Interaction of thrombospondin with platelet glycoproteins GPI_a-II_a and GPII_b-III_a

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The interaction of human thrombospondin (TSP) with GPI_a-II_a and GPII_b-III_a was studied. The binding for both proteins became time-independent after 60 min. A 7-fold excess concentration of unlabelled GPI_a-II_a added either initially, or after time-dependent binding, resulted in a 50 % inhibition of GPI_a-II_a bound to TSP. GPI_a-II_a and GPII_b-III_a specifically bound TSP since: (a) the binding of GPII_b-III_a to TSP was dependent on the presence of 1 mM MgCl₂ and 1 mM CaCl₂, whereas binding of GPI_a-II_a was ion-independent. (b) The binding was saturable, with dissociation constants of $0.69 \pm 0.17 \mu\text{M}$ and $3.77 \pm 1.02 \mu\text{M}$ for GPI_a-II_a and GPII_b-III_a respectively. (c) GPII_b-III_a and GPI_a-

II_a did not significantly bind to BSA. (d) GPII_b-III_a bound fibrinogen ion-specifically, whereas little or no binding of GPI_a-II_a was detectable. (e) Both GPII_b-III_a and GPI_a-II_a bound collagen in an ion-independent manner. (f) GPII_b-III_a did not compete with GPI_a-II_a for binding to TSP. (g) Binding of GPI_a-II_a to TSP was inhibited with anti-(GPI_a-II_a) (6F1), whereas mouse IgG and anti-(GPII_b-III_a) (AP-2) had no effect. (h) The interaction of GPI_a-II_a with TSP is 5.5-fold more favourable than that of GPII_b-III_a suggesting that GPI_a-II_a may be a preferred binding protein for TSP-mediated platelet adhesion.

INTRODUCTION

Thrombospondin (TSP) is a major platelet-secreted protein, that was first purified in its non-denatured state by Lawler and co-workers in 1978 [1]. Since then a great number of studies have been published describing its structure, biological function and tissue distribution. For example, TSP has been found to be a multi-domain protein much like fibronectin with binding sites for heparin [2], collagen [3], and fibrinogen [4]. A number of studies have demonstrated that TSP promotes cell-substratum adhesion of a variety of cells including fibroblasts [5], endothelial cells [5,6], epithelial cells [5] and various tumour cell lines such as melanoma cells [5–8], small-cell carcinoma cells [8], and osteosarcoma cells [9]. Recent studies have also postulated a role for TSP in cell proliferation [10] and angiogenesis [11].

A major interest of ours has been to define a role for TSP in haemostasis. Toward this end, we have observed that TSP promotes platelet aggregation [12] and platelet adhesion [13]. A possible mechanism which might account for our previous observations would require secreted TSP to bind to platelet receptors and cross-link platelets to themselves and to exposed collagen fibrils in the damaged vessel. A similar mechanism, which proposes that TSP stabilizes platelet aggregates by cross-linking platelet-bound fibrinogen, has been proposed previously to explain the role of TSP in platelet aggregation [14].

Our previous studies have identified two potential platelet TSP-binding proteins which could function to bind secreted TSP. The first is GPII_b-III_a, the platelet fibrinogen receptor, which reversibly binds TSP *in vitro* [15] and antibodies against which inhibit TSP-promoted platelet aggregation and adhesion [13,15]. The second is GPI_a-II_a, a platelet collagen receptor [16]. It was shown that human platelets from a patient with a bleeding disorder, showing a deficiency of glycoprotein I_a, failed to respond to collagen [17] and displayed a decreased platelet adhesion to subendothelium [18]. We have shown that anti-

(GPI_a-II_a) monoclonal antibodies (mAbs) completely inhibited the TSP-dependent adhesion of normal and thrombasthenic platelets [13]. This inhibition was dose-dependent and complete inhibition could be achieved with as little as 2 $\mu\text{g}/\text{ml}$ of antibody, while no more than 50 % inhibition of adhesion could be achieved with anti-(GPII_b-III_a) mAb at concentrations in excess of 50 $\mu\text{g}/\text{ml}$. These results suggested that the interaction of GPI_a-II_a with TSP must be more favourable than the interaction of GPII_b-III_a with TSP, despite the fact that platelets contain approx. 50 000 GPII_b-III_a receptors as measured by anti-(GPII_b-III_a) mAb binding [19], and only 800 GPI_a-II_a receptors as measured by anti-(GPI_a-II_a) mAb binding [20].

The aim of the present study was to demonstrate that TSP could interact with GPI_a-II_a in a specific and favourable manner using an *in vitro* binding assay. Our results show that TSP can specifically interact with GPI_a-II_a and that this interaction is more favourable than the interaction of TSP with GPII_b-III_a. These results suggest that GPI_a-II_a could function as the physiologically preferred platelet TSP-binding protein.

EXPERIMENTAL

Materials

All reagents, unless specified otherwise, were purchased from Sigma. Reagents for SDS/PAGE were obtained from Bio-Rad. Iodobeads were purchased from Pierce. Na¹²⁵I was purchased from Amersham. mAb Gi9, specific against human platelet GPI_a-II_a, and mAb CD41a, specific against platelet GPII_b-III_a, were purchased from Amac. mAb AP-2 was kindly provided by Dr. Thomas J. Kunicki, The Blood Center of Southeastern Wisconsin, Milwaukee, WI, U.S.A. AP-2 is directed against epitopes on both GPII_b and GPIII_a [21]. mAb 6F1, specific for human platelet GPI_a-II_a [20], was kindly provided by Dr. Barry S. Collier (Department of Medicine, State University of New York, Stony Brook, NY, U.S.A.). Microtitre plates, Removawell

Immulon 4, were purchased from Dynatech. CNBr-activated Sepharose was purchased from Pharmacia.

TSP purification

TSP was purified from Ca^{2+} ionophore (A23187)-activated human platelets as described previously [4].

Protein assays

Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay, adapted for microtitre plates, as described by Pierce. BSA was used as the standard.

PAGE

SDS/PAGE was performed using the Pharmacia Phast Gel system. Gels were stained, dried on to paper, and autoradiograms were prepared from the dried gels using intensifying screens (DuPont Cronex Lighting Plus screens mounted in Spectroline Cassettes, Reliance X-Ray, Orelan, PA, U.S.A.). Kodak X-Omat-AR film was used and developed according to the instructions provided with the film. Films were exposed overnight at -70°C .

Protein labelling

Purified $\text{GPII}_b\text{-III}_a$, $\text{GPI}_a\text{-II}_a$ and TSP were labelled with ^{125}I using Iodobeads as described previously [15]. Briefly, 50 μg of purified protein dissolved in octylglucoside buffer (see method for purification of $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$) was incubated with one Iodobead for 5 min. Unreacted iodide was removed on a small column of Sephadex G-25 equilibrated in octylglucoside buffer as previously described [22]. The specific radioactivities of protein obtained from typical labelling experiments ranged between 5×10^6 and 1×10^7 c.p.m./ μg . When comparing the binding of $\text{GPII}_b\text{-III}_a$ with $\text{GPI}_a\text{-II}_a$ specific radioactivities were adjusted to the same value (1×10^6 c.p.m./ μg).

Purification of $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$

$\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$ were purified by immunoaffinity chromatography from platelet detergent extracts. Briefly, 5 units of washed platelet pellets, obtained after the centrifugation step following ionophore stimulation to obtain TSP [4], were dissolved in 10 ml of octylglucoside buffer (50 mM Tris/HCl, pH 7.35, containing 150 mM NaCl, 100 mM octylglucoside, 100 μM leupeptin and 1 mM phenylmethanesulphonyl fluoride). Undissolved material was removed by centrifugation of the detergent extract at 4°C for 30 min at 10000 g. Platelet detergent extract (10 ml) was first passed through an anti-($\text{GPII}_b\text{-III}_a$) mAb-Sepharose column (prepared by coupling 2 mg of CD41a to 1 ml of CNBr-activated Sepharose according to the instructions provided by Pharmacia) and then over an anti-($\text{GPI}_a\text{-II}_a$) mAb-Sepharose column (prepared by coupling 2 mg of Gi9 to 1 ml of CNBr-activated Sepharose). Antibody-treated columns were washed with 10 ml of binding buffer, containing 0.5 M NaCl, and eluted with 100 mM glycine, pH 2.7, containing 50 mM octylglucoside. Fractions (0.5 ml) were collected into tubes containing 10 μl of 1 M Tris/HCl added to the collection tubes to neutralize the solution. Peak protein fractions were pooled and concentrated.

Approx. 2 mg of $\text{GPII}_b\text{-III}_a$ and 100 μg of $\text{GPI}_a\text{-II}_a$ were isolated from 5 units of washed platelets.

Binding assays

Samples (50 μl) of radiolabelled $\text{GPII}_b\text{-III}_a$ or $\text{GPI}_a\text{-II}_a$ containing approx. 100000 c.p.m. and dissolved in binding buffer (50 mM Tris/HCl, pH 7.35, containing 150 mM NaCl, 50 mM octylglucoside, 100 μM leupeptin and 1 mM phenylmethanesulphonyl fluoride) were incubated for 1 h in detachable microtitre wells (Immulon 4 Removawell) which had been previously coated overnight at 4°C with 50 μl of a 40 $\mu\text{g}/\text{ml}$ TSP solution in 20 mM Bis-Tris-propane buffer, pH 6.5, and blocked for 1 h with 200 μl of 1% (w/v) BSA. Wells were then washed three times by aspiration with 200 μl of binding buffer and bound radioactivity levels determined by counting each well in a γ -counter.

Wells were saturated with 1 μg of adsorbed protein under the conditions of our protein-coating procedure, as determined from radioactive protein studies and the BCA protein assay (results not shown). The amount of radioactive protein bound ranged from 5 to 10% of input radioactivity. Radioactive protein bound to control BSA-coated wells was, in all experiments, less than 5% of that bound to TSP or glycoproteins, and was subtracted from experimental values given for test proteins unless otherwise stated. For SDS/polyacrylamide gel analysis, bound $\text{GPI}_a\text{-II}_a$ was eluted from immobilized TSP by incubation of washed wells with 50 μl of SDS/PAGE sample buffer.

RESULTS

Analysis of purified $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$

The platelet integrins, $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$, were purified from washed human platelets by antibody-affinity chromatography. The purified integrins were radiolabelled with [^{125}I]iodine and typically specific radioactivities of 10^6 c.p.m./ μg were obtained. The silver-stained as well as the radiolabelled proteins, were characterized on SDS/polyacrylamide gels (Figure 1). The low-molecular-mass impurities in $\text{GPI}_a\text{-II}_a$ are probably proteolytic breakdown products, because they immunoprecipitate with anti-($\text{GPI}_a\text{-II}_a$) antibody (results not shown). However, they do not interfere with the binding experiments as $\text{GPI}_a\text{-II}_a$, which eluted from the binding plates, contained very little of the lower-molecular-mass material (compare lanes 2 and 3 in Figure 1). The major polypeptides bound are the undegraded α and β chains of $\text{GPI}_a\text{-II}_a$. The estimated apparent molecular masses obtained for the purified proteins on our gel system (130 and 97 kDa for $\text{GPII}_b\text{-III}_a$ and 150 and 120 kDa for $\text{GPI}_a\text{-II}_a$) were consistent with published values [19,20]. In addition, SDS/polyacrylamide gel analysis of the 6F1 and AP-2 immunoprecipitates of labelled $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$ revealed the same bands as shown in Figure 1 (results not shown).

The requirements for binding of $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$ to immobilized TSP, collagen and fibrinogen

The binding of $\text{GPI}_a\text{-II}_a$ to TSP became time-independent after 60 min and could be partially reversed with the addition of a 7-fold excess of unlabelled $\text{GPI}_a\text{-II}_a$ at time zero or after binding had proceeded for 45 min (Figure 2). $\text{GPII}_b\text{-III}_a$ could not prevent the binding of $\text{GPI}_a\text{-II}_a$ to TSP or displace bound $\text{GPI}_a\text{-II}_a$ after binding had proceeded for 45 min, indicating that $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$ do not share the same binding domains on TSP (Figure 3). The bivalent ion requirements for binding of $\text{GPI}_a\text{-II}_a$ and $\text{GPII}_b\text{-III}_a$ to TSP differ significantly (Table 1). For

Figure 1 SDS/polyacrylamide-gel analysis of GPI_a-II_a and GPII_b-III_a purified by antibody-affinity chromatography from platelets

Approx. 250 ng of unlabelled and 10 ng of labelled protein were separated under non-reducing conditions on a 7% (w/v) polyacrylamide Pharmacia SDS Phast gel. The gel was silver stained, dried and subjected to autoradiography. Lane 1, silver stain of GPI_a-II_a; lane 2, autoradiogram of ¹²⁵I-GPI_a-II_a; lane 3, autoradiogram of ¹²⁵I-GPI_a-II_a eluted from immobilized TSP; lane 4, silver stain of GPII_b-III_a; lane 5, autoradiogram of ¹²⁵I-GPII_b-III_a.

Figure 2 Time course for the binding of ¹²⁵I-GPI_a-II_a to TSP in the presence and absence of unlabelled GPI_a-II_a

TSP-coated microtitre plate wells were incubated for various timed intervals with 50 µl of a 30 µg/ml solution of ¹²⁵I-labelled GPI_a-II_a in binding buffer containing no additional GPI_a-II_a (○) or containing 200 µg/ml of unlabelled GPI_a-II_a (▽) and the bound radioactivity determined. The amount of bound radioactivity determined after addition of unlabelled GPI_a-II_a to a final concentration of 200 µg/ml after 45 min of incubation is shown by the broken line. Values given are the means of two duplicate determinations and are representative of a typical experiment.

example, maximal binding of GPII_b-III_a to TSP requires 1 mM CaCl₂ and 1 mM MgCl₂, whereas the binding of GPI_a-II_a is ion-independent. The ion dependence for the interaction of GPI_a-II_a and GPII_b-III_a with TSP is the same whether TSP is immobilized and fluid-phase glycoprotein is bound, or whether glycoprotein is immobilized and fluid-phase TSP is bound (results not shown); this suggests that the binding of TSP to glycoprotein is not dependent on conformational changes in protein caused by adsorption to microtitre wells. Both GPII_b-III_a and GPI_a-II_a were found to bind collagen in an ion-independent manner

Figure 3 The time course for the binding of ¹²⁵I-GPI_a-II_a to TSP in the presence and absence of unlabelled GPII_b-III_a

TSP-coated microtitre plate wells were incubated for various timed intervals with 50 µl of a 30 µg/ml solution of ¹²⁵I-labelled GPI_a-II_a in binding buffer (○) or containing 20 µg/ml of unlabelled GPII_b-III_a (▽) added at time zero and the bound radioactivity was determined. Values given are the means of two duplicate determinations and are representative of a typical experiment.

Table 1 The effect of bivalent cations and EDTA on the binding of radiolabelled GPI_a-II_a and GPII_b-III_a to immobilized TSP

Wells were coated with 40 µg/ml of TSP, blocked with BSA, and incubated with 30 µg/ml of radiolabelled protein for 1 h. Wells were then washed and radioactivity measured.

Additions	¹²⁵ I-labelled protein bound (c.p.m.)	
	GPI _a -II _a	GPII _b -III _a
Mg ²⁺ /Ca ²⁺	1100 ± 83	900 ± 12
Mg ²⁺ /Ca ²⁺ /Mn ²⁺	710 ± 48	760 ± 10
EDTA	1200 ± 12	100 ± 6

(Table 2). In contrast, GPII_b-III_a bound fibrinogen in an ion-dependent manner.

The effect of anti-(GPI_a-II_a) antibody on the binding of GPI_a-II_a to TSP

To establish further the specificity of the interaction of GPI_a-II_a with TSP, we assessed the capacity of anti-(GPI_a-II_a) (6F1) to block binding. mAb 6F1 has previously been shown by us to quantitatively block TSP-dependent platelet adhesion [13]. We found that 6F1 blocked the binding of GPI_a-II_a to TSP. For example in the presence of 10 µg/ml of 6F1 IgG the amount of radioactive GPI_a-II_a bound was 500 ± 160 c.p.m./well compared with 1670 ± 250 c.p.m./well in the presence of the same amount of control mouse IgG. In addition, in the presence of 10 µg/ml of AP-2, an mAb raised against GPII_b-III_a, 1430 ± 200 c.p.m./well was bound while 1600 ± 300 c.p.m./well was bound for the buffer control. Elution of ¹²⁵I-GPI_a-II_a bound to TSP revealed that the majority of the bound counts represented GPI_a-II_a and not small amounts of contaminating proteins present in the starting material (compare lanes 2 and 3 in Figure 1). These results not only confirm that GPI_a-II_a binds TSP specifically, but

Table 2 Comparison of the effect of bivalent cations and EDTA on the binding of 125 I-labelled GPI $_b$ -II $_a$ and GPII $_b$ -III $_a$ to TSP, collagen, fibrinogen and BSA

The binding of GPI $_b$ -II $_a$ and GPII $_b$ -III $_a$ to immobilized TSP, rat tail type-1 collagen (coll), human fibrinogen (FG) and BSA was compared. Binding buffer contained either 2 mM EDTA or 1 mM CaCl $_2$ and 1 mM MgCl $_2$. The concentration of radiolabelled integrins was 30 μ g/ml. Values given are the means of two duplicate determinations and are representative of a typical experiment.

Solid phase	Fluid-phase 125 I-protein bound (c.p.m.)			
	GPI $_b$ -II $_a$		GPII $_b$ -III $_a$	
	EDTA	Mg $^{2+}$ /Ca $^{2+}$	EDTA	Mg $^{2+}$ /Ca $^{2+}$
TSP	1310 $\pm$ 60	1130 $\pm$ 81	200 $\pm$ 12	870 $\pm$ 32
Coll	1400 $\pm$ 82	660 $\pm$ 66	740 $\pm$ 72	980 $\pm$ 42
FG	150 $\pm$ 42	280 $\pm$ 48	10 $\pm$ 6	1000 $\pm$ 21
BSA	10 $\pm$ 6	10 $\pm$ 6	10 $\pm$ 6	10 $\pm$ 5

Table 3 The effect of fluid-phase TSP on the binding of GPI $_b$ -II $_a$ to solid-phase TSP

TSP-coated microtitre wells were incubated for 1 h at room temperature with 50 μ l of a 1.5 μ g/ml solution of 125 I-labelled GPI $_b$ -II $_a$ in binding buffer containing various concentrations of TSP, mouse IgG and BSA. The protein-GPI $_b$ -II $_a$ solutions were preincubated for 30 min before addition to TSP-coated wells. Wells were washed and the bound radioactivity counted. Values are the means of three determinations $\pm$ S.E.M. Percentage inhibition was calculated as [c.p.m. (μ g/ml TSP) - c.p.m. (X μ g/ml TSP)]/c.p.m. (0 μ g/ml TSP) $\times$ 100, where X is 5, 10, 20, or 40.

Protein (μ g/ml)	Bound 125 I-(GPI $_b$ -II $_a$) (c.p.m.)			Percentage inhibition by TSP
	TSP	IgG	BSA	
0	2230 $\pm$ 335	2480 $\pm$ 463	2180 $\pm$ 286	0
5	1160 $\pm$ 40	2430 $\pm$ 305	2005 $\pm$ 329	48
10	1092 $\pm$ 87	2307 $\pm$ 162	1920 $\pm$ 192	51
20	669 $\pm$ 48	1988 $\pm$ 266	1965 $\pm$ 126	70
40	332 $\pm$ 44	2040 $\pm$ 180	1918 $\pm$ 207	85

also lend support to our previous studies showing that GPI $_b$ -II $_a$ may function as a platelet TSP-binding protein [13].

To establish that binding of GPI $_b$ -II $_a$ to solid-phase TSP is the same as binding to fluid-phase TSP, fluid-phase TSP was evaluated for its capacity to inhibit binding of GPI $_b$ -II $_a$ to solid-phase TSP. We found that increasing concentrations of fluid-phase TSP competed for the binding of solid-phase TSP, whereas IgG and BSA had no effect (Table 3). Therefore, fluid-phase and solid-phase TSP bind GPI $_b$ -II $_a$.

Comparison of the binding constants for the interaction of GPI $_b$ -II $_a$ and GPII $_b$ -III $_a$ with TSP

We previously observed that TSP-mediated platelet adhesion could be completely inhibited with anti-(GPI $_b$ -II $_a$) mAbs but only partially inhibited with anti-(GPII $_b$ -III $_a$) mAbs [13]. These results suggested that the interaction of GPI $_b$ -II $_a$ with TSP was more favourable than the interaction of GPII $_b$ -III $_a$ with TSP. To test this hypothesis, the apparent dissociation constant (K_d) for the interaction of TSP with GPII $_b$ -III $_a$ was measured and

Figure 4 Concentration dependence of the binding of 125 I-labelled GPI $_b$ -II $_a$ and GPII $_b$ -III $_a$ to TSP

Binding was performed to immobilized TSP in binding buffer containing 1 mM CaCl $_2$ and 1 mM MgCl $_2$. The closed and open circles depict the binding obtained with GPI $_b$ -II $_a$ and GPII $_b$ -III $_a$ respectively. Specific radioactivities for both proteins were adjusted to the same value (10 5 c.p.m./ μ g). Values given are the means of two duplicate determinations and are representative of a typical experiment. The line drawn was computer generated by a least-squares-fit to a rectangular hyperbola with calculated dissociation constants of 0.69 ± 0.17 μ M ($\pm$ S.E.M.) for GPI $_b$ -II $_a$ and 3.77 ± 1.02 μ M ($\pm$ S.E.M.) for GPII $_b$ -III $_a$ with correlation coefficients of 0.933 and 0.938 respectively (GraphPad Inplot, Version 3.1, San Diego, CA, U.S.A.).

compared with that obtained for GPI $_b$ -II $_a$ (Figure 4). We found that the K_d value for the interaction of GPI $_b$ -II $_a$ with TSP was 0.69 μ M and that for the interaction of GPII $_b$ -III $_a$ with TSP was 3.77 μ M. These results indicate that GPI $_b$ -II $_a$ binds TSP 5.5-fold more favourably than GPII $_b$ -III $_a$.

DISCUSSION

The identity of the cell receptor mediating TSP-dependent cell-adhesive interactions has been of great interest to us because of our previous work showing that antibodies raised against the platelet fibrinogen receptor, GPII $_b$ -III $_a$, inhibited TSP-dependent platelet aggregation [12] and TSP-dependent melanoma cell adhesion [23], and that GPII $_b$ -III $_a$ could bind TSP *in vitro* [15]. Similar observations were made by Lawler and co-workers who showed that (GPII $_b$ -III $_a$)-like integrin receptors could be isolated by TSP-affinity chromatography from endothelial and smooth muscle cells [24] and from platelets [25]. However, the specific functional consequence of these binding interactions remained unclear since GPII $_b$ -III $_a$ and (GPII $_b$ -III $_a$)-like integrin receptors display a broad reactivity with a number of adhesive proteins, notably fibrinogen [26]. In addition, several studies have failed to show an interaction of GPII $_b$ -III $_a$ with TSP *in vitro*. For example, Leung and Nachman [27], using an enzyme-linked immunoassay, could demonstrate an interaction between GPII $_b$ -III $_a$ and fibrinogen but not between GPII $_b$ -III $_a$ and TSP. Similarly, Pytela et al. [28], using GPII $_b$ -III $_a$ incorporated into liposomes, could not demonstrate an interaction with TSP.

Non-integrin TSP-cell binding proteins have also been described. Human lung small-cell carcinoma has been shown to bind TSP through a two-chain receptor having molecular masses of 80000 and 105000 [29]. Human lung carcinoma cells contain a 50000 molecular-mass CSVTG-specific TSP receptor [30]. Platelet GPIV or CD36 has been referred to as the platelet TSP receptor because platelet GPIV was found to bind TSP *in vitro* [31] and GPIV was found to mediate platelet-monocyte interactions in a TSP-dependent manner [32]. However, the presence

or absence of CD36 in malaria-parasitized erythrocytes had no effect on TSP binding, suggesting that CD36 is not a TSP receptor in this system [33]. Platelets deficient in GPIV bound TSP to the same extent as normal platelets containing GPIV, suggesting that GPIV is not a TSP receptor in this system as well [34].

In an effort to identify and characterize platelet TSP receptors further, we recently developed a rapid and sensitive cell-adhesion assay [35]. We have used this assay to identify possible platelet and cell-adhesion receptors. In a recent study aimed at identifying potential platelet TSP receptors, we discovered that antibodies against the platelet fibrinogen receptor, GPII_b-III_a, and GPI_b-II_b, the collagen receptor, inhibited TSP-mediated platelet adhesion [13].

To establish further that GPI_b-II_b and GPII_b-III_a could function as platelet-adhesion receptors, as suggested from our earlier antibody studies, we undertook the present study comparing the binding interactions of GPI_b-II_b and GPII_b-III_a with TSP using an *in vitro* binding assay. Our results indicate that both GPI_b-II_b and GPII_b-III_a interact with TSP in a reversible and saturable manner. The binding interaction between TSP and GPI_b-II_b differs significantly from that of GPII_b-III_a. GPI_b-II_b interacts with TSP in an ion-independent manner, whereas GPII_b-III_a requires Ca²⁺ and Mg²⁺. Although Mn²⁺ has been shown to promote fibronectin-receptor-mediated adhesion and binding [36], in this system Mn²⁺ did not potentiate the binding of either purified GPI_b-II_b or GPII_b-III_a to TSP. This result is consistent with the observation that Mn²⁺ does not promote collagen- and fibrinogen-dependent platelet aggregation (G. P. Tuszynski and M. A. Kowalska, unpublished work). Although Mg²⁺ has been shown to promote and Ca²⁺ to inhibit binding of GPI_b-II_b to collagen [37], we observed no bivalent-cation-dependent binding of GPI_b-II_b in our studies *in vitro*. A possible explanation for this difference is that we studied binding of GPI_b-II_b in detergent solution, whereas previous studies measured binding of GPI_b-II_b incorporated into lipid vesicles. These lipid vesicles could alter bivalent-cation requirement for binding. However, under physiological conditions bivalent-cation binding of GPI_b-II_b may not occur as plasma has high levels of Ca²⁺. Therefore the non-cation-dependent interaction between GPI_b-II_b and its ligands would be favoured.

GPI_b-II_b binds to different domains on TSP as GPII_b-III_a neither competes with the binding of GPI_b-II_b to TSP nor displaces bound TSP. These latter observations suggest that TSP can bind to both GPI_b-II_b and GPII_b-III_a on the platelet surface. Finally, the binding interaction between GPI_b-II_b and TSP is 5.5-fold more favourable than the binding interaction between GPII_b-III_a and TSP, suggesting that GPI_b-II_b may function as a high-affinity platelet TSP-binding protein.

The number of GPI_b-II_b sites on platelets has been reported to be 800 per platelet [20]. This number is consistent with the number of TSP molecules binding to non-stimulated platelets which was reported by Wolff et al. [38] to be 760 per platelet in the absence of added bivalent cations. In addition, consistent with the bivalent-cation-independent binding observed in our studies *in vitro*, these authors observed little or no cation dependence for binding of TSP to unstimulated platelets, finding that in the presence of EDTA, prostaglandin EM (PGE₁) and theophylline, 760 molecules per platelet were bound. Our previous studies showing inhibition of TSP-dependent platelet adhesion by anti-(GPI_b-II_b) were done using PGE₁-treated platelets [13].

Our results may help to explain the haemorrhagic diathesis of a patient who suffered a bleeding tendency, characterized by frequent petechial bleeding, post-operative haemorrhages and severe menstrual bleeding episodes, necessitating blood trans-

fusions [39]. The patient had a long bleeding time, abnormal collagen-induced adhesion and defective collagen-induced aggregation, but normal ADP-induced aggregation. Analysis of her platelets revealed the absence of GPI_b-II_b and the presence of degraded TSP. Intact, exogenously added TSP corrected the platelet-aggregation defect and when TSP and GPI_b-II_b appeared in her platelets after menopause, her bleeding tendency disappeared. One explanation for these patient data, as supported by the results of this study, is that TSP can bind either GPII_b-III_a or GPI_b-II_b and promote collagen-induced aggregation and adhesion by providing a platelet collagen binding site, as TSP interacts strongly with collagen.

This research was supported by Grant HL28149 from the Department of Health and Human Services. A preliminary report of this study was presented at the 64th Scientific Sessions of the American Heart Association.

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Received 15 March 1993/19 May 1993; accepted 26 May 1993