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The Binding Site in β_2-Glycoprotein I for ApoER2′ on Platelets Is Located in Domain V*

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Menno van Lummel[‡§1], Maarten T. T. Pennings[‡§1], Ronald H. W. M. Derksen[¶], Rolf T. Urbanus[‡§], Bianca C. H. Lutters[‖], Niels Kaldenhoven[**], and Philip G. de Groot[‡§2]

From the Departments of [‡]Haematology and Rheumatology and [¶]Clinical Immunology, University Medical Center, 3508GA Utrecht, [§]Institute of Biomembranes, Utrecht University, 3508GA Utrecht, the [**]Department of Crystal and Structural Chemistry, ABC Protein Expression Center, Utrecht University, 3584 CH Utrecht, and the [‖]Division of Biopharmaceutics, Center for Drug Research, Leiden University, 2300 RA Leiden, The Netherlands

The antiphospholipid syndrome is caused by autoantibodies directed against β_2-glycoprotein I (β_2GPI). Dimerization of β_2GPI results in an increased platelet deposition to collagen. We found that apolipoprotein E receptor 2′ (apoER2′), a member of the low density lipoprotein receptor family, is involved in activation of platelets by dimeric β_2GPI. To identify which domain of dimeric β_2GPI interacts with apoER2′, we have constructed domain deletion mutants of dimeric β_2GPI, lacking domain I (ΔI), II (ΔII), or V (ΔV), and a mutant with a W316S substitution in the phospholipid (PL)-insertion loop of domain V. ΔI and ΔII prolonged the clotting time, as did full-length dimeric β_2GPI; ΔV had no effect on the clotting time. Second, ΔI and ΔII bound to anionic PL, comparable with full-length dimeric β_2GPI. ΔV and the W316S mutant bound with decreased affinity to anionic PL. Platelet adhesion to collagen increased significantly when full-length dimeric β_2GPI, ΔI, or ΔII (mean increase 150%) were added to whole blood. No increase was found with plasma β_2GPI, ΔV, or the W316S mutant. Immunoprecipitation indicated that full-length dimeric β_2GPI, ΔI, ΔII, and the W316S mutant can interact with apoER2′ on platelets. ΔV did not associate with apoER2′. We conclude that domain V is involved in both binding β_2GPI to anionic PL and in interaction with apoER2′ and subsequent activation of platelets. The binding site in β_2GPI for interaction with apoER2′ does not overlap with the hydrophobic insertion loop in domain V.

The antiphospholipid syndrome is a non-inflammatory autoimmune disease associated with a wide variety of clinical symptoms. The main clinical features are arterial, venous, or small vessel thrombosis, both early and late pregnancy losses, and pre-eclampsia (1–4). The syndrome is diagnosed when one of the above clinical criteria is accompanied by the persistent presence of antiphospholipid antibodies (aPL)[3] (lupus anticoagulants and anticardiolipin antibodies) in the plasma of patients. These aPL are a heterogeneous group of antibodies directed to plasma proteins with affinity for anionic phospholipids (PL). We now know that the most important plasma protein, to which the aPL are directed, is β_2-glycoprotein I (β_2GPI or apolipoprotein H) (5, 6).

β_2-Glycoprotein I is abundantly present in plasma (~200 μg/ml) and is mainly synthesized in the liver, although mRNA coding for β_2GPI has been found in a variety of cells such as trophoblasts, placental cells, endothelial cells, and neurons (7–9). The mature sequence of human β_2GPI consists of 326 (44 kDa) amino acids (aa) with four *N*-linked glycosylation sites. It is composed of five repeating units that belong to the complement control protein family. The first four domains have ~60 aa residues and 4 cysteines each, with potential disulfide bridges joining the first to third and the second to fourth cysteines to contribute to a "looped-back" structure, called Sushi domains. The fifth domain is aberrant, having 82 aa and three disulfide bridges. A positively charged (multiple lysine) region between Cys^{281}–Cys^{288} in domain V is highly conserved and a critical phospholipid-binding site (10–13). The flexible loop Ser^{311}–Lys^{317}, containing Trp^{316}, which is essential for phospholipid binding (14), is located in the middle of this charged region. Domain V has also been described to interact with anionic hydrophobic ligands (15). Domain I of β_2GPI harbors another cationic region. Involvement of this region in binding to PL has also been described (16). Apolipoprotein E receptor 2 (also known as apoER2 or LRP8) is a member of the low density lipoprotein (LDL) receptor family. It has been identified by Kim *et al.* in 1996 (17) and shares structural homology with the LDL and the very low density lipoprotein (VLDL) receptors (17). With respect to restricted tissue expression (brain, testis, and placenta) and structural homology, apoER2 is closer to the VLDL receptor (45–63% aa homology) than to the LDL receptor (18). The apoER2 cDNA encodes a cluster of eight complement-type repeat domains, not all of which are translated into the polypeptide chain. Because of alternative splicing, receptors with either four or five complement-type repeat domains are produced (19). Spatial and temporal differences in the expression pattern of these proteins suggest different physiological functions for individual receptor species. It seems that apoER2 has an alternative physiological function *in vivo*, as there is firm support that this protein is involved in signaling processes (20–23).

Recently, a splice variant of apolipoprotein E receptor 2 (apoER2′ or apoER2Δ5) was identified in platelets and megakaryocytic cell lines, as a member of the LDL receptor family (24). Platelet apoER2′ mRNA encodes a 130-kDa protein including the LDL receptor class A repeats, epidermal growth factor homology repeats, *O*-linked sugar domain, a cytoplasmic domain that contains one internalization signal, and a single transmembrane region. In recent publications it has been shown that LDL and dimeric β_2GPI can interact with apoER2′ on platelets (22,

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Thrombosis and Haemostasis Laboratory, G.03.647, Dept. of Haematology, University Medical Centre Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: 31-30-2507770; Fax: 31-30-2511893; E-mail: ph.g.degroot@azu.nl.

[3] The abbreviations used are: aPL, antiphospholipid antibodies; PL, phospholipid; β_2-GPI, β_2-glycoprotein I; aa, amino acid(s); apoER2′, apolipoprotein E receptor 2′; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DM, domain deletion mutants; sh, soluble human; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PC, phosphatidylcholine; PC, phosphatidylserine; PT, prothrombin time; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

25). Until now, little is known about the interaction between (dimeric) β_2GPI and platelets. As for phospholipid binding, cationic patches might play an important role, because ligand binding to apoER2′ is dependent on electrostatic interactions (26). β_2-Glycoprotein I contains two cationic regions, located in domain I (including the interface between domain I and II) and domain V. The largest cationic patch is in domain V. One may speculate that these domains play a role in binding of dimeric β_2GPI to apoER2′ on platelets. To understand the mechanism of the interaction between apoER2′ and dimeric β_2GPI it is essential to know: (i) which domain(s) and (ii) what structures in these domains are involved in interaction with apoER2′.

EXPERIMENTAL PROCEDURES

Construction of Dimeric Constructs of β_2GPI—The dimer apple 4-β_2GPI and the apple 2-β_2GPI, which is not able to form dimers, were constructed as described previously (27). To exclude the possibility that apple 4-β_2GPI binds via the dimerization domain of factor XI (apple 4), dimer apple 4 was constructed. The sequence of dimeric apple 4 was amplified from the vector apple 4-tissue-type plasminogen activator-S478A with the primers apple 4-BglII (GCCAGATCTTTCTGCCATTCTTCA) and apple 4-XbaI (GGTCTAGACTCGAGTCCCTCCTTTGATGCGTG). The PCR product was subcloned into the vector pCR®2.1-TOPO® (Invitrogen, Breda, The Netherlands), and cloned into the vector apple 4-tissue-type plasminogen activator-S478A with BglII and XbaI (underlined in apple 4-BglII and apple 4-XbaI, respectively). The starting point for the construction of the domain deletion mutants (DM) was the full-length cDNA of apple 4-C321S-β_2GPI (in short apple 4-β_2GPI) cloned into the vector apple 4-C321S-tissue-type plasminogen activator-S478A. The domain I deletion was constructed with the primers domain II β_2GPI-XhoI forward (CCCTCGAGATCCCAGAGTATGTCCTTTTGCTG) and β_2GPI-XbaI reverse (GCTCTAGAAAACAAGTGTGACATTTTATGTGGA). For the construction of the domain II deletion a set of two primers was used: for domain I amplification the primers β_2GPI-XhoI forward (CCCTCGAGGACGGACCTGTCCCAAGCC) and domain I β_2GPI reverse (TGTACATTTCAGAGTGTTGATG) and for domain III–V amplification the primers domain III β_2GPI forward (ACTCTGAAATGTACACCCATCATCTGCCCTCCACCA) and β_2GPI-XbaI reverse. These two products served as a template in a second PCR to amplify the full-length domain I deletion using β_2GPI-XhoI forward and β_2GPI-XbaI reverse. The domain V deletion was constructed with the primers β_2GPI-XhoI forward and domain IV β_2GPI-XbaI reverse (TCTAGATCATTTACAACTTGGCATGGCAGACCA). To construct fusion proteins of apple 4 and the domain deletion mutants of β_2GPI, the PCR product was cloned with XhoI and XbaI into the vector apple 4-C321S-tissue-type plasminogen activator-S478A. In this way DM of apple 4-β_2GPI were constructed. Sequence analyze were performed to confirm correct amplification of the cDNAs.

Construction of Soluble Human ApoER2′—Mature megakaryocytes were cultured from citrated umbilical cord blood as described by Den Dekker et al. (28). cDNA was synthesized from mRNA of mature megakaryocytes using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Soluble human apoER2′ (sh-apoER2′) was then cloned from this cDNA using Phusion DNA polymerase (Finnzymes). Primer design was such that the signal peptide was omitted and the stop codon was deleted, forward primer: sh-apoER2′ BamHI, GGATCCGGGCCGGCCAAGGATTGCGAAAAGG and reverse primer: sh-apoER2′ NotI, GCGGCCGCCTTGCAGTTCTTGGTCAGTAGGTCC. Sh-apoER2′ was then cloned into PTT3-SRα-GH-HISN-TEV. This expression vector is constructed from the pTT3 (29) and the pSGHV0 expression vectors (30).

Transfection, Expression, Cell Culture, and Purification of Dimeric Constructs of β_2GPI and Sh-ApoER2′—Transfection of baby hamster kidney cells with the calcium phosphate method was performed as described previously (31). Expression of all fusion constructs was performed in conditioned serum-free medium (Dulbecco's modified Eagle's medium/F-12 medium supplemented with 0.5% UltroserG; Invitrogen). Protein expression was measured using a β_2GPI-enzyme linked immunosorbent assay (ELISA). Domain deletion mutants of apple 4-β_2GPI fusion proteins were purified from cell culture medium with a monoclonal antibody against β_2GPI (21B2) coupled to a CNBr-activated Sepharose column (Amersham Biosciences). Bound DM was eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris (pH 9). The purified proteins were further subjected to purification on a Mono S column using fast protein liquid chromatography (Amersham Biosciences). Fusion proteins were eluted with a linear salt gradient from 50 mM to 1 M NaCl. After determination of the purity of the protein fractions on a SDS 4–15% PAGE, fractions with DM of apple 4-β_2GPI were pooled, concentrated with polyethylene glycol, and dialyzed against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Apple 4 was purified using monoclonal antibody XI-1 (generous gift of Dr. J. C. M. Meijers, Academic Medical Hospital, Amsterdam), which recognizes the apple 4 domain, coupled to CNBr-activated Sepharose. Bound proteins were eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris (pH 9). For sh-apoER2′ production, HEK293-EBNA cells were transfected by the DNA-polyethyleneimine method according to Durocher *et al.* (29). Sh-apoER2′ production was done in a 1-liter suspension culture (in medium containing 90% freestyle, 10% calcium-free Dulbecco's modified Eagle's medium, 0.5% fetal calf serum, Invitrogen) for 4 days. Sh-apoER2′ was affinity purified using receptor-associated protein-Sepharose from expression medium. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce) according to the instructions of the manufacturer, and with bovine serum albumin as a standard. Purified constructs were analyzed by SDS-PAGE.

Purification of Plasma β_2GPI—Plasma β_2GPI was isolated from fresh citrated human plasma as described previously (32). In short, dialyzed human plasma was subsequently applied to the following columns: DEAE-Sephadex A50, protein G-Sepharose, S-Sepharose, and finally heparin-Sepharose (all Sepharoses were obtained from Amersham Biosciences). Bound proteins were eluted with a linear salt gradient. Afterward, β_2GPI was dialyzed against TBS. Purity of the protein was checked with SDS-PAGE analysis. Concentration of the protein was determined using the BCA protein assay.

Preparation of Phospholipid Vesicles—Phospholipid vesicles containing 20% phosphatidylserine (PS) and 80% phosphatidylcholine (PC) were prepared according to Brunner et al. (33), with some modifications as described by Van Wijnen *et al.* (34). The phospholipid concentration was determined by phosphate analysis (35).

Binding of Domain Deletion Mutants to Phospholipid Vesicles—Binding of DM of apple 4-β_2GPI to PS/PC vesicles was tested in a solid phase binding assay. High binding 96-well ELISA plates (Costar, Corning Inc., 9102) were coated with 20% PS, 80% PC (25 μM in TBS; 50 μl/well) overnight at 4 °C. Wells were blocked with TBS, 0.5% gelatin (150 μl/well) for 2 h at 37 °C. Subsequently, wells were incubated with different concentrations of DM (0.25–32 μg/ml) for 1.5 h at 37 °C, followed by incubation with monoclonal antibody 2B2 (3 μg/ml; 50 μl/well; 1.5 h at 37 °C), a generous gift of Dr. J. Arnout, Leuven, Belgium.

Apple 4-β_2GPI was used as a positive control and plasma β_2GPI as a negative control. Afterward the wells were incubated with peroxidase-conjugated rabbit anti-mouse antibody (1:1000, 50 μl/well, 1.5 h at 37 °C), followed by a staining procedure using orthophenylenediamine. Samples were diluted in TBS, 0.5% gelatin. Nonspecific binding was determined using non-coated wells. Results are expressed as mean ± S.D. ($n = 3$).

Determination of the Effect of Domain Deletion Mutants on Clotting Time—Coagulation assays were performed in a KC-10 coagulometer (Amelung, Lemgo, Germany). To detect the effect of the DM on clotting time, the prothrombin time (PT) was performed as follows: 25 μl of normal pooled plasma and 25 μl of DM, apple 4-β_2GPI, plasma β_2GPI (final concentration 100 μg/ml), or buffer were incubated for 30 min at 4 °C, followed by an incubation of 90 s at 37 °C. Clotting was initiated by the addition of 50 μl of Innovin (Dade Behring, Marburg, Germany).

Blood Collection for Perfusion Experiments—Freshly drawn venous blood was collected from healthy donors (with informed consent) into 1/10 of volume of 3.2% trisodium citrate (w/v). These donors denied taking aspirin or other platelet function inhibitors during the previous 10 days.

Perfusion Experiments—Perfusions were performed in a single-pass perfusion chamber under nonpulsatile flow conditions using a modified parallel plate perfusion with a slit width of 2 mm and a slit height of 0.1 mm. Experiments with collagen type III as a surface were performed with a perfusion time of 90 s, at a shear rate of 800 s^{-1}, which represents the flow rate in small arteries. Plasma-derived β_2GPI, apple 4, apple 2-β_2GPI, apple 4-β_2GPI, DM, or buffer were added to whole blood 5 min before the start of the perfusion at a concentration of 100 μg/ml and incubated at 37 °C. The prewarmed blood was drawn through the perfusion chamber by an infusion pump (pump 22, model 2400–004; Harvard, Natrick, MA). Afterward, the coverslips were taken from the perfusion chamber and rinsed with Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4), fixed in 0.5% glutaraldehyde in PBS, dehydrated with methanol, and stained with May-Grünwald/Giemsa as described previously. Platelet deposition was evaluated with a light microscope equipped with a JAI-CCD camera (Copenhagen, Denmark) coupled to a Matrox frame grabber (Matrox Electronic Systems Ltd., Quebec, Canada) using Optimas 6.2 software (Optimas Inc., Seattle, WA) for image analysis. Evaluation for platelet adhesion was performed on 20 fields, perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Results are expressed as mean relative coverage (mean ± S.D., $n = 3$). Statistical analysis was performed with a Student's t test.

Binding of Apple 4-β_2GPI to Immobilized Soluble Human ApoER2′—Binding of apple 4-β_2GPI to sh-apoER2′ was tested in a solid phase binding assay. All incubations were performed for 1 h at room temperature. Hydrophobic 96-well ELISA plates were coated with increasing concentrations of sh-apoER2′ in PBS (0–10 μg/ml; 50 μl/well). Wells were blocked with PBS, 4% bovine serum albumin (150 μl/well). After incubation with plasma-derived β_2GPI, apple 4, apple 2-β_2GPI, apple 4-β_2GPI, apple 4-β_2GPI-W316S, ΔV (3 μg/ml, 50 μl/well), or plasma β_2GPI (3 μg/ml) in the presence of a mouse monoclonal α-β_2GPI antibody (19H9; 1 μg/ml) in PBS, 1% bovine serum albumin, bound protein was detected using rabbit polyclonal anti-β_2GPI (1:500; 50 μl/well). Wells were incubated with peroxidase-conjugated swine anti-rabbit antibody (1:500 50 μl/well), followed by a staining procedure using orthophenylenediamine. A control protein with a C terminus growth hormone tag was used to measure nonspecific binding of apple 4-β_2GPI. Results are expressed as mean ± S.D. ($n = 3$).

Association of Apple 4-β_2GPI with Sh-ApoER2′ in the Presence of Inhibiting Peptides—Binding of apple 4-β_2GPI to sh-apoER2′ was further investigated using peptides with the following sequences: VSRGGMRK (representing a cationic patch at aa position 37–44 in domain I of β_2GPI), CKNKEKKC (representing a cationic patch at aa position 282–287 in domain V of β_2GPI), and EKCKNKCK (scrambled). Hydrophobic 96-well ELISA plates were coated with 5 μg/ml of sh-apoER2′ in PBS (50 μl/well). Wells were blocked with PBS, 4% bovine serum albumin (150 μl/well). After incubation with apple 4-β_2GPI (3 μg/ml, 50 μl/well) with or without increasing concentrations of peptides (0–500 μg/ml), wells were subsequently incubated with a rabbit polyclonal anti-β_2GPI antibody (1:500; 50 μl/well) and peroxidase-conjugated swine anti-rabbit antibody (1:500, 50 μl/well). This was followed by a staining procedure using orthophenylenediamine. Binding of apple 4-β_2GPI in the absence of peptide was set at 100%. Results are expressed as mean ± S.D. ($n = 3$).

Immunoprecipitations—500-μl aliquots of washed platelets (300,000/μl) resuspended in Hepes/Tyrode buffer were incubated for 5 min at 37 °C with buffer or with plasma β_2GPI, apple 4-β_2GPI, apple 4-β_2GPI-W316S, or DM of apple 4-β_2GPI (final concentration 100 μg/ml). Incubations were performed in the presence of 3 mM $CaCl_2$. For competition experiments, proteins (final concentration 100 μg/ml) were incubated with the inhibiting peptides (final concentration 500 μg/ml) for 5 min at 37 °C. As control, platelets were incubated with buffer and the inhibiting peptides. Afterward, platelets were lysed on ice with 1% CHAPS, containing 50 mM MES, and 150 mM NaCl (pH 7.4). Proteins were precipitated with 1 μg/ml of a polyclonal anti-apoER2′ antibody (sc-10112, Santa Cruz Biotechnology, Santa Cruz, CA) and protein G-Sepharose (Amersham Biosciences). The immunoprecipitations were incubated for 18 h at 4 °C in a top over top rotor, washed three times with lysis buffer, resuspended in non-reducing Laemmli sample buffer (0.001% (w/v) bromphenol blue, 2% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8), and boiled for 5 min. The supernatants were subjected to 10% SDS-PAGE and electroblotted onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with TBS with 0.1% (v/v) Tween 20 (TBST) containing 2% (w/v) nonfat dry milk for 1 h at room temperature. Incubation with anti-β_2GPI antibody 2B2 (3 μg/ml) was performed overnight in TBST supplemented with 1% nonfat dry milk. The membranes were washed three times and incubated with peroxidase-conjugated rabbit anti-mouse antibody (1:2500; Dako, Glostrup, Denmark) in the same buffer. Bands on blots were visualized with enhanced chemiluminescence. This experiment was performed with washed platelets from three different donors.

RESULTS

Expression and Purification of Domain Deletion Mutants—To study the effect of domain involvement of apple 4-β_2GPI on PL binding, clotting time, and platelet adhesion, dimeric constructs of β_2GPI fused to the C terminus of the dimerization domain (apple 4) of factor XI were made. Baby hamster kidney cells were transfected with expression vectors containing DM of apple 4-β_2GPI. Protein expression was confirmed by Western blotting using an anti-β_2GPI monoclonal antibody. Cell lines with the highest expression were selected using a β_2GPI ELISA. The proteins were purified using a monoclonal α-β_2GPI antibody (monoclonal antibody 2B2) column followed by further purification on a Mono S column using fast protein liquid chromatography. After purification, DM were applied on a 7.5% SDS-PAGE under non-reducing (Fig. 1, *panel A*) and reducing (*panel B*) conditions and stained with Coomassie Brilliant Blue. In the presence of SDS full-length apple 4-β_2GPI (*D*), apple 4-β_2GPI-W316S (*W*), apple 4-Δ1β_2GPI (Δ*I*), apple

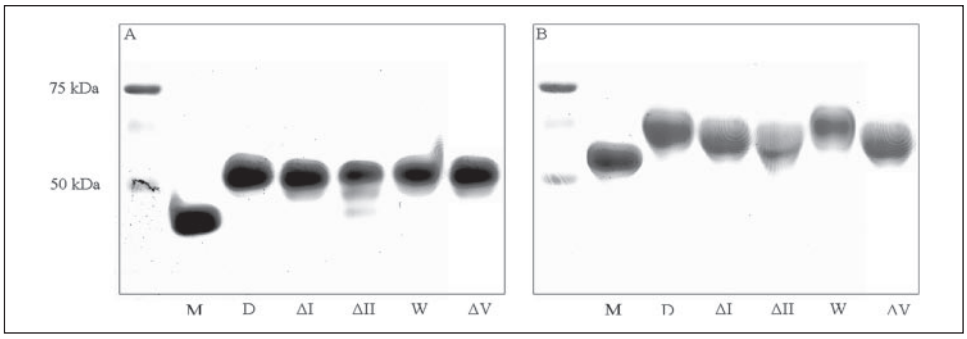

FIGURE 1. **SDS-PAGE analysis of apple 4-β_2GPI constructs.** Purified plasma β_2GPI (*M*), apple 4-β_2GPI (*D*), ΔI (Δ*I*), ΔII (ΔΘΘ), ΔV (Δ*V*), and apple 4-β_2GPI-W316S (*W*) were analyzed on a 10% SDS-PAGE under non-reducing (*A*) and reducing (*B*) conditions. Gels were stained with Coomassie Brilliant Blue. The molecular masses of prestained markers are expressed in kilodalton (kDa).

4-Δ2β_2GPI (Δ*II*), and apple 4-Δ5β_2GPI (Δ*V*) migrated as monomers with an apparent molecular mass of 50 kDa. Plasma β_2GPI (*M*) migrated with a molecular mass of 45 kDa under non-reducing conditions. Under reducing conditions, full-length apple 4-β_2GPI and apple 4-β_2GPI-W316S migrated with a molecular mass of ~62 kDa. Apple 4-Δ1β_2GPI, apple 4-Δ2β_2GPI, and apple 4-Δ5β_2GPI migrated slightly slower with a molecular mass of ~56 kDa. Plasma β_2GPI migrated with a molecular mass of ~52 kDa.

Effect of Apple 4-β_2GPI Domain Deletion Mutants on Clotting Time—To study the role of individual domains of apple 4-β_2GPI for competing with coagulation factors, we studied the effect of the DM on clotting time. For this purpose we performed the PT. Concentrations of 200 μg/ml plasma-derived β_2GPI, apple 4-β_2GPI, or DM, diluted in TBS, were mixed 1:1 with normal pooled plasma and incubated for 30 min at 4 °C (final concentration 100 μg/ml). This was followed by measurement of the PT. The clotting time in the presence of buffer was set at 100%. The observed effect for ΔI and ΔII was comparable with full-length apple 4-β_2GPI; apple 4-β_2GPI showed a relative prolongation of the clotting time to 171.3 ± 3.7%, ΔI to 163.6 ± 7.9%, and ΔII to 180.7 ± 23.8%. Results are presented in Fig. 2. The addition of plasma β_2GPI, ΔV, or apple 4-β_2GPI-W316S to normal pooled plasma did not influence the clotting time. Furthermore, the control proteins apple 4 and apple 2-β_2GPI did not influence the clotting time.

Binding of Domain Deletion Mutants to Immobilized Phospholipids—The phospholipid binding features of apple 4-β_2GPI fusion proteins were tested in a solid phase binding assay. Phospholipid vesicles (25 μM, 20% PS, 80% PC) were immobilized on 96-well ELISA plates, and binding of plasma-derived β_2GPI, and DM of apple 4-β_2GPI was measured. As shown in Fig. 3, half-maximal binding of apple 4-β_2GPI to phospholipid vesicles occurred at a concentration of 2.1 μg/ml (TABLE ONE). For the domain deletion mutants a similar interaction with phospholipid vesicles was observed: with ΔI having half-maximal binding to phospholipids at a concentration of 2.9 μg/ml and ΔII at a concentration of 4.1 μg/ml. Half-maximal binding to immobilized phospholipids of ΔV occurred at a concentration of 29.2 μg/ml. For apple 4-β_2GPI-W316S, half-maximal binding was observed at a concentration of 26.0 μg/ml. The presence of an aa substitution in the phospholipid-insertion loop explains why the W316S mutant hardly binds to anionic phospholipids. Plasma-derived β_2GPI showed little binding at a concentration of 16 μg/ml.

Effect of Domain Deletion Mutants on Platelet Deposition to Collagen Type III under Conditions of Flow—To determine which domain of apple 4-β_2GPI is involved in platelet sensitization, we performed perfusion experiments with citrated whole blood preincubated with plasma β_2GPI, apple 4-β_2GPI, or DM (final concentration 100 μg/ml). To determine basal platelet adhesion to collagen type III, whole blood was

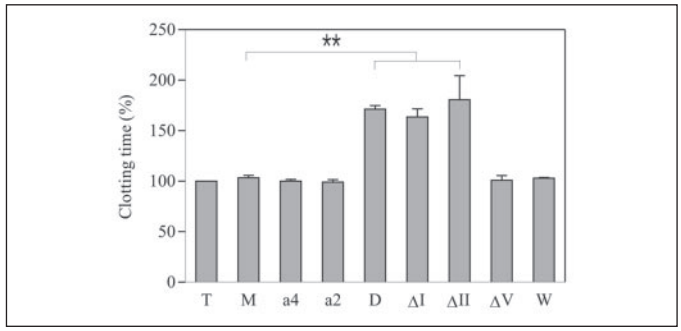

FIGURE 2. **Effect of apple-β_2GPI constructs on the PT.** Plasma-derived β_2GPI (*M*), apple 4 (*a4*), apple 2-β_2GPI (*a2*), apple 4-β_2GPI (*D*), ΔI (Δ*I*), ΔII (Δ*II*), ΔV (Δ*V*), or apple 4-β_2GPI-W316S (*W*) were 1:1 diluted with normal pooled plasma (final protein concentration, 100 μg/ml) followed by measurement of the PT. Clotting time with buffer (*T*) was set at 100%. Results represent mean clotting time ± S.D. in percentage ($n = 3$). Statistical analysis was performed using the Student's *t* test ($p \leq 0.001$). Differences between apple 4-β_2GPI, ΔI, and ΔII are not significant. **, statistical significance between D, ΔI, ΔII, and M using the Student's *t* test ($p < 0.0001$).

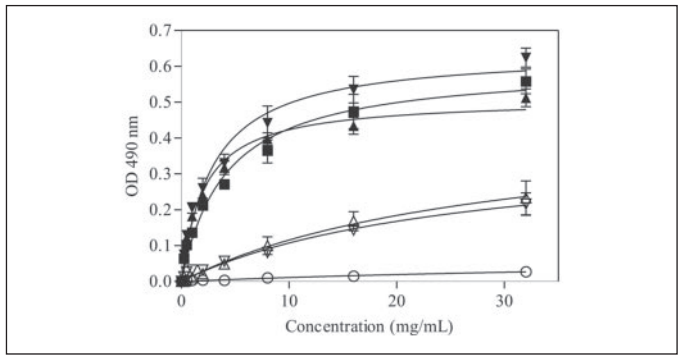

FIGURE 3. **Binding of apple 4-β_2GPI domain deletion mutants to immobilized PL.** Phospholipid vesicles (20% PS, 80% PC, 25 μM) were immobilized on high binding 96-well ELISA plates and incubated with increasing concentrations (ranging from 0.25 to 32 μg/ml) of plasma β_2GPI (○), apple 4-β_2GPI (▲), ΔI (▼), ΔII (■), ΔV (△), or apple 4-β_2GPI-W316S (▽) at 37 °C for 2 h. Afterward bound protein was detected with monoclonal antibody 2B2. Bound 2B2 was detected using the orthophenylenediamine staining procedure. Results are expressed as mean ± S.D. ($n = 3$).

preincubated with buffer. The basal platelet coverage after 90 s was 9.4 ± 2.1%, which was set at 100% (baseline). As shown in Fig. 4*A*, no increase in platelet adhesion was found when plasma β_2GPI, apple 4, apple 2-β_2GPI, or ΔV were added to whole blood (105.5 ± 11.4, 103.8 ± 15.6, 97.3 ± 16.1, and 99.5 ± 5.5%, respectively). As has also been shown by Lutters *et al.* (27), apple 4-β_2GPI-W316S did not induce increased platelet adhesion to collagen. In contrast, platelet adhesion increased significantly when full-length apple 4-β_2GPI, ΔI, or ΔII was added to whole blood (155.4 ± 11.0, 148.3 ± 8.6, and 157.5 ± 7.9%, respectively). Morphology of platelets and number of platelet aggregates were similar

TABLE ONE

Apparent dissociation constants of domain deletion mutants for PS/PC vesicles

Curves of plasma β_2GPI and apple 4-β_2GPI fusion proteins were fitted according to a one-site binding model in GraphPad. Half-maximal binding is given as apparent K_d (K_d(app)) both in μ g/ml as in nanomole/liters (nM). Results are expressed as mean ± S.D. ($n = 3$).

Apparent K_d	Plasma β_2GPI	Apple 4-β_2GPI	Apple 4-Δ1β_2GPI	Apple 4-Δ2β_2GPI	Apple 4-Δ5β_2GPI	Apple 4-β_2GPI-W316S
K_d(app) (μg/ml)	62.1 ± 24.4	2.1 ± 0.2	2.9 ± 0.4	4.1 ± 0.5	29.2 ± 2.4	26.0 ± 4.3
K_d(app) (nM)	$1.4 \times 10^3 \pm 0.6^{e3}$	18.3 ± 1.8	29 ± 4.0	41 ± 5.0	314 ± 25.8	226.0 ± 37.4

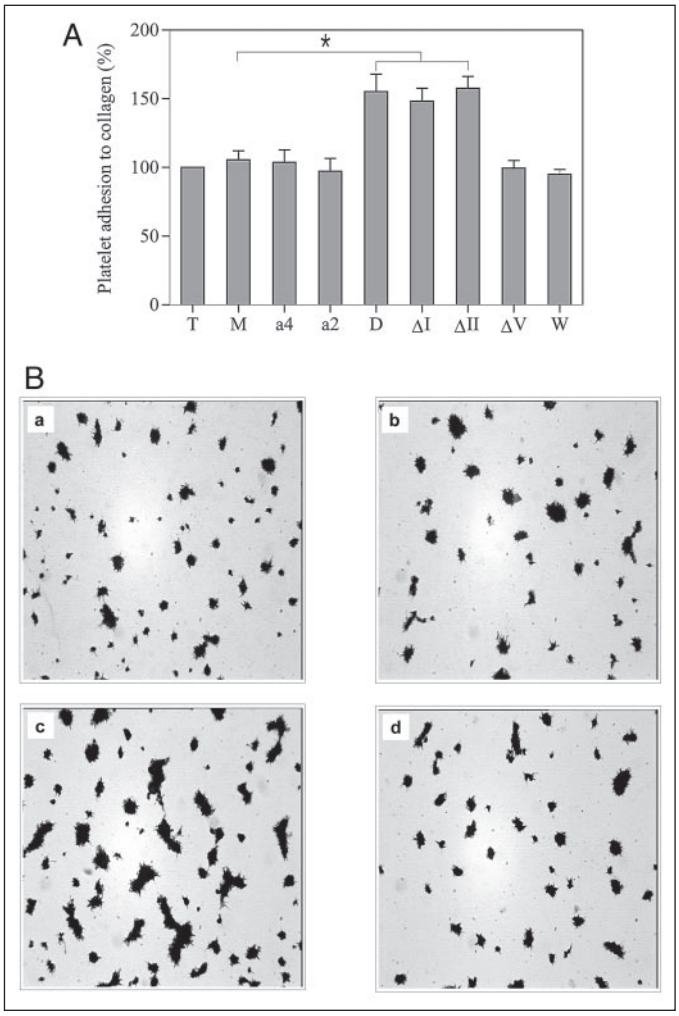

FIGURE 4. **Platelet deposition on collagen type III in the presence of domain deletion mutants of apple 4-β_2GPI.** Whole blood was preincubated at 37 °C for 5 min with buffer (*T*), plasma-derived β_2GPI (*M*), apple 4 (*a4*), apple 2-β_2GPI (*a2*), apple 4-β_2GPI (*D*), apple 4-β_2GPI-W316S (*W*) or domain deletion mutants (*ΔI*, *ΔII*, and *ΔV*, respectively) of apple 4-β_2GPI (10% v/v) with a final concentration of 100 μg/ml. Whole blood was perfused over collagen type III for 90 s at a shear rate of 800 s^{-1}. *A*, percentage of platelet coverage is expressed relative to platelet coverage in the presence of buffer (set at 100%). Data are expressed as mean ± S.D. ($n = 3$). Statistical analysis was performed using the Student's *t* test ($p < 0.005$). Differences between apple 4-β_2GPI, ΔI, and ΔII were not significant. *, statistical significance between D, ΔI, ΔII, and M using the Student's *t* test ($p < 0.005$). *B*, platelet morphology and aggregate formation in the presence of buffer (*panel a*), plasma β_2GPI (*panel b*), apple 4-β_2GPI (*panel C*), or ΔV (*panel d*). There was no difference between apple 4-β_2GPI, ΔI, and ΔII. Platelet morphology and aggregate formation in the presence of apple 4-β_2GPI-W316S was similar to buffer control.

in conditions with full-length apple 4-β_2GPI (Fig. 4*B*, *panel a*), ΔI (not shown), and ΔII (*not shown*). Plasma β_2GPI, ΔV (*panels b* and *d*, respectively), and apple 4-β_2GPI-W316S (not shown) displayed comparable effects on morphology and number of platelet aggregates as incubation with buffer (*panel a*).

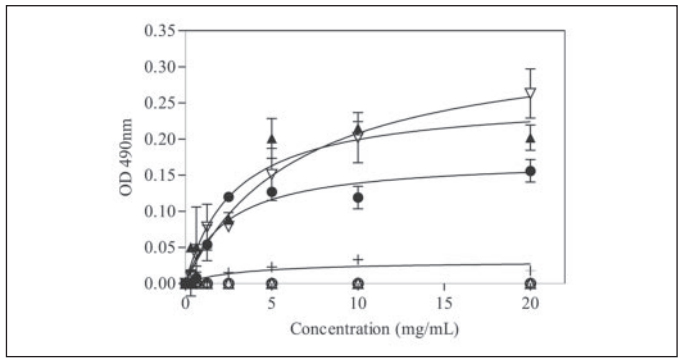

FIGURE 5. **Binding of apple 4-β_2GPI and the W316S mutant to immobilized apoER2′.** Interaction between apple 4-β_2GPI and sh-apoER2′ was measured in a solid phase binding assay. Soluble human apoER2′ was coated on a hydrophobic 96-well ELISA plate in a concentration dependent manner (0–10 μg/ml). After blocking, plasma β_2GPI (○), apple 4 (224), apple 2-β_2GPI (+), apple 4-β_2GPI (▲), ΔV (△), apple 4-β_2GPI-W316S (▽) mutant and plasma β_2GPI + 19H9 (●) were incubated (3 μg/ml). Afterward bound protein was detected with rabbit polyclonal anti-β_2GPI. Bound antibody was detected using peroxidase-conjugated swine anti-rabbit antibody. Results are expressed as mean ± S.D. ($n = 3$).

Binding of Apple 4-β_2GPI and the W316S Mutant to Immobilized Soluble Human ApoER2′—Binding of apple 4-β_2GPI and the W316S mutant to immobilized sh-apoER2′ was measured using an ELISA setup. As shown in Fig. 5, half-maximal binding of both apple 4-β_2GPI and apple 4-β_2GPI-W316S to immobilized sh-apoER2′ occurred at concentrations as low as 2.9 ± 0.7 and 6.0 ± 0.8 μg/ml, respectively (corresponds to 25 and 52 nM, respectively). Also, plasma β_2GPI in the presence of a monoclonal α-β_2GPI antibody (19H9) displayed binding to sh-apoER2′. Half-maximal binding occurred at a concentration of 2.4 ± 0.7 μg/ml (corresponds to 21 nM). No binding was found with plasma β_2GPI, apple 4, or ΔV. Apple 2-β_2GPI displayed only a slight interaction with sh-apoER2′.

Association of Domain Deletion Mutants and the W316S Mutant of Apple 4-β_2GPI with ApoER2′ on Platelets—The only member of the LDL receptor family known to be present on platelets is apoER2′. To demonstrate that a cationic patch in domain V of β_2GPI and not the hydrophobic PL-insertion loop in domain V (Ser^{311}–Lys^{317}) is responsible for interaction with apoER2′, immunoprecipitations were performed. Platelets were incubated with buffer, plasma-derived β_2GPI, full-length apple 4-β_2GPI, DM, or apple 4-β_2GPI-W316S, lysed, and subjected to immunoprecipitation with an anti-apoER2′ antibody. Afterward, Western blots were incubated with a monoclonal anti-β_2GPI antibody to detect interaction between β_2GPI and apoER2′. Association with apoER2′ was observed with apple 4-β_2GPI (Fig. 6). Hardly any association was observed when platelets were incubated with plasma β_2GPI or ΔV. To demonstrate that this finding was not the result of the inability of ΔV to bind to anionic phospholipids, immunoprecipitations were performed with apple 4-β_2GPI-W316S. The interaction of the W316S mutant with apoER2′ on the surface of platelets was similar to that of full-length apple 4-β_2GPI.

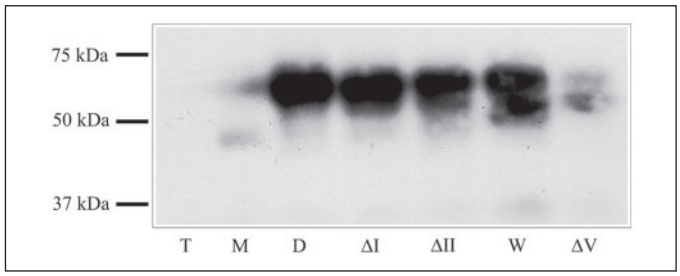

FIGURE 6. **Immunoprecipitations with domain deletion mutants of apple 4-β_2GPI and apoER2′ on platelets.** Washed platelets (300,000/μl), resuspended in Hepes/Tyrode buffer containing 3 mM $CaCl_2$ were incubated with buffer, apple 4-β_2GPI (*D*), ΔI (Δ*I*), ΔII (Δ*II*), ΔV (Δ*V*), or apple 4-β_2GPI-W316S (*W*) (final concentration 100 μg/ml; 10% v/v) for 5 min at 37 °C followed by lysis with CHAPS on ice. Lysed platelets were subjected to immunoprecipitation with an anti-apoER2′ antibody in the presence of protein G-Sepharose. Afterward Western blots were incubated with an anti-β_2GPI antibody followed by visualization using chemiluminescence. The blot represents three different experiments.

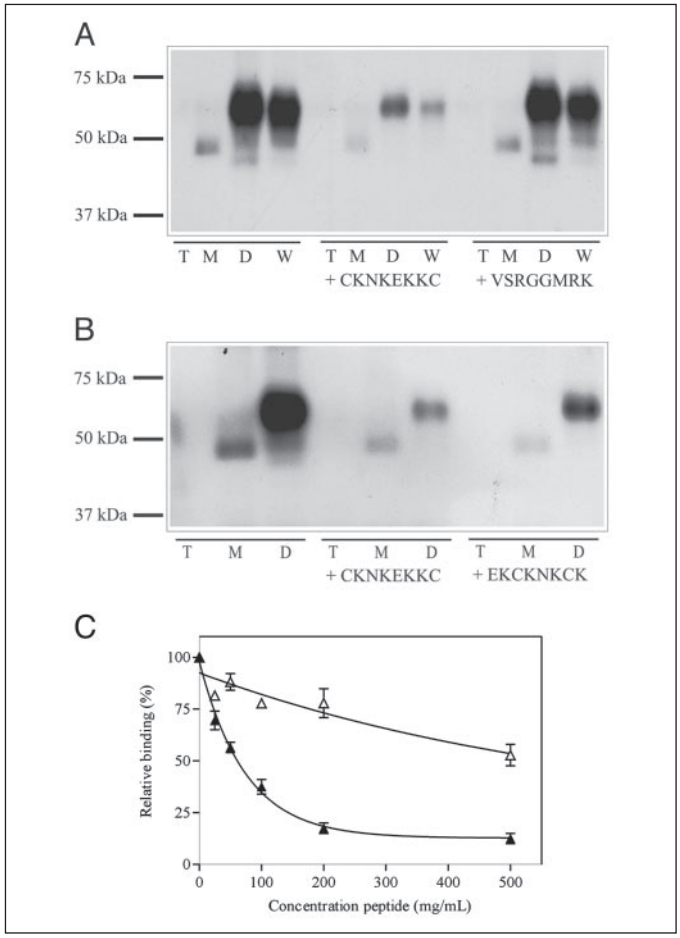

FIGURE 7. **Immunoprecipitations with apple 4-β_2GPI and apoER2′ on platelets in the presence of CKNKEKKC, EKCKNKCK, or VSRGGMRK peptides.** Competition experiments were performed to investigate electrostatic involvement between apple 4-β_2GPI and apoER2′. For this purpose washed platelets were incubated with buffer (*T*), plasma β_2GPI (*M*), apple 4-β_2GPI (*D*), and apple 4-β_2GPI-W316S (*W*) in the presence of cationic peptides; incubations were in the presence of CKNKEKKC or VSRGGMRK peptides (*panel A*) or in the presence of the EKCKNKCK peptide (*panel B*) (final peptide concentration; 500 μg/ml) for 5 min at 37 °C. Afterward immunoprecipitations were performed as described previously. Blots represent three different experiments. To show direct competition between apple 4-β_2GPI and the CKNKEKKC (▲) and EKCKNKCK (Δ) peptides, binding of apple 4-β_2GPI in the presence of the peptides was investigated in the solid phase binding assay (*panel C*).

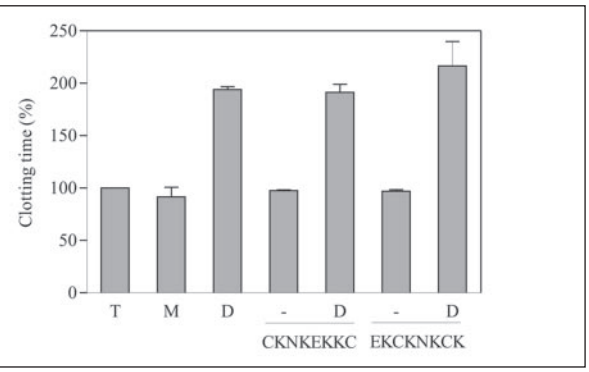

FIGURE 8. **The influence of the inhibiting CKNKEKKC and EKCKNKCK peptides on the prothrombin time.** Plasma-derived β_2GPI (*M*) and apple 4-β_2GPI (*D*), in the absence or presence of CKNKEKKC or EKCKNKCK peptides (final concentration 500 μg/ml), were 1:1 diluted with normal pooled plasma (final concentration 100 μg/ml) followed by measurement of the PT. Clotting time with buffer (*T*) was set at 100%. Results represent mean clotting time ± S.D. in percentage (n = 3).

Association of Apple 4-β_2GPI with ApoER2′ in the Presence of CKNKEKKC, EKCKNKCK, (scrambled) or VSRGGMRK Peptides—Association of ligands with members of the LDL receptor family is supported by electrostatic interactions. To investigate the possibility that association of apple 4-β_2GPI with apoER2′ is supported by a cationic patch in domain V of apple 4-β_2GPI, competition studies were performed with positively charged peptides. Incubation of washed platelets with apple 4-β_2GPI and CKNKEKKC (represents a cationic patch at aa position 282–287 in domain V of β_2GPI) peptide resulted in decreased binding of apple 4-β_2GPI to apoER2′ (Fig. 7*A*). This effect was not seen with the VSRGGMRK peptide (represents a cationic patch at aa position 37–44 in domain I of β_2GPI). The decreased binding of apple 4-β_2GPI to apoER2′ in the presence of CKNKEKKC was also seen for the W316S mutant. Incubation of washed platelets with apple 4-β_2GPI and EKCKNKCK (scrambled peptide) resulted in a minor reduction of the association between apple 4-β_2GPI and apoER2′ (Fig. 7*B*).

Binding of Apple 4-β_2GPI to Sh-ApoER2′ in the Solid Phase Binding Assay in the Presence of CKNKEKKC, EKCKNKCK (Scrambled), or VSRGGMRK Peptides—To show direct competition between apple 4-β_2GPI and the different peptides, apple 4-β_2GPI was able to interact with sh-apoER2′ in the presence of increasing concentrations of peptides VSRGGMRK, CKNKEKKC, or EKCKNKCK (scrambled). As shown in Fig. 7*C*, inhibition was observed for both the CKNKEKKC and EKCKNKCK peptides. Binding of apple 4-β_2GPI to sh-apoER2′ in the presence of the CKNKEKKC peptide was reduced to 12.5 ± 3.6%. In the presence of the scrambled peptide EKCKNKCK binding was reduced to 52.8 ± 7.4%. In the presence of the VSRGGMRK peptide no inhibition was observed (data not shown).

Effect of the CKNKEKKC and EKCKNKCK Peptides on the Prothrombin Time—The inhibiting effect of the peptides on association between apple 4-β_2GPI and apoER2′ might be because of interference with binding of the apple 4- β_2GPI constructs to phospholipids. Therefore, we investigated the influence of the peptides in the clotting time (PT), which is a phospholipid-dependent clotting assay. The clotting time in the presence of buffer was set at 100%. Apple 4-β_2GPI displayed a relative prolongation of the PT to 194.0 ± 2.8% (Fig. 8). In the presence of the CKNKEKKC or EKCKNKCK peptides (500 μg/ml) prolongation of the clotting time was observed to 191.3 ± 7.8 and 216.5 ± 23.3%, respectively. The CKNKEKKC or EKCKNKCK peptides (500 μg/ml) did not influence the clotting time in the absence of dimeric β_2GPI up to concentrations of 1 mg/ml (data not shown).

DISCUSSION

It is difficult to envision that mere binding of β_2GPI to anionic PL on the cell surface can activate these cells. Therefore, a search for a cellular

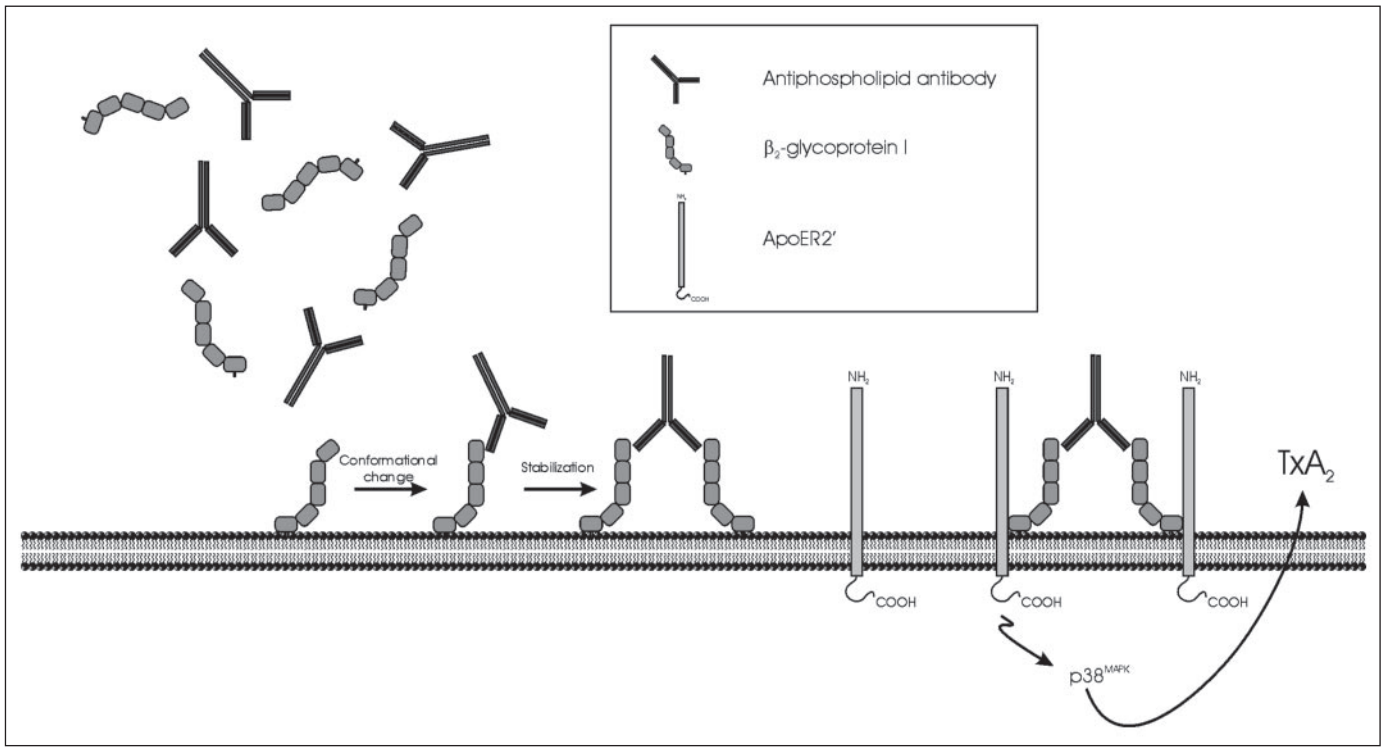

FIGURE 9. **Proposed mechanism of platelet activation via dimerization of β_2GPI by aPL.** β_2-Glycoprotein I circulates free in plasma with low affinity for phospholipids. A conformational change in domain I β_2GPI is induced after low-affinity binding to platelets. Dimerization of β_2GPI via binding of aPL occurs followed by stabilization of the complex on the platelet surfaced that subsequently results in firm adhesion of the β_2GPI-aPL complex to PL and association with apoER2′ on the surface of platelets. We cannot exclude the possibility that dimeric β_2GPI dissociates from the cellular surface after interaction with apoER2′. The interaction results in downstream signaling, thromboxane A2 (*TxA2*) synthesis, and further platelet activation.

receptor for β_2GPI on platelets was initiated. Lutters *et al.* (27) have demonstrated that dimerization of β_2GPI (either artificially by fusing β_2GPI with the apple 4 domain of factor XI, or physiologically by binding aPL to β_2GPI) results in increased affinity of β_2GPI for platelets, which results in an increased platelet deposition to collagen under conditions of flow. The increase in platelet adhesion could be blocked by the addition of receptor-associated protein, suggesting that a member of the LDL receptor family as the platelet receptor for dimeric β_2GPI was involved. This receptor has later been identified as apoER2′ (25). In the present study, the domain of β_2GPI responsible for interaction with apoER2′ has been determined by using constructs of dimeric β_2GPI lacking domains I, II, or V and a construct with an aa substitution (W316S) in the hydrophobic loop in domain V. Substitution of Trp^{316} by a serine completely abolished binding of β_2GPI to anionic phospholipids (14). In this study, we showed that a cationic patch in domain V of dimeric β_2GPI is involved in interaction with apoER2′ on platelets and that, by using the W316S mutant, the binding site for apoER2′ does not coincide with the phospholipid-binding site within domain V.

Deletion of domain I or domain II does not have an effect on the function of dimeric β_2GPI. Both domain deletion mutants (ΔI and ΔII) prolong the clotting time in the PT comparable with full-length dimeric β_2GPI (Fig. 2) and show comparable affinity for anionic phospholipids (Fig. 3). Furthermore, ΔI and ΔII cause an increased adhesion of platelets to collagen under conditions of flow comparable with full-length dimeric β_2GPI (Fig. 4*A*). Deletion of domain V or the W316S substitution abolished the prolongation of the clotting time induced by dimeric β_2GPI, which was to be expected, as domain V harbors the phospholipid-binding site. Indeed, both mutants show an approximate 15-fold decreased affinity for anionic phospholipids. Furthermore, in the perfusion model both mutants were not able to increase platelet adhesion to collagen, stressing the necessity of domain V in the activation of platelets.

Based on the observations in this article, we propose the following model for the activation of platelets by β_2-glycoprotein I (Fig. 9). β_2-Glycoprotein I binds to platelets with a low affinity. However, when bound to the platelet membrane a conformational change is induced in domain I of β_2GPI (36–38), resulting in the exposure of a cryptic epitope in this domain. Binding of anti-β_2GPI antibodies to this newly exposed epitope in domain I takes place. When one antibody interacts with two molecules of β_2GPI, the protein dimerizes resulting in an increased affinity for phospholipids on the outer surface of the platelet membrane. This increased affinity of the β_2GPI-anti-β_2GPI complexes is mimicked by our recombinant dimeric β_2GPI construct. The binding to the platelet membrane also results in concentration of β_2GPI on the cellular surface, which allows interaction with apoER2′ because of mass action effects. Stabilization of the binding of dimeric β_2GPI to phospholipids is crucial before it can bind to apoER2′, as the W316S mutant is not able to activate platelets under conditions of flow (Fig. 4*A*), despite the fact that the protein can bind to immobilized apoER2′ (Fig. 5).

We cannot exclude that also a conformational change in domain V is necessary for interaction with apoER2′, as plasma β_2GPI is not able to associate with the receptor (Fig. 6). Conformational changes in domain V of β_2GPI after binding to phospholipids have been described before (39). The interaction between β_2GPI and apoER2′ takes place via a cationic region in domain V, as peptides covering cationic amino acids present in domain V are able to inhibit the binding of dimeric β_2GPI to apoER2′ (Fig. 7, *A*–*C*). The apoER2′ interaction site does not completely overlap the phospholipid-binding domain, as the hydrophobic loop in domain V is not involved in this interaction.

After interaction with dimeric β_2GPI, dimerization of the receptor

may occur (40). The interaction of dimeric β_2GPI with apoER2′ results in downstream signaling, mediated via $p38^{MAP}$ kinases (41). This is followed by synthesis of thromboxane A2 (25). Thromboxane A2 further mediates platelet activation (42, 43, 44).

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