The Binding Site in $\beta_2$-Glycoprotein I for ApoER2’ on Platelets Is Located in Domain V*

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Mенно van Lumbee1, Maarten T. T. Penning2, Ronald H. W. M. Derksen3, Rolf T. Urbanus4, Bianca C. H. Lutters5, Niels Kaldenhoven6, and Philip G. de Groot7,8

From the Departments of 1Haematology and Rheumatology and 3Clinical Immunology, University Medical Center, 3508 GA Utrecht, the 4Department 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 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 mainly synthesized in the liver, although mRNA coding for $\beta_2$-GPI is present in a variety of cells such as trophoblasts, placental cells, endothelial cells, and neurons (7–9). The mature sequence of human $\beta_2$-GPI 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 Cys241–Cys258 in domain V is highly conserved and a critical phospholipid-binding site (10–13). The flexible loop Ser311–Lys317, containing Trp316, 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 $\beta_2$-GPI 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 LR8P) 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) receptor family. It is 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 $\beta_2$-GPI can interact with apoER2’ on platelets (22,
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25. Until now, little is known about the interaction between (dimeric) β₂-GPI and platelets. As for phospholipid binding, cationic peptides might play an important role, because ligand binding to apoER2’ is dependent on electrostatic interactions (26). β₂-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 β₂-GPI to apoER2’ on platelets. To understand the mechanism of the interaction between apoER2’ and dimeric β₂-GPI 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 β₂-GPI—The dimer apple 4-β₂-GPI and the apple 2-β₂-GPI, which is not able to form dimers, were constructed as described previously (27). To exclude the possibility that apple 4-β₂-GPI 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 (GCCAGATCTTCTGTCAGATTCCGT) and apple 4-XbaI (GGTCTAGACTCGAGTCCCTTCTTAGTCGCTG). The PCR product was subcloned into the vector pCR2.1-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-β₂-GPI (in short apple 4-β₂-GPI) cloned into the vector apple 4-C321S-tissue-type plasminogen activator-S478A. The domain I deletion was constructed with the primers domain I β₂-GPI-XhoI forward (CCCTCGAGATCCGACGATCAGATTTTATGTGGGA) and domain I β₂-GPI reverse (GCTCTAGATCCGACGATTTTATGTGGGA). These two PCR products served as a template in a second PCR to amplify the full-length domain I deletion using β₂-GPI-XhoI forward and β₂-GPI-XbaI reverse. The PCR product was cloned into the vector apple 4-β₂-GPI-XhoI forward (CCCTCGAGATCCGACGATCAGATTTTATGTGGGA) and domain I β₂-GPI reverse (GCTCTAGATCCGACGATTTTATGTGGGA). The PCR product was subcloned into the vector pCR2.1-TOPO’ (Invitrogen, Breda, The Netherlands), and cloned into the vector apple 4-β₂-GPI-S478A with BglII and XbaI (underlined in apple 4-BglII and apple 4-XbaI, respectively). For the construction of the dimer II deletion a set of two primers was used: for domain I apple 4-BglII (GCCAGATCTTCTGTCAGATTCCGT) and apple 4-XbaI (GGTCTAGACTCGAGTCCCTTCTTAGTCGCTG) and for domain III–V amplification the primers domain III TCAGAGTGTTGATG) and for domain III–V amplification the primers domain III TCAGAGTGTTGATG). The PCR product was subcloned into the vector pCR2.1-TOPO’ (Invitrogen, Breda, The Netherlands), and cloned into the vector apple 4-β₂-GPI-S478A with BglII and XbaI (underlined in apple 4-BglII and apple 4-XbaI, respectively). For the construction of the domain II deletion a set of two primers was used: for domain II amplification the primers β₂-GPI-XhoI forward (CCCTCGAGATCCGACGATCAGATTTTATGTGGGA) and domain I β₂-GPI reverse (GCTCTAGATCCGACGATTTTATGTGGGA) and for domain III–V amplification the primers domain III β₂-GPI forward (ACTCTGAAATGTACACCCCATCATGCCTCACCACCA) and β₂-GPI-XbaI reverse. These two products served as a template in a second PCR to amplify the full-length domain I deletion using β₂-GPI-XhoI forward and β₂-GPI-XbaI reverse. The domain V deletion was constructed with the primers β₂-GPI-XhoI forward and domain IV β₂-GPI-XbaI reverse (TCTAGATCATTTAACCTTGGCATGCAGCACA). To construct fusion proteins of apple 4 and the domain deletion mutants of β₂-GPI, the PCR product was cloned with XhoI and XbaI into the vector apple 4-β₂-GPI-S478A. In this way DM of apple 4-β₂-GPI 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, GGATCCGGGCGGCGCCAGATGCGCCAGGAAAGG and reverse primer: sh-apoER2’ NotI, GGCGCGGCGTTCGAGGGTGTTCTCTGGTCTCAGATGGTCC. Sh-apoER2’ was then cloned into PTT3-SRα-GH-HISN-TEV. This expression vector is constructed from the pTT3 (29) and the pSH40 expression vectors (30).

Transfection, Expression, Cell Culture, and Purification of Dimeric Constructs of β₂-GPI 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% UltrasorG; Invitrogen). Protein expression was measured using a β₂-GPI-enzyme linked immunosorbent assay (ELISA). Domain deletion mutants of apple 4-β₂-GPI fusion proteins were purified from cell culture medium with a monoclonal antibody against β₂-GPI (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-β₂-GPI 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 β₂-GPI—Plasma β₂-GPI was isolated from fresh citrated human plasma as described previously (32). In short, díalyzed human plasma was subsequently applied to the following columns: DEA-Septadex 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, β₂-GPI 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-β₂-GPI 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-β2-GPI was used as a positive control and plasma β2-GPI 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-β2-GPI, plasma β2-GPI (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 10 days.

Expression and Purification of Domain Deletion Mutants—To study the effect of domain involvement of apple 4-β2-GPI on PI binding, clotting time, and platelet adhesion, dimeric constructs of β2-GPI fused to the C terminus of the dimerization domain of factor XI were made. Baby hamster kidney cells were transfected with expression vectors containing DM of apple 4-β2-GPI. Protein expression was confirmed by Western blotting using an anti-β2-GPI monoclonal antibody. Cell lines with the highest expression were selected using a β2-GPI ELISA. The proteins were purified using a monoclonal α-β2-GPI 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 conditions. Hydrophobic 96-well ELISA plates were coated with 5 μg/ml of sh-apoER2’ in PBS (50 μl/well). Wells were blocked with PBS, 5% bovine serum albumin (150 μl/well). After incubation with apple 4-β2-GPI (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-β2-GPI 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-β2-GPI in the absence of peptide was set at 100%. Results are expressed as mean ± S.D. (n = 3).

RESULTS

Expression and Purification of Domain Deletion Mutants—To study the effect of domain involvement of apple 4-β2-GPI on PI binding, clotting time, and platelet adhesion, dimeric constructs of β2-GPI 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-β2-GPI. Protein expression was confirmed by Western blotting using an anti-β2-GPI monoclonal antibody. Cell lines with the highest expression were selected using a β2-GPI ELISA. The proteins were purified using a monoclonal α-β2-GPI 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 conditions. Hydrophobic 96-well ELISA plates were coated with 5 μg/ml of sh-apoER2’ in PBS (50 μl/well). Wells were blocked with PBS, 5% bovine serum albumin (150 μl/well). After incubation with apple 4-β2-GPI (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-β2-GPI 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-β2-GPI in the absence of peptide was set at 100%. Results are expressed as mean ± S.D. (n = 3).

Association of Apple 4-β2-GPI with Sh-ApoER2’ in the Presence of Inhibiting Peptides—Binding of apple 4-β2-GPI to sh-apoER2’ was further investigated using peptides with the following sequences: VSRG-GMKR (representing a cationic patch at aa position 37–44 in domain I of β2-GPI), CKNKEKK (representing a cationic patch at aa position 282–287 in domain V of β2-GPI), and ECKKKNCK (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, 5% bovine serum albumin (150 μl/well). After incubation with apple 4-β2-GPI (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-β2-GPI 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-β2-GPI 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 β2-GPI, apple 4-β2-GPI, apple 4-β2-GPI-W316S, or DM of apple 4-β2-GPI (final concentration 100 μg/ml). Incubations were performed in the presence of 3 mM CaCl2. 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 in 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-β2-GPI 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

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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.

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4Δ2β2GPI (ΔII), and apple 4Δ5β2GPI (ΔV) migrated as monomers with an apparent molecular mass of 50 kDa. Plasma β2GPI (M) migrated with a molecular mass of 45 kDa under non-reducing conditions. Under reducing conditions, full-length apple 4β2GPI and apple 4β2GPI-W316S migrated with a molecular mass of ~62 kDa. Apple 4Δ1β2GPI, apple 4Δ2β2GPI, and apple 4Δ5β2GPI migrated slightly slower with a molecular mass of ~56 kDa. Plasma β2GPI migrated with a molecular mass of ~52 kDa.

Effect of Apple 4β2GPI Domain Deletion Mutants on Clotting Time—To study the role of individual domains of apple 4β2GPI 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 β2GPI, apple 4β2GPI, 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 4β2GPI; apple 4β2GPI 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 β2GPI, ΔV, or apple 4β2GPI-W316S to normal pooled plasma did not influence the clotting time. Furthermore, the control proteins apple 4 and apple 2β2GPI did not influence the clotting time.

Binding of Domain Deletion Mutants to Immobilized Phospholipids—The phospholipid binding features of apple 4β2GPI fusion proteins were tested in a solid phase binding assay. Phospholipid vesicles (25 μl, 20% PS, 80% PC) were immobilized on 96-well ELISA plates, and binding of plasma-derived β2GPI, and DM of apple 4β2GPI was measured. As shown in Fig. 3, half-maximal binding of apple 4β2GPI 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 4β2GPI-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 β2GPI 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 4β2GPI is involved in platelet sensitization, we performed perfusion experiments with citrated whole blood preincubated with plasma β2GPI, apple 4β2GPI, or DM (final concentration 100 μg/ml). To determine basal platelet adhesion to collagen type III, whole blood was 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, no increase in platelet adhesion was found when plasma β2GPI, apple 4, apple 2β2GPI, 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 4β2GPI-W316S did not induce increased platelet adhesion to collagen. In contrast, platelet adhesion increased significantly when full-length apple 4β2GPI, 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.
in conditions with full-length apple 4-β_{2}GPI (Fig. 4B, panel a), Δ1 (not shown), and ΔII (not shown). Plasma β_{2}GPI, ΔV (panels b and d, respectively), and apple 4-β_{2}GPI-W316S (not shown) displayed comparable effects on morphology and number of platelet aggregates as incubation with buffer (panel a).

### TABLE ONE

| Apparent dissociation constants of domain deletion mutants for PS/PC vesicles |
|------------------------------------------------------------------------------------------------|
| Curves of plasma β_{2}GPI and apple 4-β_{2}GPI 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 μg/ml as in nanomole/liters (nM). Results are expressed as mean ± S.D. (n = 3). |
|------------------------------------------------------------------------------------------------|
| **Apparent K_{d} (μg/ml)** | **Plasma β_{2}GPI** | **Apple 4-β_{2}GPI** | **Apple 4-ΔΔβ_{2}GPI** | **Apple 4-ΔΔβ_{2}GPI** | **Apple 4-ΔΔβ_{2}GPI** | **Apple 4-ΔΔβ_{2}GPI** |
| K_{d}(app) | 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 × 10^{3} ± 0.6^{+} | 18.3 ± 1.8 | 29 ± 4.0 | 41 ± 5.0 | 314 ± 25.8 | 226.0 ± 37.4 |

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### FIGURE 4. Platelet deposition on collagen type III in the presence of domain deletion mutants of apple 4-β_{2}GPI.

Whole blood was preincubated at 37 °C for 5 min with buffer (T), plasma-derived β_{2}GPI (M), apple 4 (a4), apple 2-β_{2}GPI (a2), apple 4-β_{2}GPI (D), apple 4-β_{2}GPI-W316S (W) or domain deletion mutants (ΔI, ΔII, and ΔV, respectively) of apple 4-β_{2}GPI (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_{2}GPI, ΔI, and ΔII were not significant.* statistical significance between D, ΔI, ΔII, and M using the Student’s t test (p < 0.0005). B, platelet morphology and aggregate formation in the presence of buffer (panel a), plasma β_{2}GPI (panel b), apple 4-β_{2}GPI (panel c), or ΔV (panel d). There was no difference between apple 4-β_{2}GPI, ΔI, and ΔII. Platelet morphology and aggregate formation in the presence of apple 4-β_{2}GPI-W316S was similar to buffer control.

### FIGURE 5. Binding of apple 4-β_{2}GPI and the W316S Mutant to Immobilized apoER2.* Interaction between apple 4-β_{2}GPI 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_{2}GPI (C), apple 4 (224), apple 2-β_{2}GPI (+), apple 4-β_{2}GPI (A), ΔV (Δ), apple 4-β_{2}GPI-W316S (M) mutant and plasma β_{2}GPI + 19H9 (●) were incubated (3 μg/ml). Afterward bound protein was detected with rabbit polycional anti-β_{2}GPI. 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_{2}GPI and the W316S Mutant to Immobilized Soluble Human ApoER2—Binding of apple 4-β_{2}GPI 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_{2}GPI and apple 4-β_{2}GPI-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_{2}GPI in the presence of a monoclonal α-β_{2}GPI 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_{2}GPI, apple 4, or ΔV. Apple 2-β_{2}GPI displayed only a slight interaction with sh-apoER2*.

Association of Domain Deletion Mutants and the W316S Mutant of Apple 4-β_{2}GPI 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_{2}GPI 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_{2}GPI, full-length apple 4-β_{2}GPI, DM, or apple 4-β_{2}GPI-W316S, lysed, and subjected to immunoprecipitation with an anti-apoER2* antibody. Afterward, Western blots were incubated with a monoclonal anti-β_{2}GPI antibody to detect interaction between β_{2}GPI and apoER2*. Association with apoER2* was observed with apple 4-β_{2}GPI (Fig. 6). Hardly any association was observed when platelets were incubated with plasma β_{2}GPI 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_{2}GPI-W316S. The interaction of the W316S mutant with apoER2* on the surface of platelets was similar to that of full-length apple 4-β_{2}GPI.
Association of Apple 4-β₂GPI with ApoER2  in the Presence of CKNKKEKKC, 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-β₂GPI with apoER2  is supported by a cationic patch in domain V of apple 4-β₂GPI, competition studies were performed with positively charged peptides. Incubation of washed platelets with apple 4-β₂GPI and CKNKKEKKC (represents a cationic patch at a position 282–287 in domain V of β₂GPI) peptide resulted in decreased binding of apple 4-β₂GPI to apoER2  (Fig. 7A). This effect was not seen with the VSRGGMRK peptide (represents a cationic patch at a position 37–44 in domain I of β₂GPI). The decreased binding of apple 4-β₂GPI to apoER2  in the presence of CKNKKEKKC was also seen for the W316S mutant. Incubation of washed platelets with apple 4-β₂GPI and EKCKNKCK (scrambled peptide) resulted in a minor reduction of the association between apple 4-β₂GPI and apoER2  (Fig. 7B).

Binding of Apple 4-β₂GPI to Sh-ApoER2  in the Solid Phase Binding Assay in the Presence of CKNKKEKKC, EKCKNKCK (Scrambled), or VSRGGMRK Peptides—To show direct competition between apple 4-β₂GPI and the different peptides, apple 4-β₂GPI was able to interact with sh-apoER2  in the presence of increasing concentrations of peptides VSRGGMRK, CKNKKEKKC, or EKCKNKCK (scrambled). As shown in Fig. 7C, inhibition was observed for both the CKNKKEKKC and EKCKNKCK peptides. Binding of apple 4-β₂GPI to sh-apoER2  in the presence of the CKNKKEKKC 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 CKNKKEKKC and EKCKNKCK Peptides on the Prothrombin Time—The inhibiting effect of the peptides on association between apple 4-β₂GPI and apoER2  might be because of interference with binding of the apple 4-β₂GPI 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-β₂GPI displayed a relative prolongation of the PT to 194.0 ± 2.8% (Fig. 8). In the presence of the CKNKKEKKC 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 CKNKKEKKC or EKCKNKCK peptides (500 μg/ml) did not influence the clotting time in the absence of dimeric β₂GPI up to concentrations of 1 mg/ml (data not shown).

DISCUSSION
It is difficult to envision that mere binding of β₂GPI to anionic PL on the cell surface can activate these cells. Therefore, a search for a cellular
receptor for \( \beta_2 \)-GPI on platelets was initiated. Lutters et al. (27) have demonstrated that dimerization of \( \beta_2 \)-GPI (either artificially by fusing \( \beta_2 \)-GPI with the apple 4 domain of factor XI, or physiologically by binding aPL to \( \beta_2 \)-GPI) results in increased affinity of \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI was involved. This receptor has later been identified as apoER2 (25). In the present study, the domain of \( \beta_2 \)-GPI responsible for interaction with apoER2 has been determined by using constructs of dimeric \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI to anionic phospholipids (14). In this study, we showed that a cationic patch in domain V of dimeric \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI. Both domain deletion mutants (\( \Delta I \) and \( \Delta II \)) prolong the clotting time in the PT comparable with full-length dimeric \( \beta_2 \)-GPI (Fig. 2) and show comparable affinity for anionic phospholipids (Fig. 3). Furthermore, \( \Delta I \) and \( \Delta II \) cause an increased adhesion of platelets to collagen under conditions of flow comparable with full-length dimeric \( \beta_2 \)-GPI (Fig. 4A). Deletion of domain V or the W316S substitution abolished the prolongation of the clotting time induced by dimeric \( \beta_2 \)-GPI, 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 \( \beta_2 \)-glycoprotein I (Fig. 9). \( \beta_2 \)-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 \( \beta_2 \)-GPI (36–38), resulting in the exposure of a cryptic epitope in this domain. Binding of anti-\( \beta_2 \)-GPI antibodies to this newly exposed epitope in domain I takes place. When one antibody interacts with two molecules of \( \beta_2 \)-GPI, the protein dimerizes resulting in an increased affinity for phospholipids on the outer surface of the platelet membrane. This increased affinity of the \( \beta_2 \)-GPI-anti-\( \beta_2 \)-GPI complexes is mimicked by our recombinant dimeric \( \beta_2 \)-GPI construct. The binding to the platelet membrane also results in concentration of \( \beta_2 \)-GPI on the cellular surface, which allows interaction with apoER2 because of mass action effects. Stabilization of the binding of dimeric \( \beta_2 \)-GPI 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. 4A), 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 \( \beta_2 \)-GPI is not able to associate with the receptor (Fig. 6). Conformational changes in domain V of \( \beta_2 \)-GPI after binding to phospholipids have been described before (39). The interaction between \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI 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 \( \beta_2 \)-GPI, dimerization of the receptor...
may occur (40). The interaction of dimeric β2GPI with apoER2 results in downstream signaling, mediated via p38MAP kinases (41). This is followed by synthesis of thromboxane A2 (25). Thromboxane A2 further mediates platelet activation (42, 43, 44).

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