Human platelet glycoprotein IIIb binds to thrombospondin fragments bearing the C-terminal region, and/or the type I repeats (CSVTCG motif), but not to the N-terminal heparin-binding region

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Major blood membrane platelet glycoprotein IIIb (GPIIb), also termed GPIV or CD365, has been identified as a receptor for thrombospondin (TSP), collagen and *Plasmodium falciparum*-infected erythrocytes. The aim of the present study was to identify region(s) of TSP involved in binding of GPIIb. Proteolytic fragments of TSP (M_r 140 kDa, 120–18 kDa and 27 kDa on SDS/PAGE under reducing conditions) were purified by f.p.l.c. and identified by N-terminal gas-phase sequencing, e.l.i.s.a. and Western blots using monoclonal antibodies directed against defined domains of TSP. The 140 kDa and 120–18 kDa fragments (C-terminal region), but not the 27 kDa fragment (N-terminal region), were shown to bind to GPIIb by using e.l.i.s.a. and affinity-chromatography systems. TSP binding to a GPIIb-affinity column was Ca^{2+} -dependent and reduced by 45 % in the presence of EDTA. Moreover, TSP was only partially eluted with EDTA from a Ca^{2+} -equilibrated GPIIb column. A fragment of 68 kDa, obtained by further digestion of the 140 kDa fragment, bound to the GPIIb–Sephacryl affinity column. This fragment, or stalk-like region, bears the TSP type I repeats that show sequence similarity to regions on properdin, *Plasmodium falciparum* proteins and antistasin. Peptides (CSVTCG or SVTCGGGV) representing these repeats bound isolated GPIIb in a Ca^{2+} -independent way, but did not completely inhibit the GPIIb and TSP interaction. These studies indicate that GPIIb binds to the TSP via the C-terminal region and/or the CSVTCG motif, but not to the N-terminal region. Interaction between GPIIb and the TSP C-terminal region or the CSVTCG motif is respectively Ca^{2+} -dependent and -independent.

INTRODUCTION

Glycoprotein IIIb (GPIIb; also known as GPIV or CD36) is a major human blood platelet membrane adhesive receptor that bears the newly identified Nak^a alloantigen [1]. This highly hydrophobic glycoprotein, resistant to chymotrypsin or trypsin [2] degradation on intact platelets, has an apparent molecular-mass range of 85–95 kDa, and a pI range of 4.4–6.3 [3]. Platelets of patients with myeloproliferative disorders were observed to have an increased concentration of GPIIb associated with a decreased concentration of GPIb [4]. GPIIb-like adhesive receptors, with slightly different apparent molecular masses, have been identified on monocytes, endothelial cells, fetal erythrocytes and certain tumour-cell lines [5–8]. Recently PAS IV, a glycoprotein present in human or bovine mammary epithelial cells, was isolated from milk-fat-globule-membrane protein and was found to have immunological, as well as N-terminal amino-acid-sequence identities with GPIIb [9,10]. The cDNA of GPIIb, isolated from placenta, has been sequenced and shown to have no structural similarities to families of other glycoprotein receptors [11]. However, it remains to be seen whether all forms of GPIIb expressed on platelets, monocytes, endothelial cells and epithelial cells, have a common protein sequence that is differently glycosylated or, alternatively, share strong sequence similarities and are members of a new family of adhesive receptors.

GPIIb is known to act as a receptor to thrombospondin (TSP) [5], collagen [12] and *Plasmodium falciparum*-infected erythrocytes [13]. Moreover, GPIIb has been shown to bind to TSP in a Ca^{2+} -dependent manner [5,14]. Such GPIIb–TSP inter-

actions are thought to be one of the links mediating platelet–macrophage interactions in situations taking place during inflammation, atherosclerosis and thrombosis [15]. TSP is known to interact to resting platelets via its N-terminal heparin-binding domain [16] and to activated platelets, in the presence of Ca^{2+} , via its C-terminal domain [17]. In addition, an RGDA domain [18], located near the C-terminal domain, has been implicated in the binding of TSP to the $\alpha_v\beta_3$ integrin receptor present on endothelial cells, melanoma and platelets [19,20]. Finally, platelet adhesion to TSP was recently reported to occur via GPIa-IIa (also known as $\alpha_2\beta_1$ or VLA2) [21]. However, at this stage very little is known on region(s) of TSP involved in binding GPIIb. In the present study, interactions between purified GPIIb and well characterized isolated TSP fragments were investigated. Results show that TSP fragments bearing the C-terminal and/or the TSP type I repeats, but not the N-terminal region of TSP, bind GPIIb. The GPIIb–TSP interaction may occur via two TSP sites, one involving the C-terminal region, in the presence of Ca^{2+} , and the other involving the CSVTCG motif, present on the TSP type I repeats, in the absence of Ca^{2+} .

MATERIAL AND METHODS

Protein A–Sephacryl 4B, heparin–Sephacryl 4B gels and a complete f.p.l.c. system with Superose 12 columns were purchased from Pharmacia–LKB biotechnology (Paris, France). Leupeptin, Lubrol PX were purchased from Sigma (St. Louis, MO, U.S.A.). D-Phenylalanyl-L-prolyl-L-argininylchloromethane ('PPACK') was purchased from Calbiochem (San Diego, CA, U.S.A.).

Abbreviations used: GPIIb, glycoprotein IIIb; TSP, thrombospondin; PVDF, poly(vinylidene difluoride); MAbs, monoclonal antibodies; TFA, trifluoroacetic acid.

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Immobilon poly(vinylidene difluoride) (PVDF) membrane was from Millipore (Bedford, MA, U.S.A.). All reagents were of analytical grade.

Purification of thrombospondin and GPIIb

TSP was purified from material released by platelets on induction by thrombin by heparin–Sepharose-4B affinity chromatography as previously described [22,23]. GPIIb was purified from Triton X-114 platelet membrane extracts under non-denaturing conditions by anion-exchange and size-exclusion chromatography [14]. Isolated TSP and GPIIb were analysed by SDS/PAGE [24] and shown to be highly purified. TSP showed a single band of 160 kDa by analysis on SDS/PAGE under reducing conditions (Figs. 1a and 1b below, track 1). Purified GPIIb had an apparent molecular mass of 88 kDa by SDS/PAGE under both non-reduced and reduced conditions (results not shown).

Generation and isolation of TSP fragments

TSP was digested with chymotrypsin [25] (0.5 or 1 %, w/w) for 30 min at 37 °C or thrombin [26] (units/ml) for 4 h at 37 °C. Chymotrypsin or thrombin treatments of TSP were performed in the presence of 2 mM-CaCl₂ in 0.55 M-NaCl/10 mM-Tris/HCl, pH 7.5. TSP fragments were either purified on a Superose 12 column or its *N*-terminal fragment bound to a heparin–Sepharose 4B column connected to an f.p.l.c. system.

Gas-phase sequencing

Intact or TSP fragments were subjected to SDS/PAGE using the modification of Hunkapiller & Hood [27] and then transferred on PVDF membrane, the method of Matsudaira [28] being used. TSP and its fragments immobilized on PVDF membrane were Ponceau-stained, excised and sequenced with an Applied Biosystems gas-phase sequencer.

Binding of anti-TSP monoclonal antibodies (MAbs) to immobilized TSP or TSP fragments

Microtitration plates were coated overnight at 4 °C with TSP, TSP fragments and BSA at 2 µg/ml in 0.05 M-bicarbonate buffer, pH 9.5 [29]. The plates were then washed, blocked and incubated with MAbs directed against the *C*-terminal (MAb C6.7 [17]) or *N*-terminal (MAb A2.5 [30], LYP12 [31]) regions of TSP. Further characterization of the *N*-terminal region was performed after TSP and its fragments were Western-blotted [32] on to Immobilon PVDF membranes and incubated overnight at 2 µg/ml with LYP12.

Binding of GPIIb to immobilized TSP and TSP fragments in an e.l.i.s.a. system

Intact TSP, purified TSP fragments and BSA were coated overnight at 4 °C on microtitre wells at 2 µg/ml in 0.05 M-bicarbonate buffer, pH 9.5 [33]. Wells were then washed and treated with blocking buffer I (0.05 % Tween 20/20 mM-Tris/HCl (pH 7.4)/2 mM-CaCl₂/2 % BSA) for 2 h at room temperature. Purified GPIIb (4 µg/ml) resuspended in blocking buffer I was then added and left incubating for 3 h at room temperature. Bound GPIIb was detected by using an anti-GPIIb MAb (OKM5) or polyclonal antibody.

Binding of TSP and TSP fragments on a GPIIb-affinity column

TSP or purified TSP fragments were loaded on a GPIIb–Sepharose 4B column (HR 5/5; Pharmacia), equilibrated in 10 mM-Tris/0.15 M-NaCl/2 mM-CaCl₂ (or 5 mM EDTA), connected to an f.p.l.c. column. The column was repeatedly washed with 10 mM-Tris/1 M-NaCl, pH 7.4, and bound material was

eluted with the washing buffer containing 10 mM-EDTA. Further elution of bound material was with 50 mM-diethylamine, pH 11.5. Purified fibrinogen (IMCO, Stockholm, Sweden) was run on the GPIIb–Sepharose 4B column as a control under conditions identical with those used for TSP and its fragments.

Peptide synthesis and purification

Peptides were prepared by solid-phase synthesis [34] on an Applied Biosystems model 431A (San Jose, CA, USA) using 9-fluorenylmethoxycarbonyl- α -amino acids and *p*-hydroxymethylphenoxymethylpolystyrene resin purchased from Applied Biosystems. The assembled peptides were: (1) simultaneously cleaved from the resin and deprotected using standard trifluoroacetic acid (TFA) methods [35]; (2) precipitated and washed with diethyl ether; and (3) purified by preparative reversed-phase liquid chromatography using a C₈ Aquapore column (Brownlee; Applied Biosystems, San Jose, CA, U.S.A.) with a linear gradient of 0–60 % (w/v) acetonitrile in 0.1 % TFA and freeze-dried.

GPIIb binding to TSP-peptide-coated wells

Peptides (CSVTCG, SVTCGGGV) [35,38], derived from the TSP type I region repeats (residues 429–434 and 487–494 respectively) and controls derived from the *N*-terminal region (EDANLIP; residues 44–50) or *C*-terminal region (DHDSV; residues 917–921) were coated on microtitre plates at 40 µM in 0.05 M-bicarbonate buffer, pH 8.5, for 3 h at 37 °C. The coated e.l.i.s.a. wells were then treated with blocking buffer II (15 mM-Tris/HCl/2 mM-CaCl₂/0.5 % BSA/1 % Tween 20) for 2 h at 37 °C. The 2 mM-CaCl₂, present in blocking buffer II, was in some cases replaced by 2 mM-EDTA. Purified GPIIb, made in blocking buffer II, at 4 µg/ml was then added to the peptide-coated wells and incubated for 2 h at 37 °C. Bound GPIIb was detected by an anti-GPIIb MAb (OKM5) at 2 µg/ml.

Effect of TSP peptides on the binding of GPIIb to immobilized TSP

Microtitre wells were coated with TSP or BSA and blocked as previously described. Purified GPIIb was labelled with ¹²⁵I using the Iodobeads method [37] and was incubated overnight at 4 °C with 1 mM-TSP peptides (CSVTCG, SVTCGGGV, EDANLIP, DHDSV) in 10 mM-Tris/HCl (pH 7.4)/2 mM-CaCl₂/0.05 % Tween 20. GPIIb was then added to wells at 4 µg/ml and left incubating for 3 h at 37 °C. Bound GPIIb was detected using a γ -radiation counter.

RESULTS

Proteolysis and isolation of TSP fragments

Two major fragments, of 140 kDa and 27 kDa, resulting from the digestion of TSP with 0.5 % (w/v) chymotrypsin, were isolated by f.p.l.c. gel-exclusion chromatography (Fig. 1a). Under non-reducing electrophoretic conditions the 140 kDa fragments migrated at 420 kDa and the *M_r* of 27 kDa fragment remained unchanged (results not shown). With a higher concentration of chymotrypsin (1 %), two major (120–18 kDa, 30 kDa) and a minor fragment of 100 kDa were isolated (Fig. 1b). Disulphide-linked fragments, 120 kDa and 18 kDa, migrated under non-reducing conditions at an *M_r* close to 410 kDa. The minor 100 kDa fragment migrated at an *M_r* close to 300 kDa under non-reducing conditions.

Characterization of isolated TSP fragments

The isolated 30 kDa fragment bears the *N*-terminal region of TSP, since it can be isolated by heparin–Sepharose chromatography, shows the same *N*-terminal sequence (XRIPESGGDNSVFDIFELT) as intact TSP [38] and is recog-

Fig. 1. Separation by SDS/PAGE of purified TSP fragments obtained with different concentrations of chymotrypsin

(a) Isolated TSP digested with 0.5% (w/v) chymotrypsin at 37 °C for 30 min. Lane 1, intact TSP; lane 2, total TSP digest; lane 3, gel-filtered 140 kDa fragment; lane 4, gel-filtered 27 kDa fragment. (b) Isolated TSP digested with 1% (w/v) chymotrypsin at 37 °C for 30 min. Lane 1, intact TSP; lane 2, total TSP digest; lanes 3 and 4, gel-filtered 120–18 kDa fragment; lanes 5 and 6, gel-filtered 100 kDa fragment; lane 7, gel-filtered 30 kDa fragment.

Table 1. Binding of MAbs directed against the *N*-(A2.5, LYP12) or *C*-(C6.8) terminal region to TSP and its purified chymotryptic fragments

E.l.i.s.a. wells were coated with intact or purified TSP fragments (2 µg/ml; 140 kDa, 120–18 kDa, 30 kDa). MAbs were added to identify the presence or absence of the *N*- or *C*-terminal regions in the purified fragments. Bound antibodies were detected with a peroxidase-linked second antibody and enzyme substrate. The values shown are means ± S.D. for five experiments. Blanks corresponding to binding to BSA have been subtracted from the A_{450} values.

Antigens coated on plates	Binding of MAbs (A_{450})		
	C6.7	LYP12	A2.5
TSP	1.010 ± 0.050	0.800 ± 0.020	0.750 ± 0.025
140 kDa	1.010 ± 0.030	0.025 ± 0.010	0.020 ± 0.010
120–18 kDa	1.020 ± 0.050	0.015 ± 0.010	0.010 ± 0.005
27 kDa	0.180 ± 0.065	0.480 ± 0.018	0.500 ± 0.025

Table 2. Binding of GPIIb to intact or purified chymotryptic TSP fragments

Wells were coated with intact or purified TSP fragments (2 µg/ml; 140 kDa, 120–18 kDa, 27–30 kDa) and purified GPIIb was added at 4 µg/ml in the presence of 2 mM-CaCl₂. GPIIb binding was detected by using OKM5 (an anti-GPIIb MAb), a peroxidase-linked second antibody and enzyme substrate. The values shown are means ± S.D. for five experiments. Blanks corresponding to GPIIb binding to immobilized BSA have been subtracted from the A_{450} values.

Antigens coated on plates	Binding of GPIIb (A_{450})
TSP	0.630 ± 0.080
140 kDa	0.800 ± 0.065
120–18 kDa	0.610 ± 0.040
27 kDa	0.050 ± 0.025

nized, in e.l.i.s.a. assays (Table 1) or in Western blots (result not shown), by MAbs LYP12 or A2.5, known to be directed against *N*-terminal region of TSP. Fragments 140 kDa and 120–18 kDa bear the *C*-terminal region of TSP, since they were specifically recognized by a monoclonal C6.7 directed against that

region and not by LYP12 or A2.5 (Table 1). The *N*-terminal sequence of the 140 kDa and 120–18 kDa fragments (IGXKTKDLQAIXG) were identical with the 70 kDa chymotryptic fragment of TSP [39].

Binding of fluid phase GPIIb to immobilized fragments of thrombospondin

Purified GPIIb bound to native TSP, 140 kDa and 120–18 kDa fragments. GPIIb bound to TSP fragments bearing the *C*-terminal region, but not to the 30 kDa *N*-terminal fragment of TSP (Table 2).

Binding of TSP and TSP fragments to a GPIIb–Sepharose column

Intact TSP, but not fibrinogen, bound to GPIIb–Sepharose in the presence of 2 mM-CaCl₂ (Fig. 2 and Table 3). In addition, purified 140 and 120–18 kDa fragments, unlike the 30 kDa heparin-binding fragment, bound to GPIIb–Sepharose (results not shown). Over 55% of the bound TSP was eluted by 5 mM-EDTA, and the remaining TSP (45%) eluted at a basic pH (diethylamine, pH 11.5) (Fig. 2 and Table 3). In the presence of 5 mM-EDTA, binding of TSP to the GPIIb–Sepharose column was reduced by up to 45% (results not shown). In order to identify a smaller fragment of TSP binding to GPIIb, the 140 kDa TSP piece was further digested by thrombin (4 U/ml) for 4 h at 37 °C in the presence of 2 mM-CaCl₂, and it resulted in the appearance of a 68 kDa fragment. The 68 kDa fragment, when injected on the column, specifically bound to GPIIb and was eluted at pH 11.5 (Fig. 3).

Use of peptides to elucidate the GPIIb binding site on the 68 kDa TSP fragment

The above results indicate the existence of a domain on the 68 kDa TSP fragment capable of interacting with GPIIb in the absence of the 18 kDa *C*-terminal binding region. This 68 kDa fragment, or stalk-like region, bears the TSP type I repeats that show sequence similarity to regions on properdin, *Plasmodium falciparum* proteins (circumsporozoite protein, TRAP) and anti-stasin, a leech salivary protein [36,38]. Moreover, recent results from Asch *et al.* [40], suggested the involvement of the TSP type I repeat in GPIIb–TSP interactions. Peptides from the TSP type I repeats (CSVTCG or SVTCGGGV) and control peptides, originating from the *C*-terminal (DHDSV) or the heparin-binding region (EDANLIP) was synthesized and tested in an e.l.i.s.a. system. Purified GPIIb was observed to bind to

Fig. 2. Chromatographic profile of the binding and elution of TSP to a GPIIb-Sephacrose column on f.p.l.c.

Peaks eluted from the GPIIb-Sephacrose column were subjected under reducing conditions to SDS/10%-PAGE, Coomassie Blue-stained and are shown in the insets. Purified TSP was added to a GPIIb-Sephacrose column in the presence of Ca^{2+} (lane 1). Peak 2 (lane 2) represents flow-through material, peak 3 (lane 3) represents TSP eluted in the presence of 5 mM-EDTA and peak 4 (lane 4) represents TSP eluted with diethylamine, pH 11.5. Molecular-mass standards are shown next to lane 4.

Table 3. Binding of TSP and fibrinogen to a GPIIb-Sephacrose column on f.p.l.c.

TSP or fibrinogen (280 μg) were injected on a GPIIb-Sephacrose column in the presence of 2 mM- CaCl_2 . The different chromatographic fractions (flow-through, bound and eluted by 5 mM-EDTA or 0.1 M-diethylamine, pH 11.5) were assayed at 280 nm. The values shown are means $\pm$ S.D. for three experiments.

Fraction assayed	Total protein ($\mu\text{g}/\text{fraction}$)	
	TSP	Fibrinogen
Flow-through	29 $\pm$ 16	225 $\pm$ 25
Elution (EDTA, 5 mM)	141 $\pm$ 30	30 $\pm$ 13
Elution (pH 11.5)	110 $\pm$ 5	5 $\pm$ 6

Table 4. Binding of purified GPIIb to immobilized TSP peptides in the presence of 2 mM- CaCl_2 or 5 mM-EDTA

Wells were coated with 20 nM-TSP peptides (EDANLIP, DHDSV, CSVTCG). Purified GPIIb (4 $\mu\text{g}/\text{ml}$) was added in the presence of 2 mM- CaCl_2 or 5 mM-EDTA. After washing, bound GPIIb was measured by OKM5 (an anti-GPIIb MAb), a peroxidase-linked second antibody and enzyme substrate. The values shown are means $\pm$ S.D. for five experiments. Blanks corresponding to GPIIb binding to BSA have been subtracted from the A_{450} values.

Peptides coated on plates	GPIIb binding (A_{450}) in the presence of:	
	Ca^{2+}	EDTA
EDANLIP	0.194 $\pm$ 0.037	0.380 $\pm$ 0.040
DHDSV	0.219 $\pm$ 0.044	0.260 $\pm$ 0.040
CSVTCG	1.090 $\pm$ 0.218	1.096 $\pm$ 0.300

Fig. 3. Chromatographic profile of the binding and elution of the 68 kDa TSP fragment to a GPIIb-Sephacrose column on f.p.l.c.

Peaks eluted from the GPIIb-Sephacrose column were subjected under reducing conditions to SDS/10%-PAGE, silver-stained, and are shown in the insets. Peak 1 (lane 1) represents flow-through material and peak 2 (lane 2) represents the 68 kDa fragment eluted with 0.1 M-diethylamine, pH 11.5. Molecular-mass standards are shown next to lane 3.

Table 5. Binding of purified GPIIb to immobilized TSP in the presence or absence of TSP peptides

^{125}I -labelled GPIIb, incubated with 1 mM-TSP-derived peptides (EDANLIP, CSVTCG, or SVTCGGGV), was added to wells coated with TSP (2 $\mu\text{g}/\text{ml}$) in the presence of 2 mM- CaCl_2 . Bound GPIIb was measured, after washing the wells, in a γ -radiation counter. The values shown are means $\pm$ S.D. for five experiments. Blanks corresponding to GPIIb binding to BSA have been subtracted from the values.

Fraction added to immobilized TSP	$10^{-3} \times \text{GPIIb bound (c.p.m.)}$
GPIIb	5551 $\pm$ 1000
GPIIb + EDANLIP	4646 $\pm$ 872
GPIIb + CSVTCG	3066 $\pm$ 263
GPIIb + SVTCGGGV	2656 $\pm$ 572

CSVTCG (Table 4) in a Ca^{2+} -independent way, but not to control peptides (Table 4). These results strongly indicate that the 68 kDa TSP fragment binds to GPIIb via the CSVTCG repeats. However, CSVTCG or SVTCGGGV, on their own, poorly inhibited (in the range between 45 and 55%) the binding intact TSP (Table 5) or the binding of endogenous TSP to activated platelets (results not shown). Moreover, these peptides did not affect platelet functions induced by low concentration of agonists (results not shown).

DISCUSSION

The present study shows, for the first time, that TSP fragments bearing the C-terminal and/or the TSP type I repeats (CSVTCG), but not the N-terminal region, bind to isolated GPIIb. Several

lines of evidence support these results: (1) plastic wells coated with the 30 kDa heparin-binding region fragments of TSP did not bind isolated GPIIb; (2) the 30 kDa fragment did not bind to a GPIIb-Sepharose affinity column; (3) coated 140 kDa and 120–18 kDa fragments, bearing the C-terminal region of TSP bound isolated GPIIb; (4) the 140 kDa and 120–18 kDa fragments bound to a GPIIb-Sepharose affinity column; (5) a 68 kDa fragment, resulting from the further digestion of the 140 kDa TSP piece by thrombin, bound to a GPIIb-Sepharose affinity column; (6) the coated TSP type I repeat peptides (CSVTCG, SVTCGGGV), known to be present on the 68 kDa TSP fragment, bound isolated GPIIb; (7) in the presence of EDTA, interaction between GPIIb and the 140 kDa or 120–18 kDa fragments was greatly reduced, as was expected from previous results [5,14]. To support these data the identity of TSP fragments used in binding assays were obtained by gas-phase sequencing, Western blots and e.l.i.s.a. assays using MAbs directed against the N- or C-terminal regions of TSP.

Resting platelets express a relatively low number of binding sites (2000–3000/platelet) for endogenous (as measured by anti-TSP MAb) or exogenous TSP (as measured by purified and labelled TSP) [41–43]. Sulphated glycolipids on the surface of resting platelets are thought to act as receptors for TSP binding via its N-terminal region [16]. Such TSP-sulphated-glycolipid interaction is cation-independent and inhibited by heparin, fucoidin and anti-TSP MAb A2.5 (directed against the N-terminal heparin-binding region of TSP) [30]. Data obtained in the present study showing that GPIIb does not interact with the N-terminal heparin-binding region of TSP, are very much in line with previously mentioned results. The role of the N-terminal heparin-binding region of TSP in platelet functions has yet to be elucidated. However, it is known to be involved in the lectin-like activity of TSP in agglutinating erythrocytes and thrombin-activated fixed platelets [44]. Antibodies directed against the N-terminal domain inhibited TSP-induced agglutination of fixed platelets, but did not affect, with one exception [45], platelet aggregation [30,31].

In contrast, thrombin-activated platelets express a much larger number of binding sites for endogenous TSP (16000/platelet) or exogenous TSP (36000/platelet) [39,41]. TSP released by platelets upon stimulation [46,47] is thought to play an important role in stabilizing platelet aggregates [48,49]. The TSP-binding sites on activated platelets are cation-dependent, present on stimulated platelets of Glanzmann-thrombasthenic patients [39,41] (platelets showing a severe reduction or absence of GPIIb–IIIa) and inhibited by anti-GPIIb MAbs [50] or polyclonal antibodies [14]. Isolated platelet GPIIb was previously observed to interact with TSP in a Ca^{2+} -dependent manner [5,14]. Results obtained in the present study clearly indicate that GPIIb interacts, in the presence of Ca^{2+} , with the 140 and 120–18 kDa TSP-fragments bearing the C-terminal domain. However, in the presence of EDTA the binding of GPIIb to these TSP fragments is considerably reduced. One structure on these TSP fragments that is very sensitive to Ca^{2+} chelation is the C-terminal domain [38]. In the absence of Ca^{2+} the large globular C-terminal domain unfolds, with considerable modifications in its structural and functional properties [25,51]. Since the binding of GPIIb to TSP fragments is closely linked with a folded, Ca^{2+} -sensitive C-terminal domain, one can assume that GPIIb interacts with determinants within that domain. Moreover, the C-terminal domain was previously shown by MAb C6.7, which inhibits platelet aggregation [17], to be implicated as a site allowing TSP to bind to activated platelets.

If the C-terminal domain were the only site on TSP binding GPIIb, one would expect no interaction between these two glycoproteins in the absence of Ca^{2+} , in contrast with what was reported [5,14] and currently observed. Results obtained in the

present study show a 68 kDa TSP fragment lacking the C-terminus and capable of binding GPIIb in a Ca^{2+} -independent way. In addition, TSP type I repeats (CSVTCG or SVTCGGGV motif), known to be present on the 68 kDa, bound GPIIb in the absence of Ca^{2+} . This stalk-like region of TSP, bearing the peptide repeats is known to bind collagen in a Ca^{2+} -independent way [51]. One should note that these peptides (CSVTCG, SVTCGGGV) did not completely block the binding of TSP to GPIIb, or to activated platelets, and had no effect on platelet functions. Peptides may not completely inhibit GPIIb–TSP interactions, either because they do not have the proper conformation or length, or simply that an additional site is present on TSP to bind GPIIb. Another possible explanation for the above data could be that GPIIb interacts with just one region on TSP, the type I repeats, and that Ca^{2+} chelation affects the globular C-terminal domain and alters the TSP configuration to such an extent that GPIIb–TSP interaction is reduced, but not abolished. This last possibility, while plausible, does not provide a complete explanation of the observed results. Recent work has shown that peptides based on the TSP type I repeats and containing the CSVTCG or VTCG motif are capable of acting as an attachment site to cells [52,53]. It is conceivable that, on some of these cells, GPIIb, known to be expressed by a number of cells [54], directly interacts with peptides bearing the CSVTCG or VTCG motif. As previously indicated, these TSP type I repeats show sequence similarity to regions on properdin, *Plasmodium falciparum* proteins (circumsporozoite protein, TRAP) and antistasin, a leech salivary protein. The role of these motifs (CSVTCG, VTCG) remains poorly understood, and one of their functions may be to act as a binding site for cellular adhesive receptors such as GPIIb.

Results obtained in the present study strongly suggest that GPIIb has two binding sites on TSP: one that is Ca^{2+} -sensitive and located on the C-terminal domain, and another that is Ca^{2+} -independent and located on the stalk-like region bearing the TSP type I repeats. The presence of two binding sites on adhesive-protein ligands for an activated receptor is not uncommon and is observed in the case of fibrinogen interacting with GPIIb–IIIa via its RGD and γ -C-terminal domains [55].

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