course of tryptic digestion. Purified GPIIIa was subjected to tryptic cleavage, and the resultant fragments were separated by SDS/PAGE (Fig. 2). One gel was stained with Coomassie Brilliant Blue R-250 (Fig. 2a); the other was electrophoretically transferred to nitrocellulose and probed with HRP.CS-1 (Fig. 2b). The results revealed that intact GPIIIa (lane 1) and fragments of 65 ± 3 kDa and 49 ± 1 kDa produced by limited proteolysis were immunoreactive. Although there were fragments smaller than 49 kDa (Fig. 2a), they were not recognized by the monoclonal antibody. Furthermore, the immunoreactive 49 kDa fragment was stable to prolonged digestion with trypsin (24 h), as evidenced by the maintenance of the intensity of the 49 kDa band at 24 h (Fig. 2a, lane 8).

Fig. 2. Analysis by SDS/PAGE of the time course of trypsin digestion of GPIIIa

H.p.s.e.c.-purified GPIIIa in 10 mM-Tris/HCl (pH 7.4) 0.1% TX-100R (lane 1) and its digestion products obtained after exposure to trypsin at a 1:100 (w/w) trypsin/protein ratio for various time periods were run on 10%-(w/v)-polyacrylamide gels (25 µg/lane): 0.5 h (lane 2); 1 h (lane 3); 2 h (lane 4); 3 h (lane 5); 5 h (lane 6); 10 h (lane 7); and 24 h (lane 8). The gel stained with Coomassie Brilliant Blue R-250 shows the protein profiles of the digested material (a). Note the appearance of the 49 ± 1 kDa fragment, which represents a trypsin-resistant core. The proteins from an identical gel were transferred to nitrocellulose and developed with HRP.CS-1. The results (b) demonstrate that a 49 kDa fragment is the smallest fragment generated by tryptic cleavage of GPIIIa which is recognized by CS-1 (molecular mass estimated by comparison with prestained molecular-mass standards; lanes S).

Sequence of the tryptic fragments

To localize this 49 kDa fragment within the GPIIIa molecule, we performed *N*-terminal amino acid sequence analysis of the electroeluted 49 kDa tryptic fragment. The band was found to contain a mixture of amino acid sequences (Table 1) that were consistent with the published amino acid sequence derived from the cDNA clone of GPIIIa [3,4]. The predominant sequence (calculated initial yield 66 pmol) was the *N*-terminal sequence of native GPIIIa (Table 1, column A) and was easily interpreted without the aid of cDNA sequence. The remaining amino acids that were observed in each cycle of Edman degradation were assigned to their respective sequences using the known amino acid sequence as indicated in Table 1 (columns B, C and D) and were completely consistent with known regions of the GPIIIa molecule. These peptides resulted from cleavage after lysine residues 298 and 302 and after an arginine residue at position 352. The calculated initial yields for these secondary sequences were 16, 20 and 15 pmol respectively, the sum of which is roughly equivalent to the yield of the prominent *N*-terminal sequence.

Table 1. N-Terminal amino-acid-sequence analysis of the 49 kDa tryptic fragment of GPIIIa

The superscript numbers indicate the amino acid (AA) sequence of GPIIIa beginning at: ¹residue 1; ²residue 299; ³residue 303; ⁴residue 353.

Cycle no.	A		B		C		D	
	AA ¹	Yield (pmol)	AA ²	Yield (pmol)	AA ³	Yield (pmol)	AA ⁴	Yield (pmol)
1	G	100	L	11	N	16	S	17
2	P	51	S	19	X	ND*	K	7
3	N	62	Q	1	(N)†	ND	V	12
4	I	39	K	12	L	13	E	14
5	X	-‡	N	10	I	ND	L	15
6	T	35	I	11	F	12	E	14
7	T	46	N	2	A	10	V	5
8	R	29	L	4	V	6	(R)†	ND
9	G	31	I	1	X	ND	D	8
10	V	20	F	1	E	4	L	5
11	S	31	A	4	N	12	P	6
12	S	39	V	1	(V)†	ND	E	2

* Abbreviation: ND, not determined.

† This amino acid was present from two of the peptides and therefore could not be quantified.

‡ The protein was neither reduced nor alkylated before sequence analysis, so cysteine (C) was not determined.

Scheme 1. GPIIIa and the 49 kDa immunoreactive tryptic fragment of GPIIIa

The C-terminal proteinase-resistant domain is designated by the hatched box. The apparent molecular masses of immunoreactive peptide fragments are encircled and denoted by an asterisk. Complete reduction of the 49 kDa fragment results in the loss of immunoreactivity and the separation of the N-terminal (apparent molecular mass 19 kDa) and C-terminal (apparent molecular mass 58 kDa) portions of the 49 kDa fragment. The increase in apparent molecular mass upon reduction is attributed to the cleavage of disulphide bonds within the C-terminal proteinase-resistant core, which must result in a more extended species with decreased electrophoretic mobility.

Analysis of the reduced 49 kDa fragment by SDS/PAGE and staining with Coomassie Brilliant Blue R-250 revealed a major band with an increased apparent molecular mass of 58 ± 2 kDa and a weakly stained 19 ± 1 kDa band (results not shown). (The increase in apparent molecular mass upon reduction is attributed to the cleavage of the disulphide bonds present within the C-terminal proteinase-resistant core of GPIIIa, which must result in a more extended species with decreased electrophoretic mobility on SDS/PAGE.) By immunoblotting, neither peptide was immunoreactive. N-Terminal amino acid sequence analysis was performed on the 58 kDa peptide purified by SDS/PAGE and electroelution as described in the Materials and methods section. A clear sequence was observed that was identical with the published amino acid sequence of GPIIIa [3,4], beginning at residue 303 of the intact glycoprotein:

NINLIFAVTENV

This sequence corresponded to one of the sequences observed upon analysis of the non-reduced tryptic fragment (Table 1, column C) and is consistent with the specificity of trypsin for cleavage of the peptide bond that follows lysine-302. A minor sequence was also seen in this preparation that was consistent with cleavage after lysine-298, also seen in the mixture of peptides in the non-reduced form (Table 1, column B). The sequence following arginine-352 was not seen in this particular sequencer run.

Another peptide, of approx. 46 kDa, was also observed in the trypsin digest (Fig. 2a), but was not recognized by CS-1 (Fig. 2b). This 46 kDa non-immunoreactive fragment was electroeluted and subjected to N-terminal amino acid sequence analysis. The results were consistent with a two-chain molecule with sequences beginning at residues 1 and 423 of intact GPIIIa. Each cycle of Edman degradation contained two major residues as follows (in one-letter code): cycle 1: D,G; cycle 2: S,P; cycle 3: L,N; cycle 4: I; cycle 5: V; cycle 6: Q,T. This mixture of residues is consistent with the major N-termini present corresponding to residue 1 of GPIIIa (GPNICT...) and residue 423 of GPIIIa (DSLIVQ...). Cycle 4 contained isoleucine as the major peak, consistent with the presence of this amino acid at the fourth position of each polypeptide. The major residue in cycle 5 was

Table 2. *N*-Terminal amino acid sequence analysis of the immunoreactive α -chymotryptic fragment of GPIIIa

Cycle no.	A		B	
	AA*	Yield (pmol)	AA†	Yield (pmol)
1	G	74	(G)‡	ND§
2	P	34	K	20
3	N	27	I	22
4	I	38	R	9
5	X	—	S	13
6	T	35	K	19
7	T	43	V	10
8	R	90	E	12
9	G	16	L	9
10	V	9	E	4
11	S	10	X	ND
12	S	15	X	ND
13	X	—	D	7
14	Q	10	L	6
15	Q	15	P	4

* Amino acid sequence of GPIIIa beginning with residue 1.

† Amino acid sequence of GPIIIa beginning with residue 349.

‡ This amino acid was present in this cycle from both peptides and could not be quantified.

§ ND, not determined.

|| The protein was neither reduced nor alkylated before sequence analysis so cysteine (C) was not determined.

valine, consistent with the absence of cysteine, which would not have been detected in this sample, which had not been reduced and alkylated before sequence analysis.

Interpretation of trypsin digestion results

Our interpretation of the trypsin digestion results is presented in Scheme 1. Limited proteolysis of GPIIIa by trypsin yields a series of two-chain core fragments comprised of a 19 kDa *N*-terminal segment connected by disulphide bond(s) to one of four polypeptides, which begin at residues 299, 303, 349 or 423 or GPIIIa and may extend to the *C*-terminus of intact GPIIIa (based on molecular-mass estimates). The observation that removal of residues 300–422 of GPIIIa coincides with loss of immunoreactivity towards CS-1 strongly suggests that the epitope recognized by our monoclonal antibody is contained within this mid-molecule segment, although these data alone do not rule out the possibility that interactions between residues 300–422 and the *N*-terminus could be involved.

Limited proteolysis with α -chymotrypsin

A series of limited proteolysis and partial disulphide-bond reduction experiments were conducted to determine if the epitope for CS-1 is contained solely within the central domain of GPIIIa. To focus on this question, we purified the smallest immunoreactive fragment produced by α -chymotrypsin (approx. 49 kDa) because digestion with this proteinase resulted in the production of the 49 kDa fragment without the larger undigested fragments (Fig. 1a, lane 4). *N*-Terminal sequence analysis of this electroeluted fragment yielded a mixture of two amino acid sequences (Table 2) that could be interpreted with reference to the published amino acid sequence [3,4]. The predominant sequence (initial yield 52 pmol) corresponded to the *N*-terminus of GPIIIa (Table 2, column A). The secondary sequence corresponded to residues 349–366 of intact GPIIIa (Table 2, column B). Consistent with the cleavage specificity of α -chymotrypsin, this fragment resulted from cleavage of GPIIIa after tyrosine-348.

Fig. 3. SDS/PAGE analysis of the 49 kDa α -chymotryptic fragment under conditions of selective reduction

The purified 49 kDa α -chymotryptic fragment (30 μ g/lane, smallest immunoreactive fragment obtained) was analysed by SDS/PAGE: in the absence of reducing agent (lane 1); in the presence of 5% β -mercaptoethanol (lane 2); in the presence of dithiothreitol in a 1:1 ratio with the cysteine content (lane 4); in the presence of dithiothreitol in a 0.25:1 ratio with the cysteine content (lane 5); in the presence of dithiothreitol in a 0.5:1 ratio with the cysteine content (lane 7); and in the presence of dithiothreitol in a 2:1 ratio with the cysteine content (lane 8). One gel was stained with Coomassie Brilliant Blue R-250 (a) and the other electrophoretically transferred to nitrocellulose (b) and developed as described in the Materials and methods section. Comparison of lane 2 (fully reduced) and lane 5 (selectively reduced) indicated that the disulphide bond(s) between the *N*- and *C*-terminal components of the 49 kDa has been reduced and the *N*-terminal (19/20 kDa) and *C*-terminal (46 kDa) components separated. The difference in mobility of the larger component in lanes 2 and 5 (58 kDa under conditions of complete reduction and 46 kDa under mild dithiothreitol treatment) indicates that mild dithiothreitol treatment does not fully reduce the disulphide bonds present in the cysteine-rich *C*-terminus of GPIIIa and therefore the *C*-terminal component of the 49 kDa fragment must have a more compact conformation that migrates with an apparent molecular mass of 46 kDa.

Upon reduction, the light and heavy chains are separated and migrate with molecular masses corresponding to a $19 \pm 1/20 \pm 1$ kDa doublet and 58 ± 4 kDa (see the parenthetic note above about increase in molecular mass on reduction), respectively (Fig. 3a, lane 2). The 58 ± 4 kDa component was subsequently electroeluted from polyacrylamide gels and subjected to *N*-terminal sequence analysis. A clear sequence of amino acids was observed which was identical with the published sequence of GPIIIa beginning at residue 349 of the intact glycoprotein [3,4]. Therefore the heavy chain of the 49 kDa

immunoreactive, α -chymotryptic fragment is this 58 kDa species and is represented in Table 2 by column B. *N*-Terminal amino acid sequence analysis of the 19/20 kDa species revealed the *N*-terminal amino acid sequence of intact GPIIIa (results not shown). Thus this species represents the disulphide-linked *N*-terminal component of the 49 kDa α -chymotryptic fragment.

Partial reduction of the 49 kDa α -chymotrypsin fragment

In order to determine if the *N*-terminal portion of the 49 kDa fragment of GPIIIa contributes to formation of the epitope recognized by monoclonal antibody CS-1, we employed the technique of Calvete *et al.* [9] to reduce selectively the disulphide bond(s) between the light and heavy chains with dithiothreitol. Analysis of the gel shows that, upon complete reduction (Fig. 3, lane 2), the 58 kDa fragment and the *N*-terminal segment (19/20 kDa) of the α -chymotryptic fragment were separated. Densitometric scans revealed the presence of similar quantities of the *N*-terminal fragment after incubation with dithiothreitol concentrations corresponding to a 1:1, 0.25:1, 0.5:1, and 2:1 ratio of the half-cystine content of the 49 kDa fragment (Fig. 3, lanes 4, 5, 7 and 8 respectively).

In the presence of sub-stoichiometric concentrations of reducing agent, the heavy chain migrated with an apparent molecular mass of 46 ± 2 kDa (lanes 5, 7), faster than either the non-reduced (Fig. 3, lane 1) or fully reduced (Fig. 3a, lane 2) 58 kDa fragment. This altered electrophoretic mobility, coupled with the appearance of the *N*-terminal fragment, indicates that reduction of the disulphide bond(s) joining the two polypeptides which comprise the 49 kDa fragment had been achieved over a limited range of dithiothreitol concentrations.

A gel identical with that in Fig. 3(a) was electrophoretically transferred to nitrocellulose and probed with HRP-CS-1 to determine if the epitope recognized by the monoclonal antibody is contained within the light or heavy chains of the 49 kDa α -chymotryptic fragment. The lanes corresponding to the intact (Fig. 3b, lane 1) and selectively reduced fragment (Fig. 3b, lanes 5 and 7) were strongly immunoreactive, indicating that CS-1 recognized the larger portion of the fragment, even in the absence of the smaller *N*-terminal fragment. Moreover, it is noteworthy that immunoreactivity was nearly completely lost after incubation with higher concentrations of reducing agent (Fig. 3b, lanes 2, 4 and 8). The identity of the weakly immunoreactive bands seen in lanes 2, 4 and 8 is unknown, but they were only observed at high sample loads and were not seen in two other partial-reduction experiments (results not shown).

In order to amplify the small change in electrophoretic mobility which accompanied partial reduction, additional samples of non-reduced, partially reduced, and fully reduced 49 kDa α -chymotryptic fragment were subjected to SDS/PAGE for approximately twice the usual period, until the pre-stained 26.6 kDa molecular-mass marker reached the bottom of a 10 gel. Under these conditions, the mobility of the partially reduced band revealed by Coomassie Blue staining increased by 2.7% compared with the non-reduced sample; analysis of the corresponding immunoblot revealed a similar 2.6% increase in mobility of the partially reduced sample. These observations confirm that the main immunoreactive material in lanes 5 and 7 of Fig. 3 corresponds to the partially reduced bands revealed by Coomassie Blue staining in Fig. 3(a).

Since the possibility existed that the species recognized by CS-1 in Fig. 3b (lanes 5 and 7) was the intact 49 kDa fragment which had not yet been reduced by the mild dithiothreitol treatment, the fragment with an apparent molecular mass of 46 kDa was purified by electroelution and subjected to *N*-terminal amino acid analysis. The amino acid sequence obtained corresponded to the published sequence of GPIIIa beginning with residue 349

Scheme 2. The 49 kDa α -chymotryptic fragment under various states of reduction (fully reduced with 5% β -mercaptoethanol and partially reduced with dithiothreitol at a 0.25:1 ratio with the half-cystine content of the fragment)

This diagram shows that complete reduction results in the separation of the *N*- and *C*-terminal components (19 kDa and 58 kDa respectively), neither of which is immunoreactive (immunoreactivity is denoted by the encircled molecular masses with an asterisk). Under mild dithiothreitol treatment, the *N*- and *C*-terminal portions are also separated; however, the disulphide bonds within the *C*-terminal cysteine-rich core were not fully reduced and this portion migrated with an apparent molecular mass of 46 kDa. In addition, this *C*-terminal portion maintained immunoreactivity in the absence of the *N*-terminal component.

of the central region of GPIIIa (GKIRSKVELE; initial yield 27 pmol) and did not contain residues corresponding to the *N*-terminus of mature GPIIIa. Residues corresponding to the mature *N*-terminus would have been detected with an initial yield of 2 pmol.

Interpretation of the α -chymotrypsin digestion/partial-reduction results

Scheme 2 summarizes our results on the localization of the epitope recognized by monoclonal antibody CS-1. α -Chymotrypsin cleavage converts intact GPIIIa into a two-chain molecule composed of a 19/20 kDa *N*-terminal fragment held by disulphide bond(s) to a 58 kDa fragment which begins at residue 349 and may extend to the *C*-terminus of the intact polypeptide. Limited disulphide-bond reduction releases the *N*-terminal segment, and the resultant single-chain central fragment retains immunoreactivity toward CS-1. Therefore the α -chymotrypsin data indicate that the epitope recognized by monoclonal antibody CS-1 is contained within residues 349–762. Taken together with the results of trypsin cleavage, which show that removal of residues 300–422 correlates with complete loss of immunoreactivity (Scheme 1), we conclude that residues 349–422 contain the site on GPIIIa to which monoclonal antibody CS-1 binds. The conformation of this region of GPIIIa is evidently dependent on the presence of intrachain disulphide bonds, as extensive reduction results in loss of immunoreactivity.

We note that Calvete *et al.* [9] have shown that trypsin digestion of GPIIIa, followed by limited disulphide-bond reduction, yields a 23 kDa *N*-terminal fragment containing a single free cysteine residue and a mixture of 80/70 kDa polypeptides containing two

cysteine residues. They concluded that a single disulphide bond present within the first 17 kDa of GPIIIa linked the *N*-terminal region to a 80/70 kDa fragment [9]. Recently, Niewiarowski *et al.* [10] and Beer & Collier [8] have also presented evidence for intrachain disulphide bond(s) involving one of the first seven *N*-terminal cysteines and another cysteine residue on the large chymotryptic fragment which begins at residue 349 of GPIIIa. The data presented here establish that the disulphide bond which connects *N*-terminal and central domains of GPIIIa involves one of the seven cysteines within the *N*-terminal, 50 amino acids and one of the 42 cysteine residues present on the proteinase-resistant core formed by residues 423–762. Results of structural studies in the laboratories of Dr. J. Calvete and Dr. A. Henschen, who have been elucidating the disulphide-bond pairings in GPIIIa, are consistent with this interpretation (J. J. Calvete, personal communication; A. Henschen, personal communication).

Functional significance of the central region of GPIIIa

Biochemical and immunological data have clearly established an important role for the *N*-terminal region of GPIIIa in integrin-receptor-function. In particular, D'Souza *et al.* [5,6] have demonstrated that residues 109–171 of GPIIIa contain a site to which synthetic peptides containing the Arg-Gly-Asp sequence, common to all ligands which bind to the platelet GPIIb-IIIa complex, can be chemically cross-linked. Calvete *et al.* [9] have shown that monoclonal antibody P37 inhibits platelet aggregation and recognizes an *N*-terminal 17 kDa tryptic fragment of GPIIIa which contains the putative Arg-Gly-Asp recognition site identified by D'Souza *et al.* [5,6].

Whether this is the only region of GPIIIa involved in ligand binding remains open to question. For example, Niewiarowski *et al.* [10] have reported that a two-chain chymotryptic fragment of GPIIIa lacking residues 130–137 does not bind to immobilized fibrinogen or to immobilized synthetic peptide GRGDSPK; as intact GPIIIa was adsorbed by both these resins, they concluded that their 66 kDa chymotryptic fragment was not involved in fibrinogen binding. Conversely, immunological data suggest a possible role for the 66 kDa fragment in both fibrinogen binding and platelet aggregation [27–29].

The observation that the *N*-terminal segment of GPIIIa is held in close proximity to the central region of GPIIIa and the adjacent cysteine-rich domain by a single disulphide bond [9] suggests that regions of the molecule which are distant in linear sequence can contribute to integrin-receptor function. Our data support this postulate in that monoclonal antibody CS-1 binds to a site contained within residues 349–422 of GPIIIa (the present work) and causes a substantial inhibition of both platelet aggregation and binding fibrin(ogen) to stimulated platelets [11]. We have presented evidence that non-specific steric hindrance cannot explain these effects, since both intact monoclonal antibody and its Fab fragment are equally effective in blocking fibrinogen binding, and quantitatively similar inhibition parameters characterize the ability of CS-1 to block the binding of fibrinogen and fibrin polymers (composed of up to 20 fibrin monomers) to stimulated platelets [11]. Therefore we conclude that the mid-section of GPIIIa either contains a second site for fibrin(ogen) binding or contributes to the formation of a discontinuous ligand recognition region involving both the *N*-terminal and central regions of the molecule.

Further evidence supporting a role for residues 349–422 in integrin-receptor function comes from the use of predictive algorithms designed to identify sequences likely to be found on the exterior of a folded polypeptide [23–25]. As illustrated in Fig. 4, several regions of predicted high surface probability [23,24] and hydrophilicity [25] were identified between residues 349 and 422. For example, peptide bonds cleaved by chymotrypsin

Fig. 4. Hydrophilicity and surface probability of residues 330–440 of GPIIIa

The Figure shows a profile of hydrophilicity [25] (a) and surface probability [23,24] (b) against residue number calculated with the program PEPTIDESTRUCTURE of the GCG sequence analysis software package [26].

(348–349) and trypsin (352–353) are located within regions of GPIIIa predicted to be exposed to the solvent. In addition, the sequence QEKEK (residues 408–412) exhibited the highest surface probability (and one of the two highest hydrophilicity indices) of any region of GPIIIa and is in close proximity to the 422–423 bond cleaved by trypsin. In summary, these observations support the hypothesis that residues 349–422 constitute a ligand-recognition region, because this segment of GPIIIa is likely to be exposed to solvent and to be available for interactions with other macromolecules.

Special thanks are due to Dr. Juan J. Calvete and Dr. Agnes Henschen for their generosity in sharing unpublished results, for providing expert advice and for their critical insights concerning data interpretation. The technical assistance of Mr. Mark Morris and Ms. Denise Williams is gratefully acknowledged. This work was supported by National Institutes of Health (NIH) grant HL-32349 and North Carolina Affiliate of the American Heart Association grant-in-aid 1988-89-A-53. Amino acid sequence analyses were performed in the Protein Analysis Laboratory of the Cancer Center of Wake Forest University with support from NIH grants CA 1297 and RR-04869 and a grant from the North Carolina Biotechnology Center.

REFERENCES

1. Phillips, D. R., Charo, I. F., Parise, L. V. & Fitzgerald, L. A. (1988) *Blood* **71**, 831–843
2. Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F. & Ruoslahti, E. (1986) *Science* **231**, 1559–1562
3. Fitzgerald, L. A., Steiner, B., Rall, S. C., Lo, S.-S. & Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 3936–3939
4. Zimrin, A. B., Eisman, R., Vilaire, G., Schwartz, E., Bennett, J. S. & Poncz, M. (1988) *J. Clin. Invest.* **81**, 1470–1475
5. D'Souza, S. E., Ginsberg, M. H., Lam, S. C.-T. & Plow, E. F. (1988) *J. Biol. Chem.* **263**, 3943–3951
6. D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. & Plow, E. F. (1988) *Science* **242**, 91–93
7. Smith, J. W. & Cheresch, D. A. (1988) *J. Biol. Chem.* **263**, 18726–18731
8. Beer, J. & Collier, B. S. (1989) *J. Biol. Chem.* **264**, 17564–17573
9. Calvete, J. J., Rivas, G., Maruri, M., Alvarez, M. V., McGregor, J. L., Hew, C.-L. & Gonzalez-Rodriguez, J. (1988) *Biochem. J.* **250**, 697–704
10. Niewiarowski, S., Norton, K. J., Eckardt, A., Lukasiewicz, H., Holt, J. C. & Kornecki, E. (1989) *Biochim. Biophys. Acta* **983**, 91–99
11. Ramsamooj, P., Doellgast, G. & Hantgan, R. R. (1990) *Thromb. Res.* **58**, 577–592
12. Leung, L. L. K., Kinoshita, T. & Nachman, R. L. (1981) *J. Biol. Chem.* **256**, 1994–1997
13. Jennings, L. K. & Phillips, D. R. (1982) *J. Biol. Chem.* **257**, 10458–10466
14. Kohler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497
15. Eirin, M. T., Calvete, J. J. & Gonzalez-Rodriguez, J. (1986) *Biochem. J.* **240**, 147–153
16. Usobiaga, P., Calvete, J. J., Saiz, J. L., Eirin, M. T. & Gonzalez-Rodriguez, J. (1987) *Eur. Biophys. J.* **14**, 211–218
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 681–685
18. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
19. Hunkapillar, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236
20. Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
21. Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222
22. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444–2448
23. Emini, E. A., Hughes, J. V., Perlow, D. S. & Boger, J. (1985) *J. Virol.* **55**, 836–839
24. Janin, J., Wodak, S., Levitt, M. & Maigret, B. (1978) *J. Mol. Biol.* **125**, 357–386
25. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
26. Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
27. Kornecki, E., Tuszyński, G. P. & Niewiarowski, S. (1983) *J. Biol. Chem.* **258**, 9349–9356
28. McGregor, J. L., Clezardin, P., James, P., McGregor, L., Dechavanne, M. & Clemetson, K. J. (1985) *Eur. J. Biochem.* **148**, 97–106
29. Ruan, C., Du, X., Wan, H., Xi, X. & Li, P. (1987) *Thromb. Haemostasis* **58**, 901a

Received 3 September 1990/9 January 1991; accepted 16 January 1991