Development of auto-antibodies towards β2-glycoprotein I in the antiphospholipid syndrome

van Os, G.M.A.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 4

$\beta_2$-Glycoprotein I has a protective function in situations of hyper-responsive von Willebrand Factor: Implications for TTP

Vivian Du,
Gwen M.A. van Os,
Johanna A. Kremer Hovinga,
Ilze Dienava,
Jacques Wollersheim,
Rob Fijnheer,
Philip G. de Groot,
Bas de Laat

Submitted for publication
ABSTRACT

Recently, it has been shown that β₂-glycoprotein I (β₂GPI) binds to von Willebrand Factor (VWF) when it is in its GPIbal binding conformation (active VWF), thereby impairing the platelet binding capacity of VWF. Given the presence of active VWF multimers in TTP, we speculated on a role for β₂GPI in acute TTP. β₂GPI plasma levels were measured in patients during an acute attack of TTP (n=38) and in patients with a history of TTP (n=34). Median β₂GPI plasma levels in TTP patients during acute TTP episodes and in remission were 95 ± 46 µg/mL and 163 ± 80 µg/mL, respectively, while healthy controls showed significantly higher values (median 220 ± 99 µg/mL). In addition it was found that the increased amount of β₂GPI bound to circulating erythrocytes and platelets blood of healthy individuals incubated with active VWF or in blood from patients with an acute episode of TTP. When platelets were perfused over cultured endothelial cells, β₂GPI was able to inhibit platelet adhesion to VWF strings released from these endothelial cells. The inhibition of platelet adhesion was dependent on domain I of β₂GPI. Our findings suggest that β₂GPI can inhibit spontaneous platelet adhesion to active VWF, which might result in a decreased disease activity.
INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) was first described by Moschowitz as a life-threatening disease characterized by thrombocytopenia, microangiopathic hemolytic anaemia, fever, renal and neurological manifestations. In the majority of cases the disease is the result of a severe deficiency of ADAMTS13 activity due to the presence of circulating inhibitory autoantibodies. VWF is stored in endothelial cells within the Weibel-Palade bodies as ultra large (UL) multimers. When released, the UL multimers are processed by ADAMTS13. ADAMTS13 deficiency results in increased levels of UL VWF multimers in the circulation. These UL VWF multimers can bind platelets spontaneously via the A1 domain of VWF. Our knowledge on TTP and its association with the absence of ADAMTS13 activity has increased significantly the last years, although the pathophysiology of TTP is still not completely understood. A severe ADAMTS13 deficiency is found in the majority of patients clinically diagnosed with TTP but the absence of ADAMTS13 activity does not automatically result in TTP-like clinical symptoms. In addition, clinical remission can be achieved in patients suffering from an acute TTP episode despite a persistently severe ADAMTS13 deficiency. Together this suggests that additional factors that can regulate VWF activity.

Recently, we have shown that β2-glycoprotein I (β2GPI), a protein abundantly present in plasma, is able to inhibit platelet deposition to VWF by binding to the A1 domain of VWF, the GPIbα binding site. Furthermore, β2GPI appeared to have an approximately one-hundred fold higher affinity for VWF in its active GPIbα binding conformation than in its native conformation. It has been shown that the nanobody AU/VWFα-11, a llama derived VHH that only recognizes VWF in its GPIb binding conformation, recognized all VWF multimers from TTP patients in a gel-based assay, thereby suggesting that A1 domains present in all multimers of VWF circulating in a TTP patient are at least partly in its GPIα binding conformation.

RESULTS

β2GPI levels in plasmas of 38 patients with an acute TTP episode (UMC Utrecht population) were lower compared to healthy controls (95 ± 50 vs 220 ± 98 µg/mL, p<0.01) (FIGURE 1A). The TTP patient population from Geneva was divided into two groups based on Bethesda score. The patients with a Bethesda score > 3 has significantly lower β2GPI levels compared to patients with a Bethesda score between 1 and 2 (119 ± 74 vs. 86 ± 36 µg/mL, p<0.05) (FIGURE 1B) Patients suffering with a history of more than one TTP attack showed lowered β2GPI levels in plasma compared to patients in remission experiencing a single TTP attack (175 ± 65 vs. 138 ± 16 µg/
mL p= 0.28) (Figure 1C). β2GPI plasma levels were also lower in 34 TTP patients in remission (Utrecht cohort) than in the healthy population (163 ± 80 vs. 220 ± 99 µg/mL, p<0.05). From eight patients plasma samples were obtained both during an acute episode (95 ± 46 µg/mL) and after the remission (155 ± 40 µg/mL, p<0.05). We observed an increase in β2GPI plasma levels when these patients achieved remission (Figure 1D).

We observed a positive correlation between ADAMTS13 activity and β2GPI levels in TTP patients in remission (Pearson r=0.51, p<0.01, Figure 2A). Although no

![Diagram](image1)

**FIGURE 1.** β2GPI plasma levels in TTP patients and healthy controls. (A) Plasma β2GPI levels of patients during an episode of acute TTP, TTP patients in remission and healthy volunteers (p<0.001). (B) β2GPI levels of patients during an episode of acute TTP in relation to the Bethesda score (p<0.05). (C) β2GPI levels of 18 patients in relation to the risk of recurrence (Between 1 and 2 no significant difference p=0.28). (D) Plasma levels of β2GPI of 7 patients measured during an acute phase of TTP and when they were in remission (p<0.05).
correlation between β₂GPI levels and VWF antigen levels (Pearson r=0.11, p<0.54, data not shown) was found, an inverse correlation between β₂GPI levels and VWF in its GPIba binding conformation was observed (Pearson r=-0.37, p<0.05, FIGURE 2B). One TTP patient donated blood before developing his/her first acute TTP episode. It was possible to retrieve plasma samples withdrawn before the first TTP episode which were stored as quarantine plasma. FIGURE 2C shows that the levels of ADAMTS13 and β₁GPI correlate very well in time.

**FIGURE 2.** Correlation of β₂GPI plasma levels (34 patients UMC population) and ADAMTS13 (A) and VWF activation factor (active VWF) (B, Pearson r=0.51, p<0.001 and (B) VWF activation factor, Pearson r = -0.37, p<0.05. (C). β₂GPI concentration (µg/mL) and ADAMTS13 activity levels (%) is a patient with TTP from which plasma samples were stored from before his/her TTP episode because he/she was a blood donor.
During an acute episode of TTP, $\beta_2$GPI levels decrease about 50%. This cannot be explained by binding to and clearance via VWF alone on molar basis. Therefore alternative ways of $\beta_2$GPI clearance related to TTP must be present. TTP is characterized by low platelet number and the presence of fragmentocytes. We hypothesized that $\beta_2$GPI was cleared together with platelets and erythrocytes, therefore we measured $\beta_2$GPI binding to platelets and erythrocytes. Platelets obtained from healthy individuals showed an increase in $\beta_2$GPI binding to their surface when incubated with either VWF-R1306W (type 2B) (18.2 ± 10.6 %) or endothelial-derived VWF (34.8 ± 6.1 %, p<0.001) compared to plasma purified VWF (13.7 ± 8.4%) (FIGURE 3A). For erythrocytes we observed increased binding of $\beta_2$GPI when VWF-R1306W (type 2B) (8.4 ± 8.0 %) or endothelial-derived VWF (13.1 ± 6.7 %, p< 0.01) was added compared to plasma purified VWF (7.4 ± 6.3 %) (FIGURE 3B). The binding of $\beta_2$GPI to platelets and erythrocytes was significantly increased when the erythrocytes were incubated with endothelial cell derived VWF compared to plasma derived VWF (p<0.05).

To investigate whether the increase presence of $\beta_2$GPI on platelets and erythrocytes is also observed in patient during an acute episode of TTP, we measured $\beta_2$GPI binding to erythrocytes and platelets of healthy donors in the plasma of 7 patients with either an acute episode of TTP and three plasmas of patients in a remission state. FIGURE 4A and 4B show that during an acute TTP episode in plasma of patients VWF antigen and active VWF are not significantly different compared to patients in remission, for

**FIGURE 3.** $\beta_2$GPI binding to platelets and erythrocytes in the presence of different VWF preparations. The presence of $\beta_2$GPI on (A) platelets and (B) erythrocytes were studied with FACS analysis. Significant increased binding of $\beta_2$GPI was observed for platelets for both Type 2B and UL VWF and for erythrocytes only in the presence of UL VWF. * p<0.05, ***p<0001.
platelets 13.3 % ± 3.8 was observed to be β2GPI positive in plasma during an acute TTP phase compared to 10.7 % ± 4.8 in plasma obtained in a remission state. For the erythrocytes we observed 5.1% ± 3.8 compared to 4.2% ± 2.2. No correlation between the amount of β2GPI positive cells and VWF antigen level was observed. However the amount of VWF activation factor correlated with the amount of β2GPI positive platelets (Spearman R = 0.452, p=0.12) and erythrocytes (Spearman R = 0.652, p= 0.01).

**FIGURE 4.** β2GPI binding to platelets and erythrocytes. Washed platelets (A) and erythrocytes (B) derived from healthy volunteers were incubated with plasma from 7 TTP patients during an acute TTP attack and during remission. Binding of β2GPI to platelets and erythrocytes was detected using FACS analysis. 13.3 % ±3.8 Platelets were observed to be β2GPI positive in plasma during an acute TTP phase compared to 10.7 % ± 4.8 in plasma obtained in a remission state. For the erythrocytes we observed 5.1% ± 3.8 compared to 4.2% ± 2.2. For both platelets as erythrocytes there is no significant difference in binding of β2GPI to cells observed. The amount of β2GPI positive erythrocytes or platelets did not correlate with VWF antigen levels (platelets Spearman R= -0.083, p=0.78, erythrocytes R=0.024, p=0.93). However VWF activation factor correlates with the amount of β2GPI positive cells (platelets Spearman R= -0.452, p=0.121, erythrocytes R=0.652, p=0.0115).
Previously, we have shown that $\beta_2$GPI inhibits platelet VWF interaction via binding to the A1 domain of VWF\textsuperscript{6}. Here we explored which domain of $\beta_2$GPI is involved in the binding to VWF. Direct binding of VWF to $\beta_2$GPI was investigated by surface plasmon resonance. First, $\beta_2$GPI was coated to a Biacore CM5 chip and subsequently perfused with mutant VWF containing type 2B gain-of-function mutation, R1306W. VWF R1306W but not wt VWF was able to interact with $\beta_2$GPI (FIGURE 5A). Next

**FIGURE 5.** Binding of $\beta_2$GPI to VWF. In panel A $\beta_2$GPI was coated to a CM5 sensor chip. Binding of VWF-R1306W (0-40 nM) was measured. (B) Binding of the individual domains of $\beta_2$GPI to the A1 domain of VWF containing a gain-of-function mutation (R1306Q). Association was observed for 3 minutes under the same condition as described for Panel A and B. (C) Clockwise starting top left domain I, II, IV, V interaction with the A1 domain of VWF (R1306Q). (D) As (A) for domain I of $\beta_2$GPI.
we coated the VWF A1 domain containing the gain-of-function-mutation R1306Q to a C1-sensor chip and perfused the separate domains of β₂GPI (I, II, IV or V) over the chip. Only substantial binding with domain I was observed (Figure 5B and 5C). In addition also Domain I was coated to a CM5 chip and subsequently perfused with mutant VWF containing type 2B gain-of-function mutation, R1306W. VWF R1306W but not wt VWF was able to interact with domain I (Figure 5D).

To further establish the interference of β₂GPI in VWF/platelet interaction, VWF ristocetin induced platelet-agglutination studies with washed platelets was performed. Ristocetin was added to recombinant VWF (10 µg/mL) in the presence of either β₂GPI (2 and 4 µM), domain-deleted mutants of β₂GPI, or the antibodies RAG35 (anti-VWF A1, 50 µg/mL) and RAG201 (anti-VWF A3, 50 µg/mL). Full-length β₂GPI and mutants containing domain I of β₂GPI were able to inhibit VWF-induced platelet-agglutination, whereas β₂GPI mutants lacking domain I did not. RAG-35 also completely inhibited VWF mediated platelet agglutination while RAG-201 did not (Figure 6).

To further establish a role for β₂GPI in the regulation of VWF platelet interactions, in vitro perfusion studies were performed. Dong et al. have shown that platelets perfused over activated endothelial cells were able to form beads-on-a-string like patterns which are thought to mimic the formation of VWF-platelet microthrombi in the absence of ADAMTS13. Endothelial cells were stimulated with 25 µM histamine for 3 minutes, followed by the perfusion of platelets in the absence or presence of RAG-35 (50 and

**Figure 6.** Inhibition of platelet agglutination by β₂GPI. Agglutination of washed platelets was initiated by the addition of ristocetin and VWF in the presence of either β₂GPI (2 and 4 µM), recombinant domains of β₂GPI (1 µM) or anti-VWF antibodies RAG201 and RAG35). *p<0.05, **p<0.01, ***p<0.001.
100 µg/mL, figure 7a) or β₂GPI (2 and 4 µM, figure 7b), respectively. Both β₂GPI and RAG-35 inhibited platelet binding to endothelial-derived VWF dose-dependently. Next, we investigated if domain I of β₂GPI was involved in inhibiting platelet-binding to endothelial derived VWF. 10 µM domain I but not domain II-V added to the platelet suspension was able to block platelet adhesion to endothelial-derived VWF strings (figure 7c).

**FIGURE 7.** The influence of β₂GPI on platelet adhesion to endothelial derived VWF. For these experiments endothelial cells were activated by adding 25 µM histamine to the culture medium for 3 minutes followed by perfusion of platelets over the endothelial layer at a shear-stress of 15 dynes/cm². (A) RAG356, (B) β₂GPI, (C) domain I of β₂GPI and domains II-V of β₂GPI was added to the perfusion solution to investigate a possible effect on platelet adhesion onto endothelial-derived VWF. **p< 0.01, ***p<0.001.
To investigate whether this inhibitory effect was due to direct binding of $\beta_2$GPI to the endothelial-derived VWF, we coated latex beads of similar size and weight as platelets with purified plasma $\beta_2$GPI or albumin. First, we confirmed that for endothelial cells stimulated with histamine and perfused with platelets the beads-on-a-string patterns appeared (FIGURE 8A). When $\beta_2$GPI coated latex beads were perfused over histamine activated endothelial cells beads-on-a-string formation took place similar to the strings observed after perfusion with platelets (FIGURE 8B), while no such strings were observed after perfusion with bovine serum albumin-coated beads (data not shown). Beads coated with domain I of $\beta_2$GPI were also able to adhere to the VWF strings released by endothelial cells (FIGURE 8C).

**FIGURE 8.** (A) Endothelial cells stimulated with histamine and perfused platelets. In detail at panel C. Binding of $\beta_2$GPI coated beads to endothelial-derived VWF under flow. $\beta_2$GPI (B), or domain I of $\beta_2$GPI (D) were coated onto latex beads and added normal plasma and perfused at a shear rate of 300 sec$^{-1}$ over histamine treated endothelial cells.
DISCUSSION

We have recently reported that β 2 GPI can act as inhibitor of platelet adhesion by binding to the A1 domain of VWF in vitro, thereby preventing the interaction between VWF and platelet receptor GPIbα. This inhibition of platelet adhesion might lead to reduced thrombus formation. This was supported by the observation that lower β 2 GPI plasma levels in patients correlate with a history of myocardial infarction despite high VWF levels. These findings suggest that β 2 GPI can counteract the prothrombotic effect of VWF.

We found lower levels of β 2 GPI in patients with an acute episode of TTP and increased binding of β 2 GPI to platelets and erythrocytes in plasma of TTP patients during an acute episode. Recently we have published that β 2 GPI can function as a scavenger for LPS, by binding LPS and clearing it from the circulation. Here we propose VWF in its GPIb binding conformation as a second component that can be neutralised in the circulation by β 2 GPI. These observations were supported by the relation found between β 2 GPI plasma levels and the ADAMTS13 activity and the inverse relationship between the levels of β 2 GPI and “active” VWF in patients with TTP. Combined with a positive correlation between β 2 GPI positive cells and VWF activation factor. Combined with a positive correlation between β 2 GPI positive cells and VWF activation factor. Apparently, the more active VWF is present in the circulation, the lower level of β 2 GPI is observed, suggesting consumption of β 2 GPI when active VWF is present.

Additionally, a positive relation between ADAMTS13 activity and β 2 GPI levels was observed. One patient who was a voluntary blood donor for a long period suffered from a TTP attack. It was possible to retrieve two plasma samples stored from this person from the period before the TTP attack. The plasma β 2 GPI levels paralleled ADAMTS13 activity over the whole period of time. Whether the observed drop of β 2 GPI plasma concentration precedes or is the result of an acute TTP episode remains to be elucidated.

Due to the large variation in β 2 GPI plasma levels in a healthy population, it is difficult to use these levels as a measurement of disease state. In the present study we have found a significant decrease in β 2 GPI levels in TTP patients during an episode of TTP and to a lesser extent in patients with a history of TTP. The large variation in plasma levels β 2 GPI in healthy volunteers does not make β 2 GPI as an attractive diagnostic marker of the amount of active VWF present in the circulation although it can be informative in individuals when baseline levels are known.

It has been previously shown that β 2 GPI inhibits platelet adhesion to VWF by binding to the A1 domain of VWF. Here, we show that the binding site for VWF is located...
within domain I of \( \beta_2 \)GPI. Unfortunately, domain I of \( \beta_2 \)GPI is not an attractive potential inhibitor of platelet thrombus formation in patients with TTP because it has been shown that injection of murine domain into mice in the absence of adjuvant results in the formation of auto-antibodies\(^{10}\). Auto-antibodies against \( \beta_2 \)GPI are one of the criteria that define the antiphospholipid syndrome, a syndrome clinically characterized by thrombosis and pregnancy morbidity\(^{11}\). Arad et al. recently showed that purified auto-antibodies towards the first domain of \( \beta_2 \)GPI strongly increase a thrombotic response in a mouse thrombosis model\(^{13}\). The use of domain I as an anti-thrombotic drug has recently been suggested for use in APS\(^{13}\) but the recent insights into the aetiology of auto-antibodies against \( \beta_2 \)GPI is a strong contraindication of using domain I in patients.

An increased binding of \( \beta_2 \)GPI to platelets and erythrocytes in plasma of TTP patients during an attack compared to in remission was observed. During a TTP attack platelet count drops and erythrocytes haemolysed. We speculate that the drop in \( \beta_2 \)GPI plasma levels observed results from the clearance of \( \beta_2 \)GPI together with platelets and fragmentocytes.

During the last years insight into the disease process of TTP has increased tremendously. Severe ADAMTS\(13\) deficiency at initial presentation with an acute episode, anti-ADAMTS\(13\) antibody isotype and/or activity has been shown to be of prognostic use to predict outcome and risk of relapse\(^{14-16}\). In addition, there may be a role for high levels of coagulation factor VIII facilitating cleavage of VWF by ADAMTS\(13\)\(^{17}\). Here, we have demonstrated that \( \beta_2 \)GPI might contribute to the clinical picture of TTP.

**MATERIALS AND METHODS**

**Patients**

For this study we investigated two patient populations, a healthy population and one individual blood donor.

**Population 1** GENEVA population. Citrated blood (0.129 M) from 38 patients diagnosed with an acute episode of acquired TTP referred for ADAMTS\(13\) activity determination to the University Clinic of Hematology and Central Hematology Laboratory, Inselspital, Bern, Switzerland. All patients presented with thrombocytopenia (platelet count <150x10\(^9\)/l) and microangiopathic hemolytic anaemia with fragmented erythrocytes on the blood smear without an apparent alternative or underlying disease responsible for these findings as well as a severe ADAMTS\(13\) deficiency (<5% of the normal) due to a functional inhibitor. ADAMTS\(13\) activity and functional inhibitors were determined by the quantitative immunoblotting assay\(^{3,18}\). **Population 2**: UMC
UTRECHT population. Citrated plasma (0.109 M) was drawn from 34 patients in remission after a first acute TTP episode regularly seen at the University Medical Center in Utrecht, The Netherlands. From 8 patients plasma samples during an acute attack were obtained.

**Single blood donor:** Citrated plasma (0.109 M) was drawn from one patient during and after an acute TTP episode. This patient was a blood donor before the diagnosis of TTP and plasma samples of his/her were stored.

**Healthy volunteers:** Citrated plasma (0.109 M) was drawn from 54 healthy volunteers, who were not taking any medication.

All patients and healthy volunteers gave informed consent and the study was approved by the Medical Ethical Institutional Review Board of the hospitals involved.

**Diagnostic assays**

ADAMTS13 activity (FRETS-VWF73 assay), VWF antigen and VWF activity levels were determined as described before5,19. ADAMTS13 antibody titre were measured by a commercially available ELISA and performed according to the instructions of the manufacturer (Technoclone, Vienna, Austria). An in house enzyme-linked immunosorbtent assay (ELISA) was used to measure β2GPI plasma levels16. In short, murine monoclonal antibody 2B2 directed against β2GPI was diluted in Tris-buffered saline pH 7.4 (TBS) and coated overnight at 4°C onto an ELISA plate (Costar, New York, USA). Subsequently the plates were washed 3 times with TBS/ 0.1% Tween, blocked with a 3% bovine serum albumin (BSA)/ TBS solution for 1 hour at 37°C and washed again 3 times. Subsequently patient plasma diluted 1:1000 (v/v) in 3% BSA/TBS/0.1% Tween was added. Serial dilutions of normal pool plasma that had been calibrated against a purified plasma-derived β2GPI standard were used as reference standard. After 1 hour at 37°C the plates were washed and incubated with a polyclonal rabbit-anti human β2GPI antibody (Kordia, Leiden, the Netherlands) at a final concentration of 5 ng/mL in 3% BSA/TBS/0.1% Tween. After extensive washing, plates were incubated with a peroxidase-labelled goat-anti-rabbit antibody (DAKO, Glostrup, Denmark) for 1 hour. Staining was performed using an ortho-phenylene diamine (OPD) solution (4 mg/mL OPD diluted in 0.1 M NaH2PO4·2H2O/0.1 M Na2HPO4·H2O) and the colour reaction was stopped by the addition of 1 M H2SO4. Absorbance was measured at 490 nm.

**Proteins**

β2GPI was purified as previously described21. In short: citrated plasma (0.109 M) of three healthy blood donors was obtained from Sanquin Blood Supply Foundation (Amsterdam, the Netherlands) and dialyzed against 0.04 M Tris/0.01 M succinate/0.005% polybrene/1 mM EDTA /1 mM benzamidin /43 mM NaCl /0.02%
NaN3. Dialyzed plasma was applied to a DEAE-Sephadex column, the flow-through was collected and subjected to a Sp-Sepharose column and bound β2GPI was eluted with a linear salt-gradient. Subsequently this was subjected to a protein-G-Sepharose column for IgG depletion. The effluent pool was added to a heparin-Sepharose column and bound β2GPI was eluted with a linear salt-gradient (138 mM to 550 mM NaCl).

To achieve full purity β2GPI was applied to gel filtration column and > 99% purity was achieved.

Recombinant mutants of β2GPI (DI, D-II, DI-II-III, DI-IV, DI-V, DII-V, DIV-V) were a generous gift of La Jolla Pharmaceutical Company (La Jolla, CA, USA). The individual domains I, II, IV and V were home-made as described and expressed in HEK293E cells22.

WT-VWF and a VWF-2B mutant (VWF-R1306W) were cloned into expression vector PC-DNA 3.1 and subsequently expressed in HEK293T cells with furin activity. Expressed proteins were purified from serum-free medium using an affinity-column coupled with anti-human VWF antibody, RAG-201. Analysis by gel electrophoresis showed that all recombinant proteins were purified to homogeneity. Both wt VWF and VWF R1306W, analyzed using 0.1% SDS, 1% agarose gel electrophoresis, showed the complete set of multimers. Recombinant VWF A1-domain containing the R1306Q mutation was produced and purified as described before23.

**VWF antibodies**

Monoclonal antibodies towards VWF RAG201 and RAG35 have been previously described24. Both are monoclonal mouse-anti-human VWF antibodies, RAG201 recognizes the VWF A3 domain thereby inhibiting collagen binding. RAG35 recognizes the VWF A1 domain inhibiting GPIbα binding to VWF. Both antibodies have been purified from hybridoma medium using protein A-Sepharose column. Purity was examined by SDS-PAGE and binding of both antibodies to recombinant and plasma-purified VWF was assessed with an ELISA set up.

**β2GPI exposure on platelets and erythrocytes**

β2GPI bound to erythrocytes and platelets was detected by flow cytometric analysis (FACS). Briefly, 1 µl whole blood was added to 100 µl Hepes buffer (10 mM Hepes, 150 mM NaCl, 1 mM MgSO4•7 H2O, 5 mM KCl, pH 7.4), a FITC conjugated anti-β2GPI antibody was added to the above reaction system together with PE conjugated anti-CD235a antibody. Samples were further diluted with 0.2% formaldehyde. Totally 60 000 events were counted by FACS. Erythrocyte population was selected by PE conjugated anti-CD235a antibody, an erythroid cell marker. The platelet population was selected based on forward scatter and side scatter.
**Platelet agglutination**

Freshly drawn blood from healthy volunteers, declining the intake of any medication known to interfere with platelet function, was collected into 0.109 M trisodium citrate. Washed platelets were isolated as described before. Agglutination studies were performed on an optical aggregometer (Chrono-log Corporation, Havertown, PA, USA). Agglutination was initiated by the addition of ristocetin (1.5 mg/mL) in the presence of recombinant wild type (WT) VWF, various domains of β₂ GPI or antibodies towards VWF.

**Binding studies**

Protein interactions were studied by SPR analysis using a Biacore 3000 and a Biacore T100 biosensor system (both Biacore, Uppsala, Sweden). Recombinant full-length β₂ GPI and domain I of β₂ GPI (La Jolla Pharmaceutical Company, La Jolla, CA, USA) were covalently coupled to an activated CM5 sensor chip (Enzyme Research Laboratories, Swansea, UK) to reach a density of approximately 0.5 nM/mm². No protein was coupled to the control channel. Association of VWF-R1306W to bound β₂ GPI and domain I of β₂ GPI was recorded for 3 minutes in 100 mM NaCl, 2 mM CaCl₂, 0.005% Tween-20, 20 mM Hepes (pH 7.4) at a flow rate of 20 µl/min at 25°C. The VWF A1 domain containing the R1306Q mutation was coupled to a C1-sensor chip to reach a density of approximately 0.03 nM/mm². Subsequently full-length β₂ GPI and the separate domains of β₂ GPI were studied for association to the coupled VWF-R1306Q mutant.

**Perfusion studies**

To investigate platelet-binding to histamine-stimulated human umbilical vein endothelial cells (HUVEC) under conditions of flow, HUVEC were isolated from human umbilical cords and cultured on fibronectin-coated coverslips as described before. Twenty-four hours prior to the experiments, the endothelial cells were washed three times with prewarmed HEPES-buffered balanced salt (HBBS; 138.3 mM NaCl/ 25 mM HEPES/ 0.82 mM CaCl₂/ 5.36 mM KCl/ 0.61 mM MgCl₂/ 1 mM α,Δ-glucose, pH 7.4) followed by the addition of FCS-free medium (EBM-2, Clonetics, Walkersville, MD, USA). The next day the endothelial cells on the coverslips were transferred into a flow chamber and the endothelial cells were perfused with culture medium (EBM-2 medium, Clonetics, San Diego, CA, USA). The day after, endothelial cells were activated by adding 25 µM histamine to the culture medium for 3 minutes. Platelets or protein-coated beads in culture medium were perfused over the endothelial cell layer at a shear-rate of 15 dynes/cm² as described before. Platelet/bead-adhesion to (histamine-activated) endothelial cells was visualized using bright field microscopy (Zeiss, Jena, Germany).
**Protein-coupling to latex-beads**

To investigate binding of β₂GPI to endothelial cell-derived VWF, β₂GPI or albumin were coupled to 1 µm carboxylated modified latex (CML) beads (Invitrogen). To do so, the beads were washed 3 times with 0.1 M phosphate buffer (pH 8.1) and resuspended in 0.1 M phosphate buffer containing 2 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (Invitrogen). After overnight incubation, the beads were centrifuged and resuspended in 0.1 M phosphate buffer containing 1 mg/mL β₂GPI or 1 mg/mL albumin and incubated for 24 hours. Subsequently, the mixture was centrifuged and the supernatant buffer was removed for protein measurement to determine coupling efficiency. The beads used in these studies showed coupling efficiency of at least 90%.

**Statistics**

Measurements are presented as average ± standard deviation. Differences between groups were calculated using an unpaired two tailed t-test, Spearman correlation coefficient was calculated to determine the relationship between two parameters. A p value <0.05 was considered statistically significant. Calculations were done with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.
REFERENCES


