Platelet Receptors Involved in the Antiphospholipid Syndrome

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Platelet Receptors Involved in the Antiphospholipid Syndrome

Rol van de bloedplaatjes receptoren ApoER2′ and GPIbα in de pathologie van het antifosfolipiden syndroom

(met een samenvatting in het Nederlands)

Proefschrift

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With arms wide open
Under the sunlight
Welcome to this place
I'll show you everything
With arms wide open
Well I don't know if I'm ready
To be the man I have to be
I'll take a breath, take her by my side
We stand in awe, we've created life
With arms wide open

Creed

Vur ons Pap

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Abbreviations

aCL	Anticardiolipin antibodies
ADP	Adenosine di-phosphate
APS	Antiphospholipid syndrome
bFGF	Basic Fibroblast growth factor
CCR	Complement control protein repeats
Cys	Cysteine
DAG	diacylglycerol
ECM	Extra cellular matrix
EC's	Endothelial cells
Fb	Fibrinogen
FII	Prothrombin
FIIa	Thrombin
fl	Femtolitre
FVIIa	Activated factor VII
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII
GPIbα	Glycoprotein Ibα
GPVI	Glycoprotein VI
HMWK	High molecular weight kininogen
IP3	inositol 1, 4, 5-triphosphate
kD	Kilo dalton
LAC	Lupus anticoagulant
LPS	Lipopolysccharides
Lys	Lysine
MMP's	Metalloproteases
MPV	Mean platelet volume
NO	Nitric oxide
OCS	Open canalicular system
PAI-1	Plasminogen activator inhibitor 1
PAPS	Primary antiphospholipid syndrome
PE	Phosphatidylserine
PE	Phosphatidylethanolamine
PF4	Platelet factor 4
PGI2	Prostaglandin I2
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand 1
SAPS	Secondary antiphospholipid syndrome
SCR	Short complement repeats
Ser	Serine
SLE	Systemic lupus erythromatosus
ß2-GPI	ß2-glycoprotein I
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
tPA	Tissue plasminogen activator
Trp	Tryptophane
TXA2	Thromboxane A2
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

1

General Introduction

Haemostasis

Circulation of the blood
Blood circulation through the vascular system is of vital importance since blood is the transporter of oxygen, CO_2, nutrients and signaling molecules such as hormones. Upon vascular damage, integrity and blood flow is disturbed and must be restored as quick as possible to prevent exsanguination. The system responsible for restoring vascular integrity is known as the haemostatic system. The haemostatic system seals a leak in the vessel by building a temporary plug at the site of vessel damage. Plug formation should not occur in intact vessels. Haemostasis is therefore tightly regulated and an imbalance will lead to either bleeding or thrombosis. Restoration of normal blood flow after vascular damage can be divided in five distinct processes:

1: Vascular damage initiates vasoconstriction restricting blood flow to minimize blood loss.
2: Blood platelets start to adhere to exposed sub-endothelial structures and a platelet plug is formed, temporarily closing the damaged site of the vessel ("primary haemostatic plug").
3: Stabilization of the "primary haemostatic plug" by the formation of fibrin. This process is known as secondary haemostasis.
4: Activation of the fibrinolytic system resulting in the formation of plasmin that leads to degradation the formed blood clot.
5: Vascular repair and restoration of the normal blood flow.

Summarizing the separate haemostatic processes in this way might suggest a sequential chain of events but processes mentioned above do overlap and interplay.

Vasoconstriction
Vessel wall contraction or vasoconstriction reduces blood flow through an injured area of a vascular bed and is one of the most important systems to reduce blood loss from the microcirculation. Vasoconstriction is transient and is therefore thought to be of only minor importance in haemostasis in larger vessels.

Primary haemostasis
"Primary haemostasis" is the formation of a platelet plug at the site of vascular injury. This is induced by the exposure of the sub-endothelial matrix of the vessel to the circulation. The most important constituent of the extra cellular matrix (ECM) involved in platelet adhesion is collagen. When collagen is exposed to circulating blood, the plasma protein von Willebrand factor (vWF) binds to collagen and is thereby "activated". Activated vWF is able to transiently bind to the platelet receptor Glycoprotein Ibα (GPIbα). This interaction results in slowing down of platelets, platelets then roll along the vWF fibers. The reduced speed of platelets allows the interaction of platelets with collagen via the platelet receptors glycoprotein VI (GPVI) and $\alpha_2\beta_1$ and mediates stable adhesion. When platelets come to a halt on the damaged blood vessel, several integrins can interact with different constituents of the ECM.

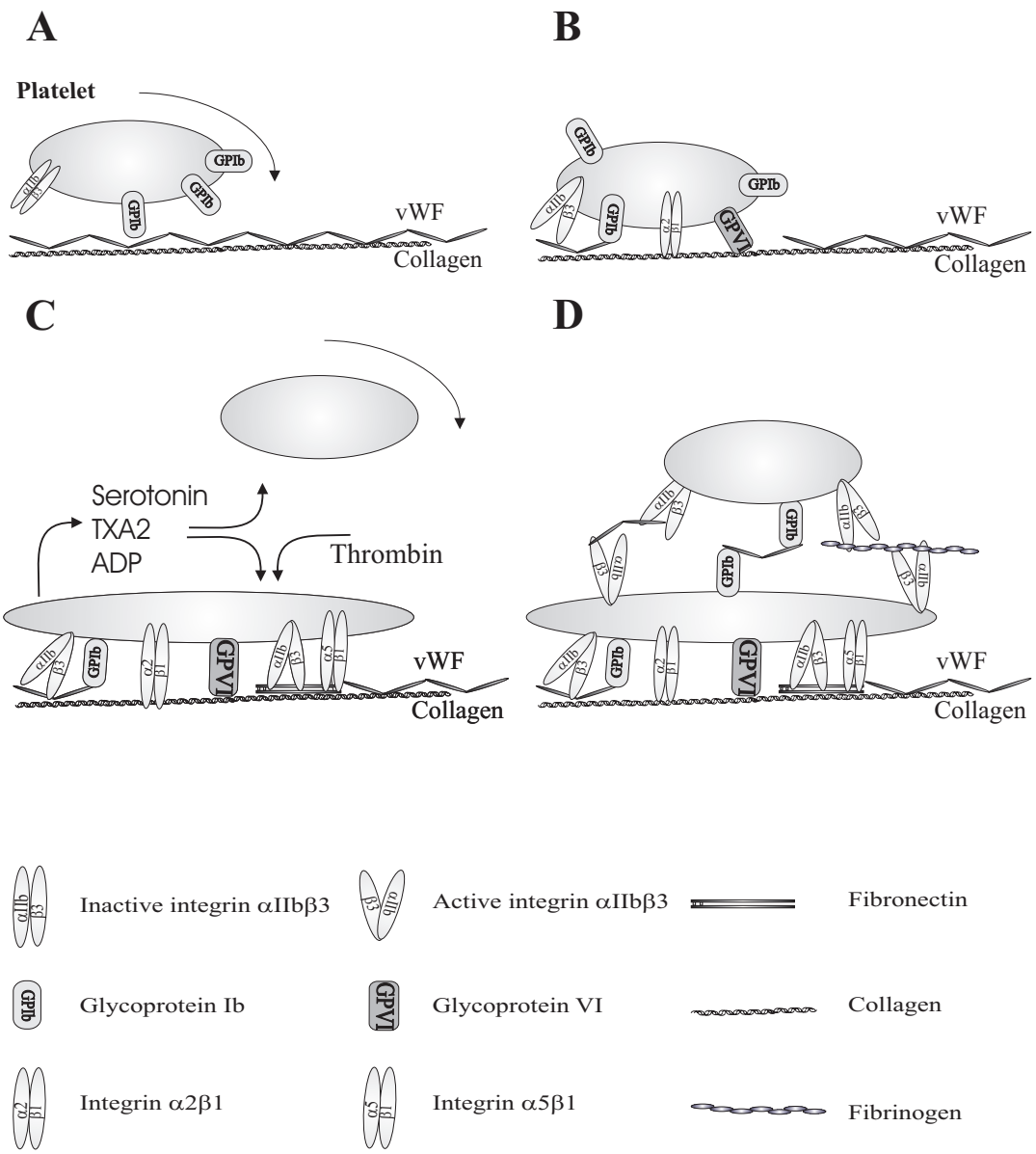

Figure 1: **Schematic representation of platelet adhesion and the formation of platelet aggregates under conditions of flow.** A: Platelets rolling over vWF bound to exposed collagen via GPIbα slowing down platelets to B: facilitate firm adhesion to collagen via GPVI and $\alpha_2\beta_1$. C: Platelets become activated and secrete their granular contents such as ADP, serotonin and TxA2 thereby activating platelets passing by. Integrin $\alpha_{IIb}\beta_3$ becomes activated enabling it to bind vWF and fibrinogen. Platelets start exposing anionic phospholipids to facilitate thrombin formation. Thrombin is then able to activate passing platelets but can also convert fibrinogen (Fb) to fibrin. D: vWF and fibrinogen start functioning as cross-bridges between passing platelets and adhered platelets thereby capturing platelets from the flow.

Some examples are the binding of $\alpha_{IIb}\beta_3$ to vWF and fibrinogen, integrin $\alpha_5\beta_1$ binding to fibronectin, of $\alpha_v\beta_3$ binding to vitronectin and of $\alpha_6\beta_1$ to laminin.
Firm adhesion of the blood platelets to the damaged vessel wall results in activation of the integrin $\alpha_{IIb}\beta_3$ and secretion of storage granules. Activated $\alpha_{IIb}\beta_3$ can bind fibrinogen and vWF present in plasma. Both fibrinogen and vWF are symmetrical molecules and able to bind two $\alpha_{IIb}\beta_3$ receptors. Fibrinogen and vWF can thus form a "bridge" between two different platelets facilitating growth of the haemostatic plug. Storage granules of platelets contain activators such as ADP and serotonin. Release of ADP from adhered platelets can activate newly arrived platelets that then can adhere to already adhered platelets via activated $\alpha_{IIb}\beta_3$ bound vWF or fibrinogen. Newly arrived platelets are then fully activated and growing of the plug continues. Besides the release of ADP, adhered platelets also synthesize thromboxane A2 ($TxA_{2)}$ from arachidonic acid. TxA2 also activates newly arrived platelets transferring activation from the site of injury higher up in the haemostatic plug. Serotonin and TxA2 are also powerful vasoconstrictors.

Another consequence of platelet activation is that platelets start to expose negatively charged phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) on their surface. Exposure of these negatively charged phospholipids provide a pro-coagulant surface supporting secondary haemostasis

Secondary haemostasis
"Secondary haemostasis" is the formation of fibrin as a consequence of the activation of a complex enzyme system known as the coagulation system. The coagulation system can be activated by two distinct pathways 1: The intrinsic pathway which starts by activation of the "intrinsic" factor XII (FXII) to active FXII (FXIIA). Intrinsic refers to the fact that blood will clot when drawn in a tube due to factors already present in the blood. This clotting reaction is relatively slow. 2: The extrinsic pathway which is initiated by the formation of a complex between activated factor VII (FVIIa) and "extrinsic" tissue factor (TF). Extrinsic refers to the fact that blood rapidly clots when "tissue homogenate" was added to blood. Both initiation pathways share a common downstream pathway starting with activating factor X to activated factor X (FXa).

The intrinsic pathway starts with the activation of FXII into FXIIa by non-physiological surfaces such as glass or kaolin in the presence of (pre)kallikrein and high molecular weight kininogen. FXIIa can activate factor XI (FXI) to active factor XI (FXIa) which in turn can activate factor IX (FIX) to active factor IX (FIXa). Together with activated factor VIII (FVIIIa), FIXa will form a "tenase" complex on a pro-coagulant surface to activate FX to FXa.

The extrinsic pathway starts with the exposure of TF to the blood flow. For long it was believed that tissue factor is not constitutively present within the vasculature and was exposed to the blood flow only upon vascular damage [1]. The current concept is that TF also circulates in a encrypted dimeric form [2] and upon activation of TF bearing cells, dimeric TF is decrypted and exposed as a monomer to the blood flow leading to the

TF-FVIIa complex formation. This complex formation on a pro-coagulant cell surface is the initiation of the extrinsic pathway that subsequently activates FX directly or via the activation of FIX and subsequent formation of a "tenase" complex.

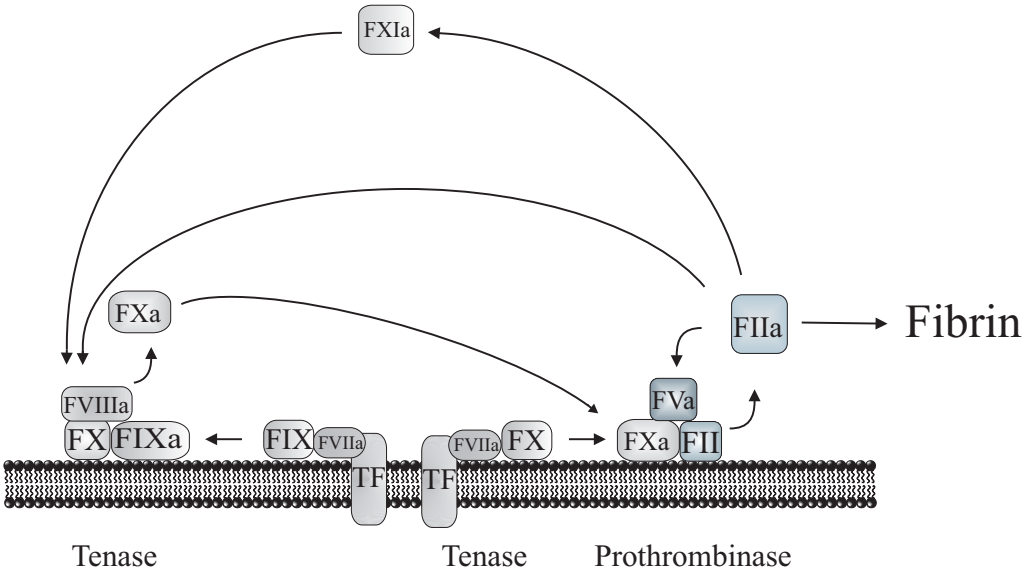

Figure 2: **Schematic representation of the common pathway in primary haemostasis.** The formation of tenase complex by either FX-FIXa-FVIIa or TF-FVIIa-FX leads to the formation of the prothrombinase complex. Prothrombin becomes activated and converts fibrinogen into fibrin. Thrombin acts as a catalyst on its own activation by enhancing FVIII, FV and FXI activation.

FXa from both the intrinsic and extrinsic initiation route forms the "prothrombinase" complex with co-factor FVa and prothrombin (FII) on a pro-coagulant surface. Activation of prothrombin results in the formation of thrombin (FIIa) [3]. Thrombin converts soluble fibrinogen into fibrin that polymerizes leading to insoluble fibrin deposition. The formed fibrin network is further stabilized by cross-linking adjacent fibrin fibrils by thrombin activated factor XIII (FXIIIa) [4] stabilizing the platelet plug.

For optimal coagulation it is required that thrombin amplifies its generation by activating FVIII, FV and FXI. Thrombin also is a very strong platelet activator and one of the links between primary and secondary haemostasis. Although differences in initiation of coagulation via both pathways remain important tools for diagnostic assays in hospital laboratories, it is now generally accepted that the intrinsic pathway does not play a significant role in initiating physiological haemostasis [5].

Secondary haemostasis is regulated by several mechanisms. Activity of the TF-FVIIa complex is inhibited by tissue factor pathway inhibitor (TFPI). TFPI interacts with FXa and this complex inhibits the activity of the TF-FVIIa complex. Propagation of coagulation is inhibited by the protein C system. Thrombin also binds to the endothelial re-

ceptor thrombomodulin and thereby loses its ability to convert fibrinogen into fibrin. Protein C and TAFI are activated by thrombomodulin bound thrombin. Activated protein C is localized on the surface of endothelial cells by endothelial cell protein C receptor (EPCR) and can, in complex with protein S, inactivate FVa and FVIIIa. The fibrin forming activity of thrombin is also directly inhibited by plasma proteins such as heparin-cofactor II and α-macroglobulin.

Fibrinolysis
When vascular integrity is restored, fibrin deposition is slowly removed by a process called fibrinolysis. Fibrinolysis starts with the conversion of plasminogen into plasmin by tissue plasminogen activator (tPA) [6]. TPA is present in small amounts in plasma but tPA is synthesized by endothelial cells and released close to the site of injury. Plasmin degrades fibrin into small soluble fragments, clearing the formed fibrin network. Another activator of plasminogen is urokinase plasminogen activator (uPA) but where tPA is the most relevant activator of plasminogen in circulation, uPA is considered to be predominantly relevant for activation of plasminogen in tissue. Both plasminogen activators are inhibited by plasminogen activator inhibitor 1 (PAI-1) but as mentioned above, thrombin activated TAFI also inhibits fibrinolysis by cleaving of C-terminal lysine and arginine residues of the formed fibrin. Cleavage of these residues prevents the binding of tPA and plasminogen to fibrin. Thus, Thrombin not only links primary and secondary haemostasis but also connects secondary haemostasis with fibrinolysis.

Vascular repair
When leakage of blood is stopped by afore mentioned haemostatic processes, the extra cellular matrix (ECM) and vascular integrity must be restored. The fibrin formed serves as a scaffold for endothelial regeneration and migration. The formed fibrin together with plasma fibronectin forms a provisional matrix for dividing endothelial cells to migrate over the damaged surface [7]. Endothelial cells start to proliferate upon stimulation by angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) both present in the α-granules of platelets. Migration is mediated by controlled degradation of the provisional matrix by metalloproteases (MMP's) and uPA activated plasmin. Once endothelial lining is restored, the provisional matrix is completely degraded and blood flow is restored.

Blood platelets
Although platelets are classified as cells, they are in fact cytoplasmic fragments derived from megakaryocytic progenitor cells in the bone marrow. Fragmentation occurs via the formation long cytoplasmic extensions and subsequent formation of pro-platelets that are shed to enter the blood flow through sinus endothelial cells in the marrow. Normal platelet counts vary between 150.000 and 440.000/μl and platelets remain in circulation 8 to 10 days. When platelet counts drop below 150.000/μl, patients are considered thrombocytopenic but only after a drop below 50.000/μl, the risk for bleeding increases. The average mean platelet volume (MPV) is 7.06±4.85 fl increases upon activation.
Platelets circulate in blood in a quiescent state. This "resting" state is maintained by inhibitory compounds produced by the endothelial cells (EC's). Endothelial cells secrete

nitric oxide (NO) and prostaglandin I_2 (PGI_2), both strong inhibitors of platelet function. The endothelial cells also express CD39 which is a membrane bound ADPase, hydrolyzing the platelet activator ADP. Furthermore, EC's are covered by negatively charged heparan sulphate moieties, preventing platelet adhesion.

Platelets are composed of a plasma membrane and internal membranes that form the so-called open canalicular system (OCS) and the dense tubular system (DTS) representing counterparts of the sarco-plasmatic reticular system in other cells. This dense tubular system is the main storage site for Ca^{2+} as well as for enzymes involved in the production of prostaglandins such as TxA2.

Platelets maintain their discoid shape by 1: A three dimensional cytoskeleton composed of actin and myosin, 2: A second two dimensional network of short actin fibers that serve as a membrane skeleton and 3: A marginal bundle of microtubules encircling the inner membrane supporting the actin membrane skeleton. The actin membrane skeleton also serves as a docking site for several platelet receptors such as integrins $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, and GPIbα.

Mitochondria, α-granules, dense granules, lysosomes and peroxisomes are present in the cytoplasm. Dense granules mainly contain Ca^{2+}, serotonin and ADP, α-granules contain proteins such as fibrinogen, vWF platelet factor 4 (PF4), fibronectin and vitronectin. Each platelet activator has at least one specific receptor on the platelet surface and when bound, results in the mobilization of signaling molecules (Ca^{2+}, diacylglycerol (DAG)

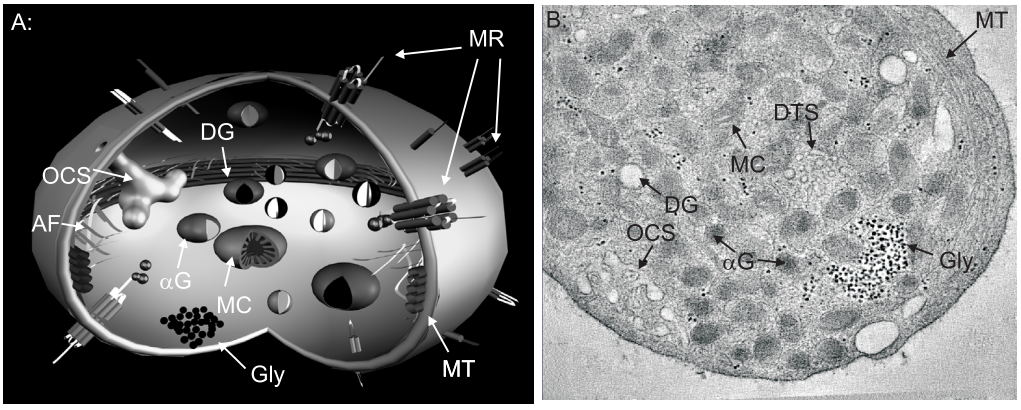

Figure 3: **A blood platelet.**
A: Schematic representation of a blood platelet (Courtesy to Arjan Barendrecht). B: Transmission Electron Microscopy image of a resting platelet (Courtesy to Hezder van Nispen tot Pannerden). MT=Microtubuli, MC=Mitochondria, αG= alfa granula, DG= Dense Granula, MR= Membrane Receptors, AF= Actin Fibers, DTS= Dense Tubular System, OCS= Open Canalicular System, Gly= Glycogen Storage Vesicles.

and inositol 1, 4, 5-triphosphate (IP_3)) from within the platelet. Subsequently, these signaling molecules will initiate shape change, aggregate formation, secretion of granular contents such as ADP and serotonin, and TxA2 synthesis.

When activated, platelets rapidly lose their discoid shape and start forming spiky pseudopods protruding from the membrane. This dramatically enlarges their surface. Activation leads to platelet aggregate formation and fusion of the dense,- and α-granules with the membranes of the OCS and ultimately to the release of serotonin and ADP, and synthesis of TxA2. Activation of platelets results in a conformational change of integrin $\alpha_{IIb}\beta_3$ so that fibrinogen and vWF can bind. Fibrinogen and vWF then can form a "bridge" between platelets resulting in aggregate formation. P-selectin, which in resting platelets is only present inside the secretory granules, becomes exposed on the outer membrane. Exposure of P-selectin mediates binding of P-selectin glycoprotein ligand 1 (PSGL-1) expressed by TF expressing cells and micro particles. Integration of TF bearing cells and micro particles in the developing thrombus initiates secondary haemostasis.

The antiphospholipid syndrome (APS)
The term "antiphospholipid syndrome" refers to patients with arterial or venous thrombosis and/or recurrent pregnancy loss who also test positive for so-called "antiphospholipid" antibodies in their plasma on a minimum of 2 occasions at least 6 week apart[8]. The presence of antiphospholipid antibodies correlates with a multitude of other clinical manifestations of disease such as heart valve abnormalities, livedo reticularis, chorea, nephropathy, thrombocytopenia and hemolytic anemia [9]. These clinical manifestations are not included in the criteria defining APS.

The antiphospholipid syndrome was first described in patients suffering from systemic lupus erythromatosus (SLE) and the association between APS and SLE was referred to as "secondary antiphospholipid syndrome" (SAPS). Later, APS has been detected in many patients without another autoimmune disease and this was then called primary antiphospholipid syndrome (PAPS). The term antiphospholipid syndrome arose when some 21 years ago from an observation made by Harris and Hughes. They summarized the finding that, the presence of a prolonged clotting assay or the presence of anticardiolipin antibodies in patients with thrombosis and/or recurrent pregnancy morbidity, often coincides with SLE patients that tested positive for syphilis in a VDRL-flocculation test without having syphilis. The main antigen in this test was a phospholipid called cardiolipin extracted from beef heart [10]. The antibodies responsible for this false positive reaction in the syphilis assay were called anticardiolipin antibodies (aCL).

In 1952, J. E Moore found in a large retrospective study using the patient population that tested false positive in the VDRL flocculation test, that one group was only transiently positive but a second group was found to be persistently positive [11]. The incidence of autoimmune diseases and especially SLE in this chronically positive group was high whereas transient false positive signal correlated with the presence of infection. In that same year Conley *et al.* described a circulating anticoagulant in two SLE patients that could not be corrected by dilution with normal plasma to overcome factor deficiencies [12]. This anticoagulant was termed lupus anticoagulant (LAC) since it was predominantly found in SLE patients and surprisingly was not associated with a bleeding tendency. In later studies, several groups found that lupus anticoagulant was not restricted to SLE patients but could also be found in association with certain drug use, infections, malignancies and sometimes in healthy individuals [13-15].

The clinical relevance of lupus anticoagulant only became clear in 1980 when the presence of LAC was associated with thrombosis, thrombocytopenia and fetal loss [16]. In this observation lies the first paradox in the antiphospholipid syndrome: the apparent correlation between thrombosis in patients with in-vitro prolongation of clotting time (LAC). The presence of a prolonged clotting time is usually associated with a bleeding tendency. The second paradox lies in the fact that not only secondary haemostasis is dys-regulated [17]. Antiphospholipid patients also have a prolonged bleeding time in combination with the occurrence of thrombosis suggesting dys-regulation of the primary haemostatic system.

Starting from 1988, several groups have been able to deplete anticardiolipin antibodies from single plasma samples without correcting the prolonged clotting time (LAC) demonstrating anticardiolipin antibodies and antibodies inducing LAC not always to be the same [18-20].

The term antiphospholipid syndrome is in fact a misnomer since these so-called "antiphospholipid" antibodies are not directed against phospholipids but to proteins bound to phospholipids [21;22]. In 1990 three groups showed anticardiolipin antibodies to be directed against the plasma protein β2-Glycoprotein I (β2-GPI) [23-25]. Antibodies against many other "co-factors' have been described in relation to the antiphospholipid syndrome such as high and low molecular weight kininogen in complex with phosphatidylserine (PE) [26-28], protein C [29], protein S, [29-31], annexin V [32-38], FXII [39-43], plasminogen[44] and prothrombin (FII) [45-48].

It is now generally accepted that antibodies directed against β2-GPI are clinically the most relevant "antiphospholipid antibodies" in the antiphospholipid syndrome and more specifically, those anti β2-GPI auto antibodies that elicit LAC activity [49;50]. Some research groups have described specific epitopes on β2-GPI for auto antibodies to bind to in order to have pathogenic properties [51-54]. Several physiological systems affected by anti phospholipid antibodies have been described and are reviewed in table 1.

β2-GPI

β2-GPI or apolipoprotein H circulates in blood as a monomeric glycoprotein at concentrations varying between 10-300 µg/ml. A small portion (4-13%) is bound to circulating lipoproteins [55]. It is mainly synthesized in the liver [56] although mRNA has been detected in different cell types such as endothelial cell (EC's) [57] and placental cells [58].
β2-GPI is expressed as a single chain polypeptide with a calculated molecular weight of approximately 37 kD. It has four N-glycosylation sites that comprise 20% of the total molecular weight of 45kD observed using Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [59;60]. It consists of five so-called sushi domains, also known as short complement repeats (SCR) or complement control protein repeats (CCR) [61] of which domains I, II, III and IV are structurally similar. Domain III contains 2 N-glycosylation sites and 1 O-linked glycosylation site. Domain 4 has one N-glycosylation site and both domains are probably prevented from proteolysis.

System affected	Mechanism	Ref.
Complement	Infusion of patient IgG's in LPS pre-treated mice induces thrombus formation in a C5 and C6 dependent manner. When IgG pool was depleted from anti β2-GPI antibodies, thrombus formation was lost.	55
	When the C5b receptor was knocked out in pregnant mice, no fetal resorption was observed.	56
	Complement activation may lead to inflammatory infiltration of the placenta. Throphoblasts in placental tissue can express PS and β2-GPI.	56;57
Cellular activation		
Endothelial cells	Binding of anti β2-GPI- β2-GPI complexes to endothelial cell β2-GPI presumably activates endothelial cells by "cross-linking" annexin A2, inducing a pro-coagulant phenotype.	58
	Anti β2-GPI- β2-GPI complexes activate EC's via Toll like receptor 2 (TLR2) and 4 (TLR4). Direct interaction between TLR2/4 and dimerized β2-GPI has not been demonstrated yet.	59;60
	Annexin A2 allows tPA and plasminogen to co-localize on the endothelial surface. Possibly dimerization of β2-GPI influences the interactions.	61
Platelets	Dimerization of β2-GPI induces binding to ApoER2' and subsequent sensitization for second stimuli such as exposed collagen or thrombin.	62
	Binding of β2-GPI to GPIbα primes platelets to produce TxA2 and activation of PI3-kinase/Akt pathway.	63
	Adhesion of blood platelets to dimerized β2-GPI could be blocked with soluble GPIbα but also by cleavage of GPIbα from the platelet surface. Adhesion could also be blocked by inhibition of dimeric β2-GPi binding to ApoER2'	64
	GPIbα allows thrombin and FXI binding to co-localize on the platelet surface. Possibly, dimerization of β2-GPI influences the interactions.	65
Monocytes	Incubation with anti β2-GPI- β2-GPI complexes induces TF expression.	66;67
Other cellular receptors	Apart from ApoER2', dimerized β2-GPI is able to bind other members of the LDL-receptor family. Megalin LRP1,and VLDL-r are all able bind dimerized β2-GPI to elicit signaling cascades on a multitude of cell types	68

Coagulation/fibrinolysis		
Activated Protein C	Anti β2-GPI- β2-GPI complexes inhibit the activation of protein C by either disruption of the APC complex or by competition for phospholipid binding.	69
FXa	Anti β2-GPI- β2-GPI complexes inhibit inactivation of FXa by protein Z/protein Z-dependent inhibitor and TFPI.	70;71
FXI	β2-GPI inhibits activation of FXI by thrombin and FXIIa. Inhibition is lost upon proteolytic clipping of β2-GPI.	72
TFPI	Anti β2-GPI antibodies stimulate TF induced FX activation	71
Protein S	In combination with anti β2-GPI antibodies, anti protein S antibodies associate with increase history of thrombosis	73
Plasminogen	Clipped β2-GPI inhibits plasminogen conversion to plasmin by tPA.	74
Annexin V	Disruption of anticoagulant protective annexin V shield present on endothelial cells.	75;76

Table 1:
A schematic overview of physiological systems affected by anti β2-GPI antibodies

Domain V of β2-GPI is different in that it consists of 82 amino acids instead of 60. It contains a large positive patch, composed of 12 lysine side chains, on arginine and one histidine residue located at the outside of domain V. Four of these lysines are delimited by the cysteine residues Cys^{281}-Cys^{288} [62]. Two positively charged lysine residues at position 308 and 324 are important for phospholipid binding [63;64]. A flexible loop in the middle of this positively charged region (Ser^{311}-Lys^{317}) serves as a phospholipid insertion loop[65;66]. In this phospholipid insertion loop Trp^{316} is essential for phospholipid binding[67]. Human, bovine, canine and rabbit β2-GPI all have 5 domains with 60-80% amino acid homology with domain V being the most conserved [68;69].

The physiological role of β2-GPI has not been established but several possible functions have been postulated and are mostly related to the haemostatic system. Both pro-coagulant [70;71] and anti-coagulant [72;73] activity has been attributed to β2-GPI. In our laboratory, we observed inhibition of platelet adhesion to collagen when reconstituted blood was perfused over vWF in the presence of β2-GPI. This inhibition was a result of reduced binding of vWF to GPIbα due to β2-GPI binding to vWF. A similar inhibition was observed in GPIbα dependent aggregation experiments (unpublished data) suggesting an inhibitory role for β2-GPI in platelet adhesion. Interestingly, deficiency of β2-GPI does not contribute to a thrombotic phenotype in humans [74] and normal concentrations of haemostatic and fibrinolytic factors were measured [75]. Also, β2-GPI knock-out mice do not show increased susceptibility to thrombosis although slightly impaired thrombin activation upon activation of the extrinsic pathway and impaired reproductivity was observed [76]. A direct interaction between β2-GPI and thrombin has also been demonstrated[77]. They showed inhibition of thrombin mediated FXI activation by anti

β2-GPI antibodies. *In vitro* studies also suggest an inhibiting effect of β2-GPI in ADP induced platelet aggregation [72] and a modulating function on the integrin $\alpha_{IIb}\beta_3$ platelet microparticle membranes [78].

As β2-GPI is also isolated with chylomicron, very low density and high density lipoproteins, β2-GPI involvement in lipoprotein metabolism has been proposed [79]. Also, β2-GPI was first termed apolipoprotein H, suggesting a role in lipoprotein lipase activation [80]. Furthermore, recent studies have shown β2-GPI to have an inhibitory effect on low density lipoprotein oxidation and cholesterol accumulation in macrophages [81] suggesting a protective role in the development of atherosclerosis.

Aim of this thesis

Background
The mechanism by which auto-antibodies directed against β2-GPI attribute to the development of clinical manifestations observed in the antiphospholipid syndrome is not clear. β2-GPI has no clear function because no clear phenotype is present when it is absent in humans or mice. It was hypothesized that dimerization of β2-GPI after binding of auto-antibodies could lead to alterations in binding properties and function of β2-GPI. Indeed, several groups demonstrated that dimerization of β2-GPI is accompanied by a dramatic increase in its affinity for negatively charged surfaces [82-84]. This increase in affinity for negatively charges surfaces could explain the prolongation of the phospholipid dependent clotting time (LAC) observed in APS patients. Dimerization of β2-GPI leads to competition with coagulation factors FII, FVII, FIX and FX for binding to negatively charged phospholipids thereby prolonging the *in- vitro* clotting time. Addition of extra phospholipids could correct this prolongation excluding a clotting factor deficiency [85].

To further investigate the dimerization hypothesis Lutters *et al.* [86] constructed a chimeric recombinant protein consisting of β2-GPI fused to the dimerization domain apple4 of coagulation factor XI (apple4-β2-GPI). As controls they constructed 1: chimeric recombinant construct with a non dimerizing apple2 domain (apple2-β2-GPI) and 2: apple4-β2-GPI-W316S mutated in the phospholipid binding domain ($Trp^{316} \rightarrow S^{316}$) preventing binding to negatively charged phospholipids and 3: apple4 alone. Only dimerized β2-GPI (apple4-β2-GPI) showed LAC activity and phospholipid binding was required since apple4-β2-GPI-W316-S did not show prolongation of clotting times. Addition of monoclonal antibodies directed against β2-GPI to phospholipid dependent clotting assays also result in a prolonged clotting time. When $F(ab)_1$ fragments of these antibodies were used, prolongation was no longer detected. All the data presented above support the hypothesis that dimerization of β2-GPI by auto-antibodies is responsible for the prolonged clotting time observed in plasma of patients.

These findings do explain the laboratory observation in plasma of patients with the antiphospholipid syndrome, however, they do not explain the clinical symptoms pre-

sented in these patients. We hypothesized that dimerization of β2-GPI can also results in altered binding properties and function on a cellular level and that such consequence might affect cell function and properties.

To better understand the development of thrombosis in APS patients the recombinant constructs of β2-GPI were used in perfusion experiments. When whole blood was perfused over collagen in the presence of dimeric β2-GPI, surface coverage was increased by 50%. Monomeric plasma β2-GPI, apple2-β2-GPI or apple4-W316S were not able to induce increased platelet deposition on collagen. Anti β2-GPI antibodies isolated from patients or monoclonal anti β2-GPI antibodies also increased platelet adhesion and aggregate formation on collagen. These results suggest a role for dimerization of β2-GPI in the development of clinical symptoms in APS patients. A possible role for conformational changes in β2-GPI could not be excluded. However, the addition of recombinant apple2-β2-GPI to blood did not result in increased adhesion. Increased platelet deposition to collagen was shown to be TxA2. Using receptor associated protein (RAP), it was demonstrated that a member of the LDL-receptor family plays a role in this process[87]. Sofar, the only member of the LDL-receptor family identified on platelets is a truncated splice variant of ApoER2 known as ApoER2′ or ApoER2Δ5 [88].

The aim of this thesis was to elucidate the interaction of dimerized β2-GPI with its platelet receptor ApoER2′ and to establish whether ApoER2′ is the only receptor for dimeric β2-GPI on platelets. In chapter 2, the interaction of dimeric β2-GPI with the platelet receptor ApoER2′ will be studied and described using recombinant soluble ApoER2′ and domain deletion mutants of apple4-β2-GPI. This is done to identify the binding site of dimeric β2-GPI to ApoER2′. Chapter 3 describes the interaction of dimeric β2-GPI with other members of the LDL-receptor family. It analyses the biochemical properties of the interaction of dimeric β2-GPI with Megalin, LRP, LDL-receptor and VLDL-receptor. In chapter 4 the binding domain for dimeric β2-GPI on ApoER2′ is determined and two other splice variants of ApoER2 expressed by blood platelets are identified and cloned. Chapter 5 describes a new platelet receptor for dimeric β2-GPI. Experiments designed to test platelet adhesion to dimeric β2-GPI under different conditions of flow, showed GPIbα to be 1: a second platelet receptor for dimeric β2-GPI and 2: present in complex with ApoER2′ on the platelet surface. Chapter 6 describes signaling pathways initiated by dimeric β2-GPI binding to both platelet receptors. Interaction between the two receptors and subsequent signaling cascades are further studied.

In Short:

Chapter 1: General introduction.

Chapter 2: Study the interaction between ApoER2′ and dimeric β2-GPI. Epitope mapping on dimeric β2-GPI

Chapter 3: Study the interaction between dimeric β2-GPI and different splice variants of ApoER2′ expressed by platelets. Epitope mapping on ApoER2′

Chapter 4: Study the interaction between dimeric β2GPI and other members of the LDL-receptor family.

Chapter 5: Study the interaction between dimeric β2-GPI and blood platelets. GPIbα is a second platelet receptor for dimeric β2-GPI.

Chapter 6: Study the downstream signaling events upon binding of dimeric β2-GPI to blood platelets.

Chapter 7: General Discussion

Chapter 8: Nederlandse Samenvatting

Chapter 9: Appendices

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The binding site on β2-GPI for ApoER2′ on platelets is located in domain V

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Abstract

The antiphospholipid syndrome (APS) is caused by auto-antibodies directed against β2-glycoprotein I (β2-GPI). Dimerization of β2-GPI results in an increased platelet deposition to collagen. We found that Apolipoprotein E receptor 2′ (ApoER2′), a member of the LDL receptor family, is involved in activation of platelets by dimeric β2-GPI. To identify which domain of dimeric β2-GPI interacts with ApoER2′, we have constructed domain deletion mutants of dimeric β2-GPI, lacking domain I (delta I), II (delta II) or V (delta V), and a mutant with a Trp316Ser substitution in the PL-insertion loop of domain V. Delta I and delta II prolonged the clotting time, as did full length dimeric β2-GPI; delta V had no effect on the clotting time. Secondly, delta I and delta II bound to anionic PL, comparable to full length dimeric β2-GPI. Delta V and the Trp316Ser mutant bound with decreased affinity to anionic PL. Platelet adhesion to collagen increased significantly when full length dimeric β2-GPI, delta I or delta II (mean increase 150%) was added to whole blood. No increase was found with plasma β2-GPI, delta V or the Trp316Ser mutant. Immunoprecipitation indicated that full length dimeric β2-GPI, delta I, delta II and the Trp316Ser mutant can interact with ApoER2′ on platelets. Delta V did not associate with ApoER2′. We conclude that domain V is involved in both binding β2-GPI to anionic PL and in interaction with ApoER2′ and subsequent activation of platelets. The binding site in β2-GPI for interaction with ApoER2′ does not overlap with the hydrophobic insertion loop in domain V.

Introduction

The antiphospholipid syndrome (APS) 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; lupus anticoagulants (LAC) and anticardiolipin antibodies (aCL)) 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 beta2-glycoprotein I (β2-GPI or Apolipoprotein H) [5;6]. Beta2-glycoprotein I is abundantly present in plasma (approximately 200 μg/mL) and is mainly synthesized in the liver, although m-RNA coding for β2-GPI has been found in a variety of cells such as trophoblasts, placental cells, endothelial cells and neurons [7-9]. The mature sequence of human β2-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 (CCP) family. The first four domains have approximately 60 aa residues and 4 cysteines each, with potential disulphide 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 disulphide bridges. A positively charged (multiple lysine) region between cys281-cys288 in domain V is highly conserved and a critical phospholipid-binding site [10-13]. The flexible loop Ser311-Lys317, containing Trp316 that 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 β2-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 LRP8) is a member of the low density lipoprotein (LDL) receptor family. It has been identified by Kim *et al*. in 1996 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 (CR) domains, not all of which are translated into the polypeptide chain. Due to alternative splicing, receptors with either four or five CR-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 (EGF) homology repeats, *O*-linked sugar domain, a cytoplasmic domain that contains one internalization signal and a single transmembrane region (TM). In recent publications it has been shown that LDL and dimeric β2-GPI can interact with ApoER2'on platelets [22;25]. Until now, little is known about the interaction between

(dimeric) β2-GPI and platelets. As for phospholipid binding, cationic patches might play an important role, because ligand-binding to ApoER2′ is dependent on electrostatic interactions [26]. Beta2-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′.

Methods

Construction of dimeric construc of β2-GPI.
The dimer apple4-β2-GPI and the apple2-β2-GPI, which is not able to form dimers, were constructed as described previously [27]. To exclude the possibility that apple4-β2-GPI binds via the dimerization domain of factor XI (apple4), dimer apple4 was constructed. The sequence of dimeric apple4 was amplified from the vector tissue plasminogen activator (tPA)-S478A with the primers apple4-*Bgl* II (GCC AGA TCT TTC TGC CAT TCT TCA) and apple4-*Xba* I (GGT CTA GAC TCG AGT CCC TCC TTT GAT GCG TG). The PCR product was cloned into the vector apple-4 tPA S478A with *Bgl* II and *Xba* I (underlined in apple4-*Bgl* II and apple4-*Xba* I, respectively). The starting point for the construction of the domain deletion mutants (DM) was the full-length cDNA of apple4-C321S β2-GPI (in short apple4-β2-GPI) cloned into the vector apple4-C321S tPA S478A. The domain I deletion was constructed with the primers domain II β2-GPI-*Xho* I forward (CC CTC GAG AT CCC AGA GTA TGT CCT TTT GCT G) and β2-GPI-*Xba* I reverse (GC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG A). For the construction of the domain II deletion a set of two primers was used; for domain I amplification the primers β2-GPI-*Xho* I forward (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and domain I β2-GPI reverse (TGT ACA TTT CAG AGT GTT GAT G) and for domain III-V amplification the primers domain III β2-GPI forward (ACT CTG AAA TGT ACA CCC ATC ATC TGC CCT CCA CCA) and β2-GPI-*Xba* I reverse. These two products served as a template in a second PCR to amplify the full-length domain I deletion using β2-GPI-*Xho* I forward and β2-GPI-*Xba* I reverse. The domain V deletion was constructed with the primers β2-GPI-*Xho* I forward and domain IV β2-GPI-*Xba* I reverse (TCT AGA TCA TTT ACA ACT TGG CAT GGC AGA CCA). To construct fusion proteins of apple4 and the domain deletion mutants of β2-GPI, the PCR product was cloned with *Xho* I and *Xba* I into the vector apple4-C321S-tPA-S478A. In this way DM of apple4-β2-GPI were constructed. Sequence analyse 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 stopcodon was deleted. Forward primer: sh-ApoER2′ *BamH* I GGA TCC GGG CCG GCC AAG GAT TGC GAA AAG G. Reverse primer: sh-ApoER2′ *Not* I GC GGC CGC CTT GCA GTT CTT GGT CAG TAG GTC C. Sh-ApoER2′ was then cloned into pTT3-SRα-GH-HISN-TEV. This expression vector is constructed from the pTT3 expression vector [29] and the pSGHV0 expression vector [30].

Transfection, Expression, Cell Culture and Purification of dimeric constructs of β2-GPI and sh-ApoER2′.

Transfection of baby hamster kidney (BHK) 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; Life Technologies, Inc., Paisley, United Kingdom). Protein expression was measured using a β2-GPI ELISA. Domain deletion mutants of apple4-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 Pharmacia Biotech, Uppsala, Sweden). 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 FPLC (Amersham Pharmacia Biotech). Fusion proteins were eluted with a linear salt gradient from 50 mM NaCl to 1 M NaCl. After determination of the purity of the protein fractions on a 4-15% polyacryl amide gel electrophoresis (SDS-PAGE), fractions with DM of apple4-β2-GPI were pooled, concentrated with polyethylene glycol (PEG) and dialysed against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Apple4 was purified using monoclonal antibody XI-1 (generous gift of Dr. J.C.M. Meijers, Academic Medical Hospital, Amsterdam), which recognizes the apple4 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-PEI method according to Durocher et al [29]. Soluble ApoER2′ production was done in a 1 liter suspension culture (in medium containing 90% freestyle, 10% calcium free DMEM, 0.5 % fetal calf serum, Invitrogen) for 4 days. Soluble ApoER2′ was affinity purified using RAP-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 (BSA) 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, 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 Pharmacia Biotech). 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 apple4-β2-GPI to PS/PC vesicles was tested in a solid phase binding assay. High binding 96 wells ELISA plates (Costar, Corning Incorporated, 9102) were coated with 20% PS/PC 80% (25 μM in TBS; 50 μL/well) overnight at 4°C. Wells were blocked with TBS/0.5% gelatine (150 μL/well) for two hours at 37°C. Subsequently, wells were incubated with different concentrations of DM (0.25-32 μg/mL) for 1.5 hours at 37°C followed by incubation with Moab 2B2 (3 μg/mL; 50 μL/well; 1.5 hours at 37°C). Apple4-β2-GPI was used as a positive control and plasma β2-GPI as a negative control. Afterwards the wells were incubated with peroxidase-conjugated rabbit anti-mouse antibody (RAMPO) (1:1000; 50 μL/well; 1.5 hours at 37°C), followed by staining procedure using orthophenylenediamine (OPD). Samples were diluted in TBS/0.5% gelatine. Non-specific binding was determined using non-coated wells. Results are expressed as mean ± SD (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, apple4-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% tri-sodium 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 β2-GPI, apple4, apple2-β2-GPI, apple4-β2-GPI, 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 ± SD, n = 3). Statistical analysis was performed with a Student T-test.

Binding of apple4-β2-GPI to immobilized soluble human ApoER2'.
Binding of apple4-β2-GPI to soluble human (sh) ApoER2' was tested in a solid phase binding assay. All incubations were performed for one hour at room temperature. Hydrophobic 96 well ELISA plates were coated with increasing concentrations of sh-ApoER2' in PBS (0-10 μg/mL; 50μL/well). Wells were blocked with PBS/4% BSA (150 μL/well). After incubation with plasma-derived β2-GPI, apple4, apple2-β2-GPI, apple4-β2-GPI, apple4-β2-GPI-Trp316Ser, delta V (3 μg/mL, 50μL/well) or plasma β2-GPI (3 μg/mL) in the presence of a mouse monoclonal a-β2-GPI antibody (19H9; 1 mg/mL) in PBS/1%BSA, bound protein was detected using rabbit polyclonal anti-β2-GPI (1:500; 50μL/well). Wells were incubated with peroxidase-conjugated swine anti-rabbit antibody (SWARPO) (1:500 50μL/well), followed by staining procedure using OPD. A control protein with a C-terminus growth hormone-tag was used to measure non-specific binding of apple4-β2GPI. Results are expressed as mean ± SD (n = 3).

Association of apple4-β2-GPI with sh-ApoER2' in the presence of inhibiting peptides.
Binding of apple4-β2-GPI 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 β2-GPI), CKNKEKKC (representing a cationic patch at aa position 282-287 in domain V of β2-GPI) and EKCKNKCK (scrambled). Hydrophobic 96 well ELISA plates were coated with 5 μg/mL of sh-ApoER2' in PBS (50μL/well). Wells were blocked with PBS/4% BSA (150μL/well). After incubation with apple4-β2-GPI (3 μg/mL, 50μL/well) with or without increasing concentrations of peptides (500 μg/mL), wells were subsequently incubated with a rabbit polyclonal anti-β2-GPI antibody (1:500; 50μL/well) and SWARPO (1:500, 50μL/well). This was followed by a staining procedure using OPD. Binding of apple4-β2-GPI in the absence of peptide was set at 100 %. Results are expressed as mean ± SD (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, apple4-β2-GPI, apple4-β2-GPI-Trp316Ser or DM of apple4-β2-GPI (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. Afterwards, platelets were lysed on ice with 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid), con-

taining 50 mM MES (2-(N-Morpholino) ethanesulfonic acid) 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 Biociences). 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) non-fat 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% non-fat dry milk. The membranes were washed three times and incubated with RAMPO (1:2500; Dako, Glostrup, Denmark) in the same buffer. Bands on blots were visualized with enhanced chemiluminescence. This experiment was performed with washed platelets form three different donors.

Results

To study the effect of domain involvement of apple4-β2-GPI on PL-binding, clotting time and platelet adhesion, dimeric constructs of β2-GPI fused to the C terminus of the dimerization domain (apple4) of factor XI were made. BHK cells were transfected with expression vectors containing DM of apple4-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 enzyme linked immunosorbent assay (ELISA). The proteins were purified using a monoclonal anti β2-GPI antibody (Moab 21B2) column followed by further purification on a mono S column using FPLC. After purification DM were applied on a 7.5 % SDS-PAGE under non-reducing (*Figure 1, panel A*) and reducing (*Panel B*) conditions and stained with Coommassie Brilliant Blue. In the presence of SDS full-length apple4-β2-GPI (*D*), apple4-β2-GPI-Trp316Ser (*W*), apple-4-Δ1β2-GPI (Δ*I*), apple4-Δ2-β2-GPI (Δ*II*) and apple4-Δ5-β2-GPI *(*Δ*V*) migrated as monomers with an apparent molecular mass of 50 kDa. Plasma β2-GPI (*M*) migrated with a molecular mass of 45 kDa under non-reducing conditions. Under reducing conditions full-length apple4-β2-GPI and apple4-β2-GPI-Trp316Ser migrated with a molecular mass of approximately 62 kDa. Apple4-Δ1-β2-GPI, apple4-Δ2-β2GPI and apple4-Δ5-β2-GPI migrated slightly slower with a molecular mass of approximately 56 kDa. Plasma β2-GPI migrated with a molecular mass of approximately 52 kDa.

To study the role of individual domains of apple4-β2-GPI for competing with coagulation factors, we studied the effect of the DM on clotting time. For this purpose we performed the prothrombin time (PT). Concentrations of 200 µg/mL plasma derived β2-GPI, apple4-β2-GPI or DM, diluted in TBS, were mixed 1:1 with normal pooled plasma (NPP) and incubated for 30 minutes 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 delta I and delta II was comparable with full length apple4-β2-GPI; apple4-β2-GPI showed a relative prolongation of the clotting time

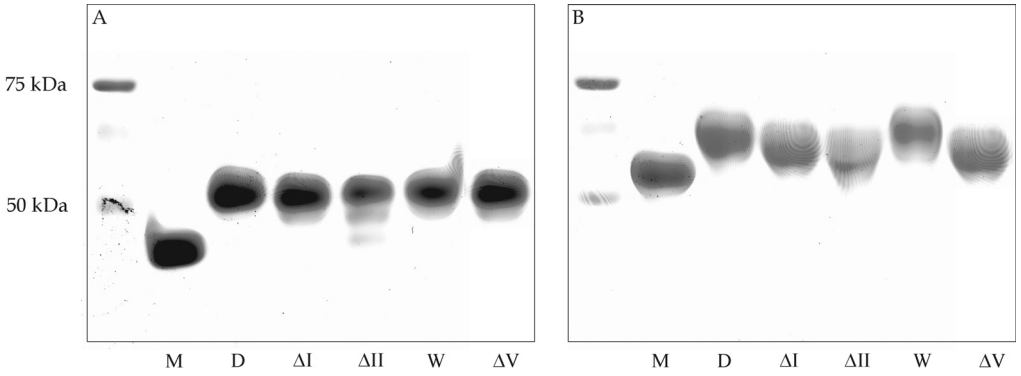

Figure 1. **SDS-PAGE analysis of apple4-β2-GPI constructs.**
Purified plasma β2-GPI (M), apple4-β2-GPI (D), delta I (Δ1), delta II (ΔII), delta V (ΔV) and apple4-β2-GPI-Trp316Ser (W) were analysed on a 10% SDS-PAGE under non-reducing- (A) and reducing (B) conditions. Gels were stained with Coommassie Brilliant Blue. The molecular masses of prestained markers are expressed in kilodalton (kDa).

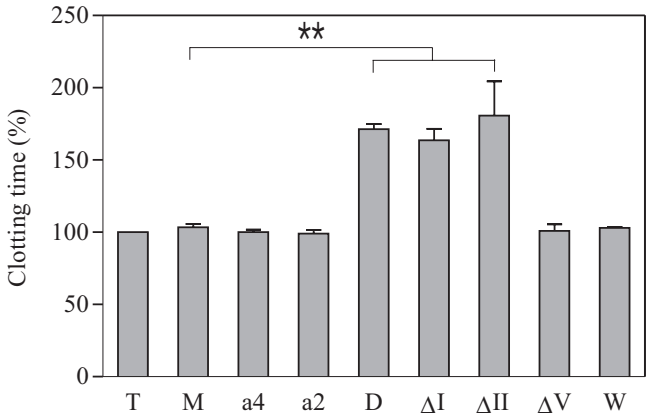

Figure 2. **Effect of apple4-β2-GPI constructs on the prothrombin time (PT).**
Plasma-derived β2-GPI (M), apple4 (a4), apple2-β2-GPI (a2), apple4-β2-GPI (D), delta I (ΔI), delta II ΔII), delta V (ΔV) or apple4-β2-GPI-Trp316Ser (W) were 1:1 diluted with normal pooled plasma (final protein concentration 100 µg/mL) followed by measurement of the PT. Clotting time with buffer (T) was set at 100%. Results represent mean clotting time ± SD in percentage (n = 3). Statistical analysis was performed using the Student T-test ($p \leq 0.001$). Differences between apple4-β2-GPI, delta I and delta II are not significant.

to 171.3 ± 3.7 %, delta I to 163.6 ± 7.9 % and delta II to 180.7 ± 23.8 %, respectively. Results are presented in *Figure 2*. The addition of plasma β2-GPI, delta V or apple4-β2-GPI-Trp316Ser to NPP did not influence the clotting time. Furthermore, the control proteins apple4 and apple2-β2-GPI did not influence the clotting time.

The phospholipid-binding features of apple4-β2-GPI fusion proteins were tested in a solid phase binding assay. Phospholipid vesicles (25 µM, 20% PS/PC 80%) were immobilized on 96-well ELISA plates, and binding of plasma-derived β2-GPI and DM of

apple4-β2-GPI was measured. As shown in *Figure 3*, half-maximal binding of apple4-β2-GPI to phospholipid vesicles occurred at a concentration of 2.1 μg/mL (*Table 1*). For the domain deletion mutants a similar interaction with phospholipid vesicles was observed: with delta I having half-maximal binding to phospholipids at a concentration of 2.9 μg/mL and delta II at a concentration of 4.1 μg/mL. Half-maximal binding to immobilized phospholipids of delta V occurred at a concentration of 29.2 μg/mL. For apple4-β2-GPI-Trp316Ser, half-maximal binding was observed at a concentration of 26.0 μg/mL. The presence of an amino acid substitution in the phospholipid-insertion loop

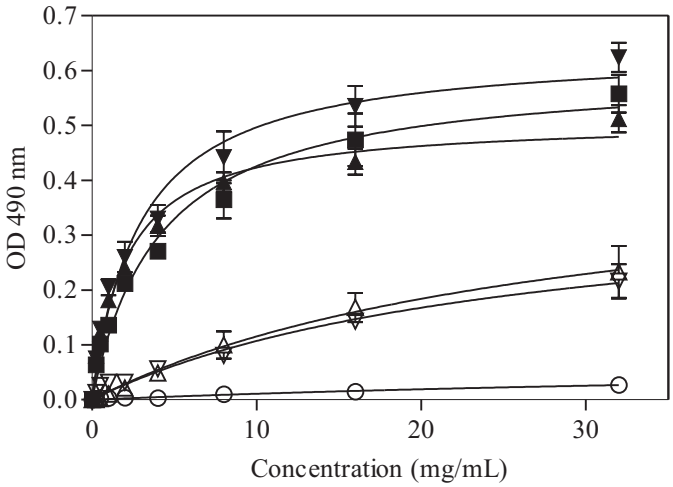

Figure 3. **Binding of apple4-β2-GPI domain deletion mutants to immobilized PL**
Phospholipid vesicles (20% PS/PC 80%, 25μM) were immobilized on high binding 96-well ELISA plates and incubated with increasing concentrations (ranging from 0.25-32 μg/mL) of plasma β2-GPI (❍), apple4-β2-GPI (▲), delta I (▼), delta II (■), delta V (Δ) or apple4-β2-GPI-Trp316Ser (▽) at 37°C for 2 hours. Afterwards bound protein was detected with Moab 2B2. Bound 2B2 was detected using OPD staining procedure. Results are expressed as mean ± SD (n = 3).

Apparent K_d	Plasma β2-GPI	Apple4β2-GPI	Apple4-Δ1-β2-GPI	Apple4-Δ2-β2-GPI	Apple4-Δ5-β2-GPI	Apple4-β2-GPI-Trp316Ser
$K_{d(app)}$ (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^{e3} ± 0.6^{e3}	18.3 ± 1.8	29 ± 4.0	41 ± 5.0	314 ± 25.8	226.0 ± 37.4

Table 1. **Apparent dissociation constants of domain deletion mutants for PS/PC vesicles**
Curves of plasma β2-GPI and apple4-β2-GPI fusion proteins were fitted according to a one-site binding model in GraphPad. Half-maximal binding is given as apparent Kd (Kd(app)) both in μg/mL as in nmol/L (nM). Results are expressed as mean ± SD (n = 3).

explains why the Trp316Ser mutant hardly binds to anionic phospholipids. Plasma-derived β2-GPI showed little binding at a concentration of 16 μg/mL.
To determine which domain of apple4-β2-GPI is involved in platelet sensitization, we performed perfusion experiments with citrated whole blood pre-incubated with plasma β2-GPI, apple4-β2-GPI or DM (final concentration 100 μg/mL). To determine basal

platelet adhesion to collagen type III, whole blood was pre-incubated with buffer. The basal platelet coverage after 90 s was 9.4 ± 2.1 %, which was set at 100 % (baseline). As shown in *Figure 4A*, no increase in platelet adhesion was found when plasma β2-GPI, apple4, apple2-β2-GPI or delta V was 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], apple4-β2-GPI-Trp316Ser did not induce increased platelet adhesion to collagen. In contrast, platelet adhesion increased significantly when full length apple4-β2-GPI, delta I or delta II 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 apple4-β2-GPI (*Figure 4B, Panel a*), delta I (*not shown*) and delta II (*not shown*). Plasma β2-GPI, delta V (*Panel b and d, respectively*) and

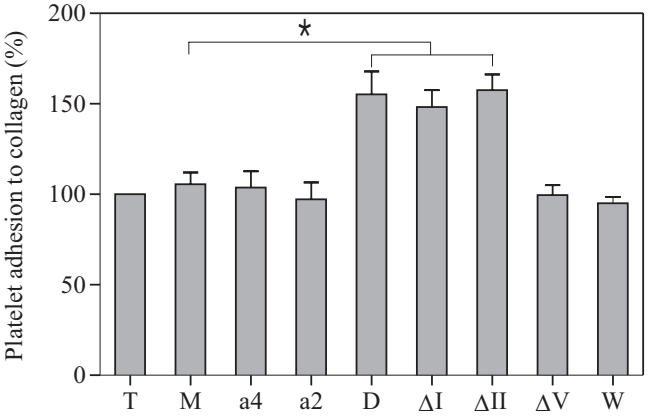

Figure 4A. **Platelet deposition on collagen type III in the presence of domain deletion mutants of apple4-β2-GPI.** Whole blood was pre-incubated at 37°C for 5 minutes with buffer (T), plasma-derived β2-GPI (M), apple4 (a4), apple2-β2-GPI (a2), apple4-β2-GPI (D), apple4-β2-GPI-Trp316Ser (W) or domain deletion mutants (ΔI, ΔII and ΔV, respectively) of apple4-β2-GPI (10% v/v) with a final concentration of 100 µg/mL. Whole blood was perfused over collagen type III for 90 sec. at a shear rate of 800 s^{-1}. Figure 4A; Percentage of platelet coverage is expressed relative to platelet coverage in the presence of buffer (set at 100%). Data is expressed as mean ± SD (n = 3). Statistical analysis was performed using the Student T-test ($p < 0.005$). Differences between apple4-β2-GPI, delta I and delta II were not significant.

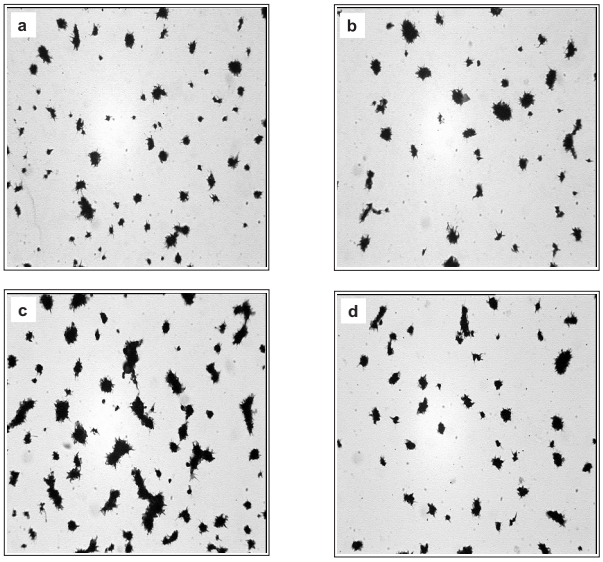

Figure 4B. **Platelet morphology and aggregate formation.** In the presence of buffer (Panel A), plasma β2-GPI (Panel B), apple4-β2-GPI (Panel C) or delta V (Panel D). There was no difference between apple4-β2-GPI, delta I and delta II. Platelet morphology and aggregate formation in the presence of apple4-β2-GPI-Trp316Ser was similar to buffer control.

apple4-β2-GPI-Trp316Ser (*not shown*) displayed comparable effects on morphology and number of platelet aggregates as incubation with buffer (*Panel a*).
Binding of apple4-β2-GPI and the Trp316Ser mutant to immobilised soluble human (sh)-ApoER2′ was measured using an ELISA setup. As shown in *Figure 5*, half-maximal binding of both apple4-β2-GPI and apple4-β2-GPI-Trp316Ser 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 β2-GPI in the presence of a monoclonal α-β2-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 β2-GPI, apple4 or delta V. Apple2-β2-GPI displayed only slight interaction with sh-ApoER2′.

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 β2-GPI and not the hydrophobic PL-insertion loop in domain V (Ser311-Lys317) is responsible for interaction with ApoER2′, immunoprecipitations were performed. Platelets were incubated with buffer, plasma-derived β2-GPI, full length apple4-β2-GPI, DM or apple4-β2-GPI-Trp316Ser, lysed and subjected to immunoprecipitation with an anti-ApoER2′ antibody. Afterwards, Western blots were incubated with a monoclonal anti-β2-GPI antibody to detect interaction between β2-GPI and ApoER2′. Association with ApoER2′ was observed with apple4-β2-GPI (*Figure 6*). Hardly any association was observed when platelets were incubated with plasma β2-GPI or delta V. To demonstrate that this finding was not the result of the inability of delta V to bind to anionic phospholipids, immunoprecipitations were performed with apple4-β2-GPI-Trp316Ser. The interaction of

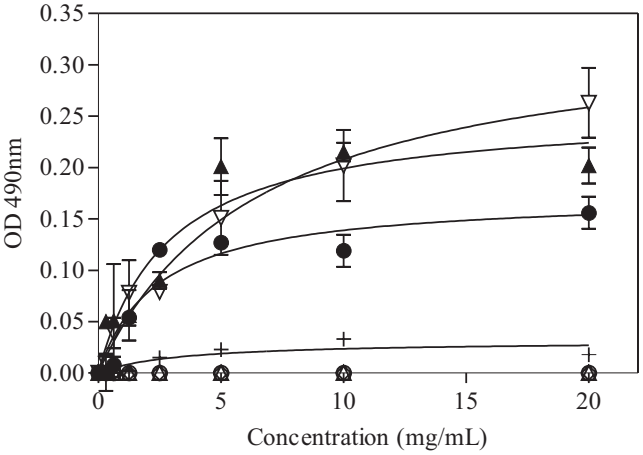

Figure 5. Binding of apple4-β2-GPI and the Trp316Ser mutant to immobilized ApoER2′
Interaction between apple4-β2-GPI and sh-ApoER2′ was measured in a solid phase binding assay. Soluble human ApoER2′ was coated on a hydrophobic 96 well ELISA plate in a concentration dependent manner (0-10 μg/mL). After blocking, plasma β2-GPI (❍), apple4 (◊), apple2-β2-GPI (+), apple4-β2-GPI (▲), delta V (△), apple4-β2-GPI-Trp316Ser (▽) mutant and plasma β2-GPI + 19H9 (•) were incubated (3 μg/mL). Afterwards bound protein was detected with rabbit polyclonal anti-β2-GPI. Bound antibody was detected using SWARPO. Results are expressed as mean ± SD (n = 3).

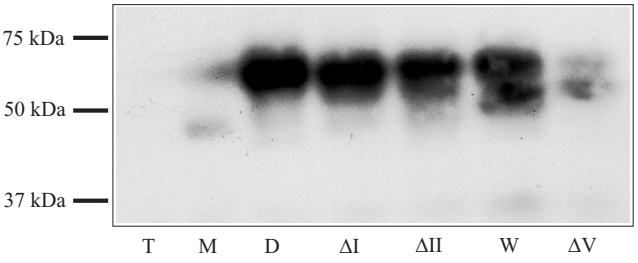

Figure 6. **Immunoprecipitations with domain deletion mutants of apple4-β2-GPI and ApoER2′ on platelets.** Washed platelets (300.000/μl), resuspended in Hepes/Tyrode buffer containing 3 mM CaCl2 were incubated with buffer, apple4-β2-GPI (D), delta I (ΔI), delta II (ΔII), delta V (ΔV) or apple4-β2-GPI-Trp316Ser (W) (final concentration 100 μg/mL; 10% v/v) for 5 minutes 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. Afterwards Western blots were incubated with an anti-β2-GPI antibody followed by visualization using chemiluminescence. Blot represents three different experiments.

the Trp316Ser mutant with ApoER2′ on the surface of platelets was similar to that of full length apple4-β2-GPI.

Association of ligands with members of the LDL receptor family is supported by electrostatic interactions. To investigate the possibility that association of apple4-β2-GPI with ApoER2′ is supported by a cationic patch in domain V of apple4-β2-GPI competition studies were performed with positively charged peptides. Incubation of washed platelets with apple4-β2-GPI and CKNKEKKC (represents a cationic patch at aa position 282-287 in domain V of β2-GPI) peptide resulted in decreased binding of apple4-β2-GPI to ApoER2′ (*Figure 7A*). This effect was not seen with the VSRGGMRK peptide

(represents a cationic patch at aa position 37-44 in domain I of β2-GPI). The decreased binding of apple4-β2-GPI to ApoER2′ in the presence of CKNKEKKC was also seen for the Trp316Ser mutant. Incubation of washed platelets with apple4-β2-GPI and

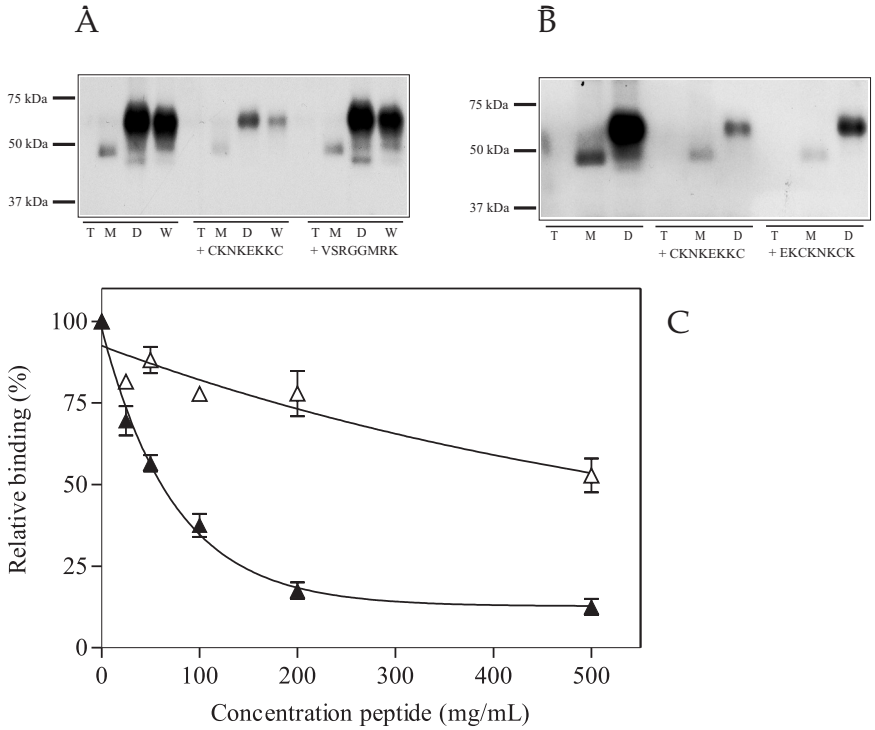

Figure 7. **Immunoprecipitations with apple4-β2-GPI and ApoER2′ on platelets in the presence of CKNKEKKC, EKCKNKCK or VSRGGMRK peptides.** Competition experiments were performed to investigate electrostatic involvement between apple4-β2-GPI and ApoER2′. For this purpose washed platelets were incubated with buffer (T), plasma β2-GPI (M), apple4-β2-GPI (D), apple4-β2-GPI-Trp316Ser (W) in the presence of cationic peptides; incubations in the presence of CKNKEKKC or the VSRGGMRK peptide (Panel A) or in the presence of the EKCKNKCK peptide (Panel B) (final peptide concentration; 500 μg/mL) for 5 minutes at 37°C. Afterwards immunoprecipitations were performed as described previously. Blots represent three different experiments. To show direct competition between apple4-β2-GPI and the peptides CKNKEKKC (▲) and EKCKNKCK (△), binding of apple4-β2-GPI in the presence of the peptides was investigated in the solid phase binding assay (Panel C).

EKCKNKCK (scrambled peptide) resulted in a minor reduction of the association between apple4-β2-GPI and ApoER2′ (*Figure 7B*).

To show direct competition between apple4-β2-GPI and the different peptides, apple4-β2-GPI was able to interact with sh-ApoER2′ in the presence of increasing concentrations peptides VSRGGMRK, CKNKEKKC or EKCKNKCK (scrambled). As shown in *Figure 7C*, inhibition was observed for both the CKNKEKKC and the EKCKNKCK peptide. Binding of apple4-β2-GPI 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 VSRGGMRK peptide no inhibition was observed (*data not shown*).

The inhibiting effect of the peptides on association between apple4-β2-GPI and ApoER2′ might be due to interference with binding of the apple4-β2-GPI 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 %. Apple4-β2-GPI displayed a relative prolongation of the PT to 194.0 ± 2.8 % (*Figure 8*). In the presence of the peptides CKNKEKKC or EKCKNKCK (500 μg/mL) prolongation of the clotting time was observed to 191.3 ± 7.8 % and 216.5 ± 23.3 %. The peptides CKNKEKKC or EKCKNKCK (500 mg/mL) did not influence the clotting time in the absence of dimeric β2-GPI up to concentrations of 1 mg/mL.

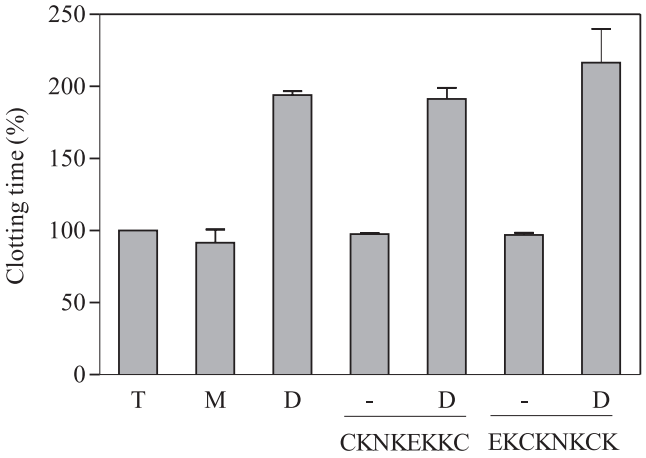

Figure 8. **The influence of the inhibiting peptides CKNKEKKC and EKCKNKCK on the prothrombin time.** Plasma-derived β2-GPI (M), apple4-β2-GPI (D), in the absence or presence of CKNKEKKC or EKCKNKCK peptide (final concentration 500 μg/mL), were 1:1 diluted with normal pooled plasma (final concentration 100 μg/mL) followed by measurement of the PT. Clotting time with buffer (T) was set at 100%. Results represent mean clotting time ± SD in percentage (n = 3).

Discussion

It is difficult to envision that mere binding of beta2-glycoprotein I (β2-GPI) to anionic phospholipids (PL) on the cell surface can activate these cells. Therefore, a search for a cellular receptor for β2-GPI on platelets was initiated. Lutters *et al.* have demonstrated that dimerization of β2-GPI (either artificially by fusing β2-GPI with the apple4 domain of factor XI, or physiologically by binding aPL to β2-GPI) results in increased affinity of β2-GPI for platelets [27] 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 (RAP), suggesting that a member of the LDL receptor family as the platelet receptor for dimeric β2-GPI was involved. This receptor has later been identified as ApoER2′ [25]. In the present study, the domain of β2-GPI responsible for interaction with ApoER2′ has been determined by using constructs of dimeric β2-GPI lacking domain I, II or V and a construct with an aa substitution (Trp316Ser) in the hydrophobic loop in domain V. Substitution of Trp316 by a serine completely abolished binding of β2-GPI to anionic phospholipids [14]. In this study, we showed that a cationic

patch in domain V of dimeric β2-GPI is involved in interaction with ApoER2' on platelets and that, by using the Trp316Ser 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 β2-GPI. Both domain deletion mutants (delta I and delta II) prolong the clotting time in the prothrombin-time (PT) comparable to full length dimeric β2-GPI (*Figure 2*) and show comparable affinity for anionic phospholipids (*Figure 3*). Furthermore, delta I and delta II cause an increased adhesion of platelets to collagen under conditions of flow comparable to full length dimeric β2-GPI (*Figure 4A*). Deletion of domain V or the Trp316Ser substitution abolished the prolongation of the clotting time induced by dimeric β2-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 paper, we propose the following model for the activation of platelets by beta2-glycoprotein I (*Figure 9*). Beta2-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 β2-GPI [36-39], resulting in the exposure of a cryptic epitope in this domain. Binding of anti-β2-GPI antibodies to this newly exposed epitope in domain I takes place. When one antibody interacts with two molecules of β2-GPI, the protein dimerizes resulting in an increased affinity for phospholipids on the outer surface of the platelet membrane. This increased affinity of the β2-GPI /anti-β2-GPI complexes is mimicked by our recombinant dimeric β2-GPI construct. The binding to the platelet membrane also results in concentration of β2-GPI on the cellular surface, which allows interaction with ApoER2' due to mass action effects. Stabilization of the binding of dimeric β2-GPI to phospholipids is crucial before it can bind to ApoER2', as the Trp316Ser mutant is not able to activate platelets under conditions of flow (*Figure 4A*), despite the fact that the protein can bind to immobilized ApoER2' (*Figure 5*). We cannot exclude that also a conformational change in domain V is necessary for interaction with ApoER2', as plasma β2-GPI is not able to associate with the receptor (*Figure 6*). Conformational changes in domain V of β2-GPI after binding to phospholipids have been described before [40]. The interaction between β2-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 β2-GPI to ApoER2' (*Figure 7A, B and 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 β2-GPI, dimerization of the receptor may occur [41]. The interaction of dimeric β2-GPI with ApoER2' results in downstream signaling, mediated via $p38^{MAP}$ kinases [42]. This is followed by synthesis of thromboxane A2 [25]. Thromboxane A2 further mediates platelet activation [43-45].

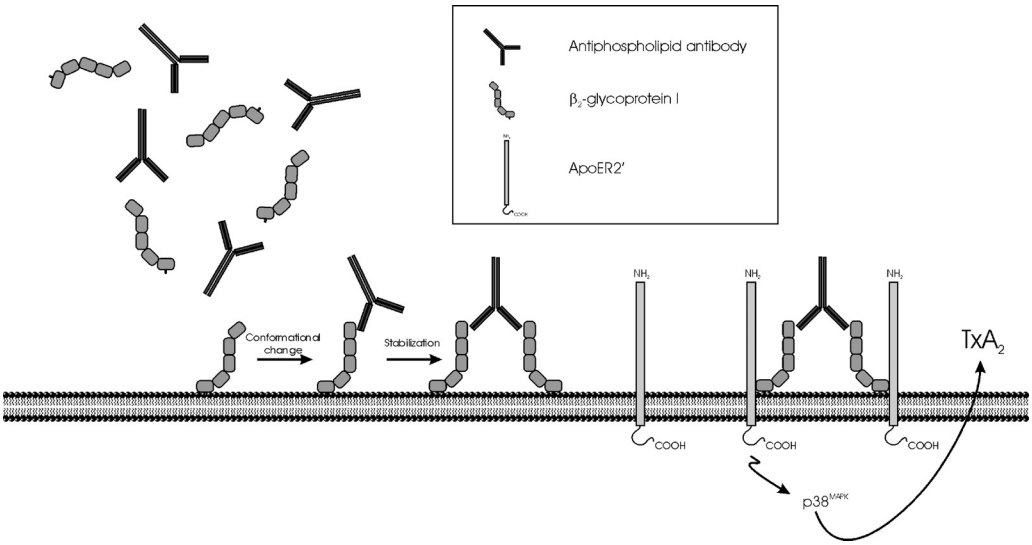

Figure 9. **Proposed mechanism of platelet activation via dimerization of β2-GPI by aPL.** Beta2-glycoprotein I circulates free in plasma with low affinity for phospholipids. A conformational change in domain I β2-GPI is induced after low-affinity binding to platelets. Dimerization of β2-GPI via binding of aPL occurs followed by stabilization of the complex on the platelet surfaced which subsequently results in firm adhesion of the β2-GPI/aPL complex to PL and association with ApoER2′ on the surface of platelets. We cannot exclude the possibility that dimeric β2-GPI dissociates from the cellular surface after interaction with ApoER2′. The interaction results in down-stream signaling, TxA2 synthesis and further platelet activation.

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3

Platelets express three 3 different splice variants of ApoER2 that are all involved in signaling.

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Abstract

Introduction: β2-Glycoprotein I is the most relevant antigen in the antiphospholipid syndrome. We have shown that binding of dimerized β2-GPI to platelets via ApoER2′ sensitizatizes platelets for second activating stimuli. Aim: Determine the region of ApoER2 involved in the binding of dimeric β2-GPI. Methods: Cultured human megakaryocytes and three different human megakaryocytic cell lines were used for mRNA isolation to clone and express recombinant soluble platelet ApoER2. Domain deletion mutants of ApoER2 were constructed to identify the binding site for dimeric β2-GPI. The presence of ApoER2 splice variants on in platelets was demonstrated by immuno-blotting. Results: Three different mRNA splice variants were isolated from all four types of megakaryocytic cells used. Sequence analysis identified the splice variants: 1) shApoER2Δ5 lacking LDL binding domains 4, 5 and 6; 2) shApoER2Δ4-5 lacking LDL binding domains 3, 4, 5, 6 and 3) shApoER2Δ3-4-5 lacking LDL binding domains 3, 4, 5, 6 and 7. The presence of three splice variants of ApoER2 on platelets could be confirmed by immuno-blotting, with ApoER2Δ4-5 being the most abundantly expressed splice variant. Upon stimulation with dimeric β2-GPI, all three splice variants were translocated to the cytosol, however, ApoER2Δ4-5 translocation was most prominent. Dimeric β2-GPI binds platelet ApoER2 variants via LDL-binding domain 1. Conclusions: Three different ApoER2 mRNA splice variants were isolated from megakaryocytes and platelets express all three splice variants. All splice variants showed to be functional by translocation upon stimulation with dimeric β2-GPI. All three splice variants express LDL-binding domain 1.

Introduction

Patient studies have shown that in the antiphospholipid syndrome the presence of anti-β2-GPI antibodies in plasma strongly correlates with the presence of thrombosis [1,2]. The mechanism by which anti-β2-GPI antibodies are involved in the patho-physiology of the antiphospholipid syndrome is not fully understood. When β2-GPI interacts with anti β2-GPI antibodies, β2-GPI becomes dimerized and conformational changes are introduced into the structure of β2-GPI resulting in an enhanced affinity for anionic phospholipids[3,4]. Previous research has demonstrated that dimerization of β2-GPI by auto antibodies can also induce activation of several cell types such as monocytes [5,6], endothelial cells [7-9] and blood platelets [10-12] via interaction with cellular receptors such as TLR4 [13], GPIbα [11,12] and several members of the LDL-receptor family [14]. These three cell types are considered to participate in the development of thrombotic complications. One of the first cellular receptors identified for β2-GPI antibody complexes was ApoER2', a receptor also present on the membrane of blood platelets [10]. ApoER2' is a splice variant of ApoER2 in which exon 5 is omitted during mRNA splicing and therefore lacks LDL-binding domains 4, 5 and 6. Binding of auto-antibodies to β2-GPI results in dimerization of β2-GPI which can subsequently bind to ApoER2' via a cationic patch in its domain V [15]. In neuronal cells it has been shown that adaptor proteins attached to ApoER2 determine its cellular localization [16-18] and ligand binding can alter proteolytic cleavage of ApoER2 [19]. It is now clear that ApoER2 is not merely an endocytic receptor mediating uptake of ApoE but ApoER2 mainly has signaling properties as reviewed by Stolt *et al.* [20]. The intracellular portion of ApoER2 contains the NPxY motif localizing it to caveolae, cholesterol and sphingolipid rich micro domains with known signaling properties [21]. The extracellular part of ApoER2 consists of three distinct functional and structural region. These are: 1) the type A-binding repeats or LDL-binding domains of ± 40 residues displaying a negative charged surface, responsible for receptor-ligand interactions. 2) The type B repeats which are homologous to regions in the epidermal growth factor precursur and 3: the protein stack comprised of modules of 50 residues of O-linked sugar domains spacing the LDL-binding domains from the cellular surface. Moreover, blood platelets play a major role in the development of arterial thrombosis. Blood platelets can be used as a model cell system to study cell activation by dimeric β2-GPI because platelets are easily isolated from humans and they respond strongly to many stimuli. To better understand the interaction of dimeric β2-GPI with blood platelets via ApoER2', we started to investigate which LDL-binding domain of ApoER2' is involved in binding of dimeric β2-GPI. Here we found that platelets express three splice variants of ApoER2 and all three variants are able to signal via β2-GPI-antibody complexes.

Methods

Reagents

Polyclonal rabbit anti β2-GPI antibody was produced by immunization of rabbits with human β2-GPI. Plasma was obtained after sequential boosting by plasmaphereses. Monoclonal mouse anti human β2-GPI antibody 21B2 was a generous gift from Prof J.

Arnout (Leuven, Belgium). For purification of dimeric β2-GPI, 21B2 was coupled to CNBr-Sepharose according to manufacturer's protocol (Amersham Pharmacia Biotech, Uppsala Sweden). We produced a monoclonal mouse anti human ApoER2 antibody (MP4-3) by immunizing mice with a peptide derived from the LDL binding domain 1 (WRCDEDDDCLDHSDED). Hybridoma cell lines were produced using a standard PEG fusion protocol with SP2.0 cells and specificity was tested using solid phase binding assays and immuno-blotting procedures.

Purification of plasma β2-GPI
Plasma β2-GPI was isolated from fresh citrated human plasma as described previously[22]. In short, dialyzed human plasma was applied to the following columns in consecutive order: DEAE-Sephadex A50, protein G-Sepharose, S-Sepharose, and finally heparin-Sepharose (all Sepharoses were obtained from Amersham Pharmacia Biotech). Bound proteins were eluted with linear salt gradients. Afterwards, β2-GPI was dialyzed against TBS. Purity of the protein was checked by 4-15% SDS-PAGE showing a single band of 47 kD under reduced conditions. Protein concentration was determined using the BCA protein assay.

Cell culture and blood platelet isolation
Human megakaryocytes were isolated as described previously [23]. Megakaryocytic cell lines Meg01, Dami and CHRF were cultured as described by Den Dekker *et al*.[24]. Human blood platelets were isolated as previously described [25].

Cloning and expression of dimeric β2-GPI
Recombinant dimeric β2-GPI was constructed and purified as described previously [26]. In short, the sequence encoding the mature β2-GPI protein was amplified with the primers β2-GPI-*Xho*I (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and β2-GPI-*Xba*I (GC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG A) by PCR. To construct chimerical fusion proteins of the dimerization domain of factor XI (apple4) and β2-GPI the PCR product was cloned with *Xho*I and *Xba*I into the vector zPL7-apple4-tissue-type plasminogen activator (tPA)-S478A [27] replacing tPA. The dimeric β2-GPI construct was then transfected into BHK cells and stable transfectants were selected in MTX containing medium. Dimeric β2-GPI was affinity purified using monoclonal anti β2-GPI antibody 21B2 and further purified using ion exchange (MonoQ, Amersham).

Cloning of soluble human ApoER2'
Mature megakaryocytes were cultured from citrated umbilical cord blood as described previously [23]. Messenger RNA from freshly isolated megakaryocytes and three megakaryocytic cell lines (Meg-01, Dami and CHRF) was isolated using Nucleospin® RNA II (Macherey-Nagel) and cDNA was synthesized using superscript II reverse transcriptase (Invitrogen,) according to the manufacturer's instructions. CopyDNA was amplified using Phusion DNA polymerase (Finnzymes, Finland). Primer design was such that, to obtain soluble receptor, forward primer started at the ATG sequence of the signal peptide adding a kozak sequence and a *Hin*DIII site for cloning and expression. Reverse primer started amplifying at the 2nd EGF domains thereby omitting the protein

stack, transmembrane portion and intra-cellular tail adding an *Eco*R1 site. Forward primer: Sol. H. ApoER2 *Hin*DIII forward: TATAA AAGCTT GCCACC ATGGGCCTC-CCCGAGCCGG. Reverse primer: Sol. H. ApoER2 *Eco*RI reverse: TAATA GAATTC CTTGCAGTTCTTGGTCAGTAGG . Amplified cDNA products were separated using agarose gel electrophoreses and cloned into cloning vector Topo®-blunt (invitrogen) according to manufacturers protocol for sequence analysis. Sequence analysis was performed using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's protocol. When secretion of expressed recombinant proteins failed using pcDNA6-V5-HIS, constructs from the cloning strategy were used as template for growth hormone fusion to recombinant protein thereby omitting the signal peptide starting directly at the beginning of LDL-binding domain 1: Forward primer: shApoER2' *Bam*HI GGA TCC GGG CCG GCC AAG GAT TGC GAA AAG G. Reverse primer: shApoER2' *Not*I GC GGC CGC CTT GCA GTT CTT GGT CAG TAG GTC C.

Cloning LDL-binding domain deletion mutants of soluble human ApoER2'
Constructs containing either LDL-binding domain 1 or LDL binding domain 2 were constructed from cDNA coding for shApoER2-650. For the deletion of LDL-binding domain 2 (shApoER2-BD1) two PCR's were performed which were combined by a third PCR afterward. PCR 1: LDL-binding domain 1 was amplified using primer 1: shApoER2'-BD1 *Bam*HI Forward TAATAGGATCC GGGCCGGCCAAGGATTGC G and primer2: shApoER2' ex2 overlap ex7 reverse primer GTTGTGCAGACACTC GTTCAGCCTGGGGCAGTCGTCCTCGTCGCGCTGTG. The EGF domains were amplified with PCR 2 using primer3: shApoER2' ex7 overlap ex2 forward CACAGC-GACGAGGACGACTGCCCCAGGCTGAACGAGTGTCTGCACAAC and primer4: shApoER2'+EGF *Not*I reverse TAATA GCGGCCGCCTTGCAGTTCT TGGTCAGTAG-GTCC. Both PCR products were then combined with PCR 3 using primers 1 and primer 4 to obtain a construct containing the LDL-binding domain 1 fused to the EGF domains of ApoER2'. For deletion of domain 1 (shApoER2-BD2), one PCR was performed using primer 5: shApoER2'-BD2+EGF *Bam*HI TAATA GGATCCCCCAAGAAGACCTGT-GCAGAC and primer 4: shApoER2' *Not*I reverse TAATA GCGGCCGC CTTGCAGTTCTT GGTCAGTAGGTCC. PCR products were analyses by agarose gel electrophoreses to determine product size, excised from the agarose and purified using a DNA extraction kit (Macherey-Nagel) and cloned into cloning vector Topo®-blunt (Invitrogen) according to manufacturers protocol for sequence analysis. Sequence analysis was performed using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's protocol.

Expression of soluble human ApoER2' splice variants and LDL-binding domain deletion mutants
Soluble human ApoER2 splice variants and domain deletion mutants were cloned into the expression vector PTT3-SRα-GH-HISN-TEV. The expression vector is constructed from the pTT3 [28] expression vector and the pSGHV0 expression vector [29]. HEK293-EBNA cells were transfected with the constructs using the DNA-PEI method according to Durocher et al [28]. Soluble human ApoER2 splice variants and domain deletion mutants were grown to produce in a 1 liter suspension culture (medium containing 90% freestyle, 10% calcium free DMEM, 0.1 % foetal calf serum, (Invitrogen)) for 4 days. Soluble

human ApoER2 splice variants and domain deletion mutants were affinity purified using NiNTA Sepharose and subsequent RAP-Sepharose. Concentration was measured with a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Protein purity and molecular weight were assessed by SDS- polyacrylamide gel electrophoreses followed by PageBlue™ Protein Staining Solution (Fermentas) according to manufacturer's protocol.

Immuno-blotting of platelet lysate
Washed platelets of 3 different donors were prepared as described by Weeterings *et al*.[25], set at 200.000/µl and 20 µl was then lysed by adding 5 µl 5x reducing Laemmli sample buffer (62.5 mM tris HCl pH6.8, 10% glycerol, 2% SDS and 5% β-mercaptoethanol). Samples were separated on a 8% SDS-polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris buffered saline (25mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween 20 (TBST) containing 2% (w/v) BSA (Sigma) for 1 h at room temperature. The blots were then incubated with mouse anti ApoER2 antibody (MP4-3, 1µg/ml) overnight in TBST with 1% BSA and washed three times with TBST. After incubation with peroxidase labeled rabbit anti mouse antibodies for 1 h at RT (1:2500; Dako, Glostrup, Denmark), blots were washed again with TBST and developed with enhanced chemiluminescence reagent plus (Perkin Elmer Life Sciences, Boston USA)

Solid phase binding assay
Binding of dimeric β2-GPI to different splice variants and domain deletion mutants was measured in a solid phase binding assay. Recombinant soluble human ApoER2 splice variants and domain deletion mutants were coated at 10 and 5 µg/ml in TBS (50 mM Tris, 150 mM NaCL pH = 7.4) in soft well ELISA plates (Vinyl Costar 96 wells Protein Assay plates). TBS treated wells were included as a negative control. Wells were then blocked using 4% Protifar (Nutricia, Zoetermeer, the Netherlands) for 1 hour at 37°C. Plates were washed 3 times with TBS+0.1% Tween20 (Riedel-de Haën) and wells were incubated with rabbit anti β2-GPI (1:500) antibody in TBS+1% Protifar for 1 hour at 37°C. After washing the plates 5 times with TBS+0.1% Tween20, wells were incubated with Goat anti Rabbit HRP 1:1000 (DAKO) in TBS+1% Protifar for 1 hour at 37°C. Plates were washed again with TBS+0.1% Tween20 and wells were incubated with Rabbit anti Goat HRP 1:1000 (DAKO) in TBS+1% Protifar for 1 hour at 37°C. Finally plates were washed for the last time and wells were incubated with Ortho-PhenyleneDiamide (OPD) for staining.

Translocation of ApoER2'
500 µl aliquots of washed platelets (220.000/µL) resuspended in Hepes/Tyrode buffer pH 7.3 were incubated with TBS or dimeric β2-GPI at 50 µg/µl for 0.5, 1, 3, 5 or 10 minutes at 37°C in the presence or absence of inhibitors. Platelets were incubated with either TBS or dimeric β2-GPI for 5 minutes while stirring and lysed by adding 1/10th volume of 10x Triton lysis buffer (10% Triton-100, 200 mM Tris, 50 mM EGTA and EDTA free proteinase inhibitor cocktail tablets according to manufactures protocol (Sigma)). Lysates were spun for 30' at 13.200 rpm and supernatant was aspirated (cytosolic fraction).

The precipitate (cytoskeleton fractions) was washed twice with 1x Triton lysis buffer. The cytoskeleton fraction was dissolved in 40 µl of 1x reducing Laemmli sample buffer (0.001% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8 and 5% β2-mercaptoethanol), and boiled for 5 min. Five µl of 5x reducing Laemmli sample buffer was added to 20 µl of the cytosolic fraction. Samples were separated on an 8% SDS-polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris buffered saline (25mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween 20 (TBST) containing 2% (w/v) BSA (Sigma) for 1 h at room temperature. The blot was then incubated with mouse anti ApoER2 antibody (MP4-3, 1 µg/ml) overnight in TBST with 1% BSA and washed three times with TBST. After incubation with peroxidase labeled rabbit anti mouse antibodies for 1 h at RT (1:2500; Dako, Glostrup, Denmark) blots were washed again with TBST and developed with enhanced chemiluminescence reagent plus (Perkin Elmer life sciences, Boston USA)

Results

To identify the binding site for dimeric β2-GPI on ApoER2′ expressed on blood platelets we isolated mRNA from freshly isolated human megakaryocytes and three different megakaryocytic cell lines. This mRNA served as a template to amplify the extra cellular LDL-binding and EGF region of ApoER2′. Three different mRNA forms were isolated and evaluated using sequencing analysis to confirm alternative splicing.

A

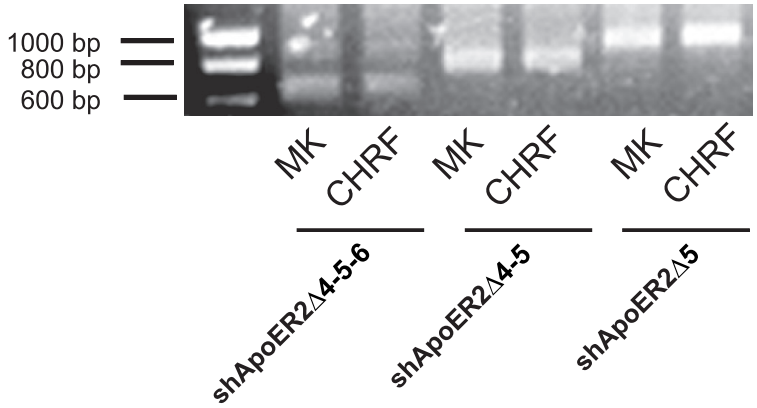

Figure 1a: **Freshly isolated megakaryocytes and three different megakaryocytic cell lines contain mRNA coding for three different splice variants of ApoER2.** Freshly isolated megakaryocytes (MK) and the megakaryocytic cell lines CHRF, Meg01 and CHRF were used to isolate mRNA and subsequent cDNA synthesis. PCR was performed to amplify soluble ApoER2′ and cDNA was cloned into TOPO blunt for further analysis. Three different forms of soluble ApoER2′were isolated from all three cell lines (data shown only for MK and CHRF)

Apart from the soluble form of ApoER2Δ5 or ApoER2'lacking LDL-binding domains 4, 5 and 6, the presence of which on blood platelets is well described [30], we also isolated two shorter forms of ApoER2, shApoER2Δ4-5 lacking LDL-binding domains 3-4-5 and 6 and shApoER2Δ4-5-6 lacking LDL-binding domains 3, 4, 5, 6 and 7. We also cloned and expressed two additional domain deletion mutants shAPoER2-BD-1 and shAPoER2-BD2. Figure 1b is a schematic representation of the mRNA splice variants.

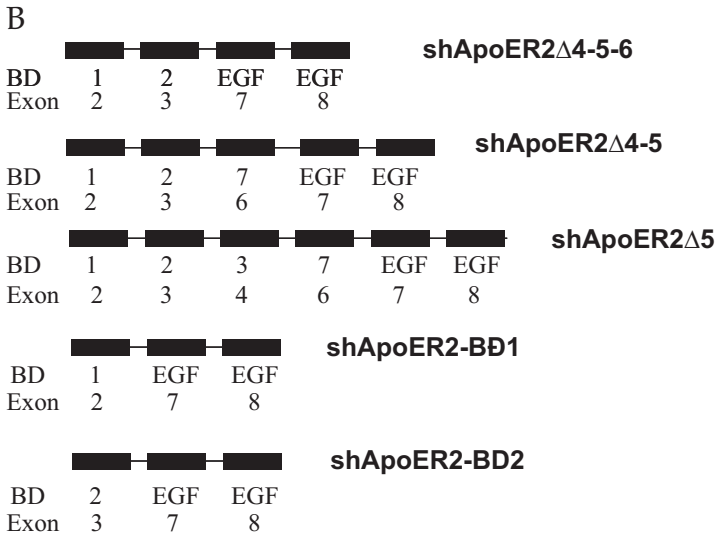

Figure 1b: **Schematic representation of the 3 isolated splice variants and 2 constructed domain deletion mutants of ApoER2.**

The mRNA splice variants isolated from megakaryocytic cells of all used origin (freshly isolated megakaryocytes, CHRF, Meg01 and DAMI) were identical (Figure 1a, data only shown for CHRF and freshly isolated megakaryocytes). To confirm the presence of different splice variants of ApoER2 on the membrane surface of blood platelets, platelet lysates from 3 different donors were analysed by immuno-blotting using monoclonal antibody MP4-3 directed against the LDL-binding domain 1 of ApoER2. Three protein bands were detected with a molecular weight of approximately 80kDa, 100kDa and 120kDa (resp. ApoER2Δ3-4-5, ApoER2Δ4-5 and ApoER2Δ5, fig 1c). ApoER2Δ4-5 seems to be the dominant splice variant expressed in all four donors.

The three mRNA splice variants isolated from megakaryocytes and the two LDL-binding domain deletion mutants that only express either binding domain 1 or binding domain 2, were cloned into an expression vector for eukaryotic expression in 293EBNA cells and recombinant proteins were affinity purified. The soluble form of ApoER2' (shApoER2Δ5) was detected at a molecular weight of 47kD. The shorter splice variant shApoER2Δ4-5 had a molecular weight of 42kD and shApoER2Δ3-4-5 was 38kD (figure 2). Both domain deletion mutants we constructed (shApoER2-BD1 and shApoER2-BD2) had a similar molecular weights of approximately 34kD.

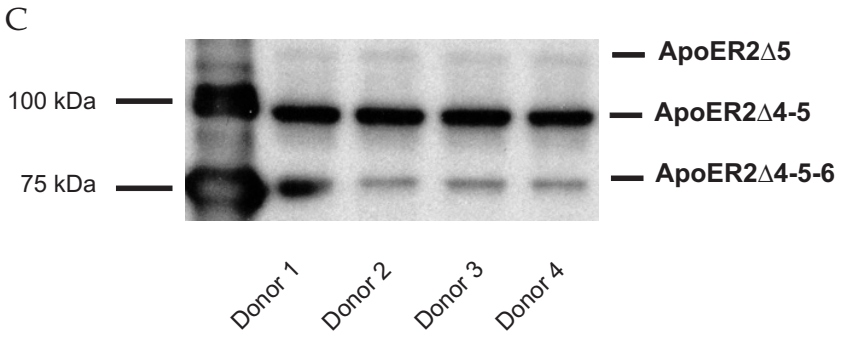

Figure 1c: **Platelets express three different splice variants of ApoER2.** Platelet lysate of three different donors was analysed by immuno-blotting using a monoclonal antibody directed against a peptide stretch present in the LDL-binding domain 1 of ApoER2 (MP4-3). Platelets express three different forms of ApoER2.

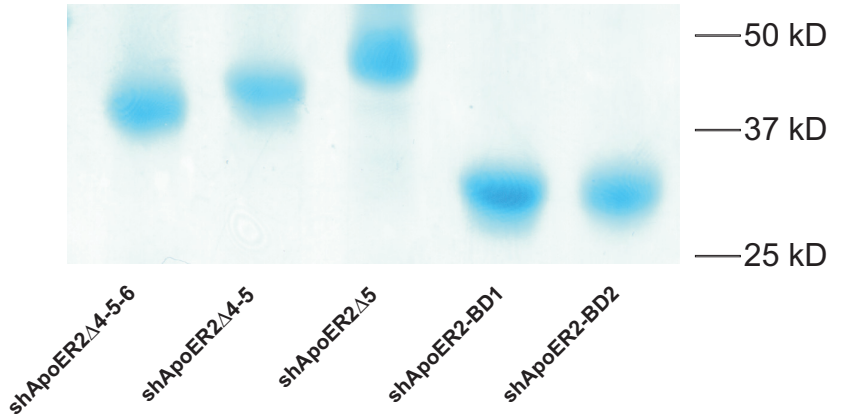

Figure 2: **Expression and purification of the 3 isolated mRNA splice variants and domain deletion mutants of ApoER2.** Recombinant proteins were expressed by 293EBNA cells. Proteins were purified by NiNTA and subsequentl RAP sepharose affinity purification. Purified proteins were run on a 10% SDS-polyacryl amide gel to check protein purity. Each recombinant protein showed only 1 band.

To characterize the binding site for dimeric β2-GPI on apoER2, we used the purified splice variants and domain deletion mutants in a solid phase binding assay. All three cloned splice variants of ApoER2 were able to bind dimeric β2-GPI in a similar fashion. Upon deletion of LDL-binding domain 1, (shApoER2-BD2), binding of dimeric β2-GPI was lost (fig 3).

To study the cellular re-distribution of ApoER2 splice variants after stimulation with dimeric β2-GPI, platelets were incubated with either TBS or dimeric β2-GPI. Platelets were lysed and the RIPA soluble cytoskeleton fraction was separated from the Tx100 soluble cytosolic fraction. ApoER2Δ5, ApoER2Δ4-5 and ApoER2Δ3-4-5 time dependently dissociated from the cytoskeleton and ended up in the cytosolic fraction however, of the three splice variants expressed, ApoER2Δ4-5 seems to be the dominant signaling receptor. (Figure 4, blots representative for 3 separate experiments).

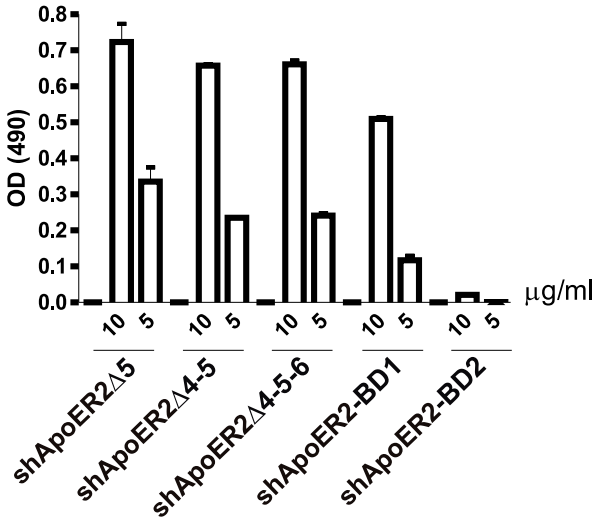

Figure 3: **Dimeric β2-GPI binds to ApoER2′ via the LDL-binding domain 1 on ApoER2′.** Purified splice variants and domain deletion mutants were coated at 10 or 5 µg/ml and wells were blocked. Coated wells were incubated with dimeric β2-GPI and binding detected using in-house rabbit polyclonal antiserum raised against β2-GPI. Upon deletion of LDL-binding domain1, binding of dimeric β2-GPI was completely abolished.

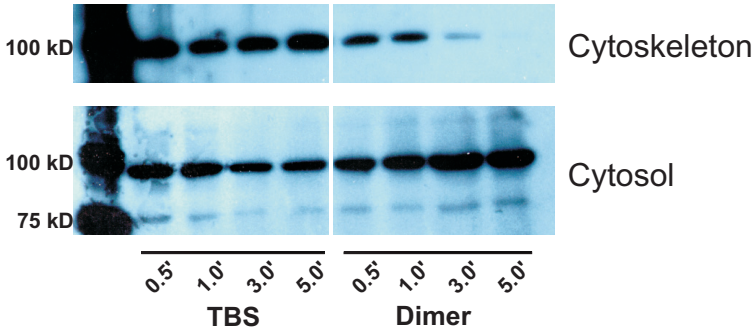

Figure 4: **Incubation of platelets with dimeric β2-GPI shows a time dependent translocation of ApoER2 splice variants away from the platelet cytoskeleton.** Platelets were stimulated with either TBS or dimeric β2-GPI. When cytosolic and cytoskeletal fractions of were separated and blotted using MP4-3, a time dependent dissociation from ApoER2-Δ5, ApoER2Δ4-5 and ApoER2-Δ3-4-5 away from the cytoskeleton was observed with ApoER2-Δ4-5 translocation being the most prominent.

Discussion

There is now ample evidence that antibodies directed against β2-GPI correlate best with the clinical manifestations in the antiphospholipid syndrome. No physiological function has been described for β2-GPI sofar. However, after complex formation with auto-antibodies, dimerized β2-GPI is able to activate a whole variety of cells, including blood platelets [13;31-34]. The interaction of β2-GPI-antibody complexes with platelets has been described to be mediated in part by ApoER2′ [10], sofar the only member of the LDL-receptor super family known on platelets [30]. The binding site for dimeric β2-GPI on

ApoER2' has not been identified. In our search for the binding site for dimeric β2-GPI on ApoER2' we have isolated three different mRNA splice variants of ApoER2 expressed by freshly isolated megakaryocytes and three different megakaryocytic cell lines. The largest variant lacks exon 5 resulting in a splice variant depleted of LDL-binding domains 4, 5 and 6 (shApoER2Δ5). Expression of this splice variant by platelets has been described previously [30]. The second variant lacks exon 4 and 5 resulting in the absence of LDL-binding domains 3, 4, 5 and 6 (shApoER2Δ4-5). The shortest splice variant of ApoER2 lacks exons 4, 5 and 6 and therefore misses LDL-binding domains 3, 4, 5, 6 and 7 (shApoER2Δ4-5-6). Both splice variants ApoER2Δ4-5 and ApoER2Δ4-5-6 have not been described before and might be platelet and megakaryocyte specific. Possible expression of ApoER2 splice variants was studied on circulating platelets. We found platelets express three splice variant of ApoER2 but based on the intensity on the bands in western blotting, ApoER2-Δ4-5 is expressed most prominent. All variants contain LDL-binding domain 1 since monoclonal antibody MP4-3 used for detection, was raised against a peptide present in LDL-binding domain 1. All three cloned mRNA splice variants of ApoER2 were able to bind dimeric β2-GPI but when LDL-binding domain 1 was omitted, binding of dimeric β2-GPI was lost. We furthermore show translocation of ApoER2Δ5, ApoER2Δ4-5 and ApoER2Δ4-5-6 away from the cytoskeleton to the cytosol upon stimulation with dimeric β2-GPI. Trafficking and re-location of ApoER2 in neuronal cells has been described during neuronal development and appears to be essential for correct development [16;17;35]. This is the first report of ApoER2 translocation in blood platelets and we assume that translocation of ApoER2 is part of the signal pathway in platelets after stimulation with β2-GPI-antibody complexes. As described in neurons, translocation of ApoER2 could be part of a physiological function of ApoER2 splice variants in platelets (table 1).

Name	**Cytosol**	**Cytoskeleton**	**Signaling**
ApoER2-120kDa	+/-	-	+
ApoER2-100kDa	++++	++++	++++
ApoER2-80kDa	+	+	+/-

Table 1: **Localization and signaling properties of ApoER2 splice variants expressed on platelets**

Dimerization of the VLDL-R and ApoER2 has been implicated in the initiation of signaling via VLDL-R and ApoER2 by the group of Strasser et al. [36]. It is possible that dimerization o f β2-GPI leads to dimerization or even clustering of ApoER splice variants thereby, transmitting a signal across the platelet membrane inside the platelet. Why blood platelets express three different ApoER2 variants is still unclear since we demonstrate binding of dimeric β2-GPI to all three splice variants isolated followed by translocation of all three spice variants. Besides differences in expression levels, no differences between ApoER2 splice variants were observed. In general, the presence of different splice variants on platelets could play a role in binding of other receptor ligand like LDL. Differences for ligand binding by ApoER2 splice variants varying in the LDL-

binding region has been described before [37]. In neuronal cells, splicing of the intracellular domain has also been described [38]. Since there is no evidence for the occurrence of circulating immuno-complexes of β2-GP and antibodies directed against β2-GPI, we assume that these complexes are formed on the platelet surface upon exposure of anionic phospholipids. Further investigations are required to elucidate the exact sequence of events leading to the formation of β2-GPI-anti-β2-GPI complexes and binding to GPIbα and ApoER2 splice variants. In conclusion we have cloned and expressed soluble forms of three different splice ApoER2 splice variants from cells of megakaryocytic origin varying in the extra-cellular LDL-binding region. We identified LDL-binding domain 1 to be the binding moiety for dimeric β2-GPI. We furthermore show the presence of three different splice variants of ApoER2 on human blood platelets which all translocate to the cytosol upon stimulation with dimeric β2-GPI. The most abundant splice variant of ApoER2 present on platelets is ApoER2Δ4-5 lacking LDL-binding domains 3, 4, 5, 6.

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4

Interaction of β2-Glycoprotein I with members of the Low Density Lipoprotein Receptor family

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Abstract

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease characterized by arterial and/or venous thrombosis and/or pregnancy morbidity in the presence of autoantibodies that recognize beta2-glycoprotein I (β2-GPI) bound to phospholipids. We have previously demonstrated that dimerization of β2-GPI by autoantibodies induces platelet activation, involving the platelet receptor Apolipoprotein E receptor 2′ (ApoER2′) a receptor belonging to the low-density lipoprotein receptor (LDL-R) family. Here, we show that dimeric β2-GPI, but not monomeric β2-GPI interacts with four other LDL-R family members: the LDL-R related protein (LRP), megalin, the LDL-R and the very-low density lipoprotein receptor (VLDL-R). Interaction between dimeric β2-GPI and LDL-R, ApoER2′ and VLDL-R was best described with a one-site binding model (half-maximal binding; ~ 20 nM for ApoER2′ and VLDL-R and ~ 300 nM for LDL-R), whereas the interaction between dimeric β2-GPI and LRP or megalin was best described with a two-site binding model, representing a high- (~ 3 nM) and a low-affinity site (~ 0.2 mM). Binding to all receptors tested was unaffected by a tryptophane to serine (W316S) substitution in domain V of β2-GPI, which is known to disrupt the phospholipid binding site of β2-GPI. Also deletion of domain I or II left the interaction with the receptors unaffected. Deletion of domain V, however, significantly decreased the affinity for the receptors. In conclusion, our data show that dimeric β2-GPI can interact with different LDL-R family members. This interaction is dependent on a binding site within domain V of β2-GPI, which does not overlap with the phospholipid-binding site within domain V.

Introduction

The antiphospholipid syndrome (APS) is non-inflammatory autoimmune disease. APS has been identified as the association of arterial and/or venous thrombosis and/or pregnancy morbidity with the presence of antiphospholipid antibodies (aPL) in plasma of affected patients [1,2]. Assays that depend on the presence of antibodies against beta2-glycoprotein I (β2-GPI) display the highest correlation with thrombosis [3]. The cause of haemostatic dysbalance induced by *β2*–GPI in the presence of β2-GPI antibodies *in vivo* still remains unraveled. Several possibilities have been proposed, of which cell activation by β2-GPI/anti-β2-GPI antibodies has received most attention. The involvement of cellular receptors that can bind these immune complexes is essential in all these hypotheses [4-7]. β2-glycoprotein I is an abundant plasma protein (approximately 200 mg/mL). At present, the physiological function of β2-GPI is unclear, but the protein has been described to have both pro- and anti-coagulant properties *in vitro* [8-11]. In previous studies we have shown that β2-GPI/antibody complexes can activate platelets. This effect could be mimicked by a recombinant form of dimeric β2-GPI. A splice variant of the Apolipoprotein E receptor 2 (ApoER2), that was recently identified as ApoER2′ on human platelets [12] has been demonstrated to be involved in the binding of dimeric β2-GPI to human platelets [13]. ApoER2 (also known as LDL receptor-related protein-8 (LRP-8) is among others expressed in brain, placenta and testis [14;15] and shares structural homology with other LDL receptor family members [16]. In general, these multiligand receptors consist of a number of discrete domains and their extracellular component contains ligand-binding domains that consist of complement-type repeats. Differential clustering of these repeats within a domain may expose specificity with respect to ligand recognition. These repeats are separated by one or more epidermal growth factor (EGF) precursor homology domains that contain YWTD motifs, responsible for ligand dissociation. The receptors are anchored in the membrane via an intracellular tail that harbors motifs for endocytosis and signaling. At present, twelve members of the LDL-receptor family have been identified in mammals, including the LDL-receptor related protein (LRP), megalin, the low-density lipoprotein receptor (LDL-R), ApoER2′ and the very-low density lipoprotein receptor (VLDL-R). These receptors are expressed on an array of cell types. The concept that they are multiligand receptors does not necessarily mean that they recognize identical ligands. In contrast, ligand binding may be tightly regulated between different members of the LDL-receptor family. In the present study we address a number of issues. Apart from ApoER2′, other LDL-R homologues (LRP, megalin, LDL-R, VLDL-R) might recognize dimeric β2-GPI with different affinities. Furthermore, ApoER2′-mediated platelet activation by β2-GPI only occurred in the presence of anti-β2-GPI antibodies. Since these antibodies possess the property to dimerize antigens, we investigated whether anti-β2-GPI antibody induced dimerization of β2-GPI also promotes the interaction with other members of the LDL-R family. It has been proposed that positive electrostatic surface potentials (mainly on domain I and V of β2-GPI), not the primary sequences, in different ligands constitute receptor-binding domains. Therefore, we used domain deletion mutants of dimeric β2-GPI to identify which domain of β2-GPI is involved in the interaction with the receptors. Finally, to investigate whether the molecular interactions between phospholipid binding and binding to the receptors is similar, we tested binding of a non-phospholipid binding β2-GPI mutant to LRP, megalin, ApoER2′ and the VLDL-R.

Methods

Purification of plasma β2-GPI.
Plasma β2-GPI was isolated from fresh citrated human plasma as described previously[17]. In short, dialyzed human plasma was applied subsequently to the following columns: DEAE-Sephadex A50, protein G-Sepharose, S-Sepharose, and finally heparin-Sepharose (all Sepharoses were obtained from Amersham Pharmacia Biotech). Bound proteins were eluted with a linear salt gradient. Afterwards, β2-GPI was dialyzed against TBS. Purity of the protein was checked on a 4-15% SDS-PAGE. Plasma β2-GPI migrated with a molecular mass of 45 kDa under non-reducing conditions. Concentration of the protein was determined using the bicinchoninic acid (BCA) protein assay.

Construction of dimeric β2-GPI
Apple4-C321S-β2-GPI (further referred to as dimeric *β2*–GPI) and the non-dimeric apple2-β2-GPI were constructed as described previously [18]. Domain deletion mutants of dimeric β2-GPI were constructed using full-length apple4-C321S-β2-GPI cDNA as a template [19]. In short, for deletion of domain I (delta I) one set of primers was used to construct domain II - V. For deletion of domain II (delta II) two sets of primers was used; one set to construct domain I and one set to construct domain III - V. The two created PCR products served as a template to obtain the full-length domain II deletion. For the domain V deletion mutant (delta V) one set of primers was used to construct domain I - IV. To construct chimaeric fusion proteins of the dimerization domain of factor XI (apple4) and β2-GPI, the PCR product was cloned into the vector apple4-C321S-tissue-type plasminogen activator (tPA)-S478A. In the experiments we also used the non-phospholipid binding mutant, in which a tryptophane has been substituted to a serine (W316S). Construction of this mutant has been described previously [20]. As a control, we also used dimeric apple4 [19]. Sequence analysis was performed to confirm correct amplification of the β2-GPI cDNA.

Construction of soluble VLDL-receptor
Soluble human (sh) ApoER2′ was constructed as described previously [19]. Construction of sh-VLDL-receptor (VLDL-R) was performed as follows. Freshly isolated umbilical vein mRNA was prepared from endothelial cells to synthesize cDNA. This cDNA was the starting point for cloning sh-VLDL-R using Phusion DNA polymerase (Finnzymes). The signal peptide was excluded and the stopcodon was deleted. The following primers were used. Forward primer sh-VLDL-R; TA ATA GGA TCC GGG AGA AAA GCC AAA TGT GAA CCC. Reverse primer sh-VLDL-R; TA ATA GCG GCC GCA GTA GCA AGA TCC ATT TGA TAG CC. The VLDL-R was subsequently cloned into the vector PTT3-SRα-GH-HISN-TEV. This expression vector is constructed from the pTT3 expression vector [21] and the pSGHV0 expression vector [22]. The sh-LDL-R was a generous gift of Dr. K. Mertens, Sanquin Bloodbank, Amsterdam, the Netherlands.

Transfection, Expression, Cell Culture and Purification of Constructs
Transfection of BHK cells with the calcium phosphate method was performed as described previously [13]. Expression of all constructs was performed in conditioned serum-

free medium (Dulbecco's modified Eagle's medium/F-12 medium supplemented with 0.5% UltroserG; ITK Diagnostics, the Netherlands). Protein expression was measured using a β2-GPI-ELISA [20]. Full-length and domain deletion mutants of dimeric β2-GPI were purified from cell culture medium using a monoclonal antibody (21B2; a generous gift of Dr. J. Arnout, Leuven, Belgium) column. The purified proteins were further subjected to purification on a mono S column using fast performance liquid chromatography (FPLC) (Amersham Pharmacia Biotech). Apple4 was purified using a monoclonal antibody (α-XI-1; a generous gift of Dr. J.C.M. Meijers, Academic Medical Center, Amsterdam). Transfection of HEK293E cells with sh-ApoER2' and sh-VLDL-R was performed using the PEI transfection method according to Durocher *et al*. Production of sh-ApoER2 and sh-VLDL-R was done in a 1 liter suspension culture (in medium containing 90% freestyle, 10% calcium free DMEM, 0.1 % fetal calf serum, Invitrogen). Receptors were affinity purified using RAP-sepharose or Ni-NTA sepharose and purity of the protein fractions was determined on a 4-15% polyacryl amide gel electrophoresis (SDS-PAGE), subsequently followed by dialyzation against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce). Purified constructs were analyzed on a 7.5% SDS-PAGE. LRP fragments cluster II and cluster IV were purified as described previously [23]. LRP was obtained from BioMac (Instruchemie, the Netherlands). Megalin was kindly provided by Dr. H. Pannekoek, Academic Medical Center, Amsterdam, the Netherlands.

Solid-phase immunosorbent assay for the interaction between β2-GPI and ApoER2' and the VLDL-R

Binding of β2-GPI and the domain deletion mutants to ApoER2' and the VLDL-R was measured using a solid-phase immunosorbent assay. Briefly, increasing concentrations (0 - 10 μg/mL in PBS) of receptors were immobilized on hydrophobic 96 wells plates (Costar). After blocking with PBS/4%BSA (150μL/well), proteins (3 μg/mL in PBS/1%BSA, 50μL/well) were allowed to interact with the receptors. After incubation, bound β2-GPI was detected using subsequently a rabbit polyclonal anti-β2-GPI antibody and a peroxidase-labeled swine anti-rabbit polyclonal antibody (SWARPO, 1:500 in PBS/1%BSA, 50μL/well), followed by a staining procedure using ortho-phenylenediamide (OPD).

Surface plasmon resonance analysis for the interaction between β2-GPI and LRP, megalin and the LDL-R

Surface plasmon resonance (SPR) binding assays were performed employing a Biacore 2000 system (Biacore AB, Uppsala, Sweden). LRP, cluster II or IV of LRP or megalin were immobilized on different CM5 sensor chips using the amine-coupling kit (Biacore AB, Uppsala, Sweden). The LDL-R was immobilized on a C1 sensor chip using the amine-coupling kit (Biacore AB, Uppsala, Sweden). Approximately 10 fmol/mm^2 LRP, 26 fmol/mm^2 cluster II, 16.5 fmol/mm^2 cluster IV, 19.3 fmol/mm^2 megalin and 29.4 fmol/mm^2 LDL-R were immobilized. For each chip one channel was activated and blocked in the absence of protein and afterwards its signal (<5% of binding to coated channels) was used to correct the signal from the coated channels for a-specific binding. SPR analysis was performed in buffer containing TBS/0.005% Tween 20, 1 mM $CaCl_2$

with a flow rate of 20 µl/min at 25°C. Association and dissociation was followed for a period of 2 minutes. Regeneration of the surface was performed by application of 0.1 M sodium citrate containing 1 mM of EDTA and 1 M of NaCl, pH 5.0.

Binding of a β2-GPI/anti-β2-GPI antibody complex to LDL-receptor family members
Binding of plasma β2-GPI in the presence of a monoclonal anti-β2-GPI antibody (3B7) that recognizes domain I of β2-GPI and prolongs the clotting time (lupus anticoagulant; LAC), was investigated for LRP, megalin, the LDL-R, ApoER2'and the VLDL-R. For SPR analysis plasma β2-GPI (100 nM) was incubated with 3B7 (200nM) for 30 minutes at 37°C. Afterwards, interaction of the immune complex with immobilized LRP, megalin and the LDL-R was investigated. Association and dissociation was followed for a period of two minutes. Analysis of binding of the immune complex to the immobilized receptors was measured in TBS/0.005% Tween 20, 1 mM $CaCl_2$ with a flow rate of 20 µl/min at 25°C. Because of the complexity of the interaction between antibody/β2-GPI complexes and immobilized receptor, the kinetic parameters of this interaction cannot be calculated using the available software programs. Interaction between β2-GPI/anti-β2-GPI antibody complex and coated ApoER2' and the VLDL-R (5 µg/mL) was measured using the solid-phase immunosorbent assay. In this assay, plasma β2-GPI (3 µg/mL) was incubated with 3B7 (1 µg/mL) in PBS/1%BSA. Bound β2-GPI was visualized as described previously.

Inhibition studies in the presence of heparin or RAP
Inhibition experiments were performed using SPR (LRP and megalin) analysis and with the solid-phase immunosorbent assay (ApoER2' and VLDL-R). For heparin inhibition studies, dimeric β2-GPI was pre-incubated with increasing concentrations of heparin (0 - 2 mg/mL) in TBS/0.005% Tween 20 containing 1 mM $CaCl_2$ for 30 minutes at 37°C prior to injection. For RAP inhibition studies, a pre-injection with increasing concentrations of RAP (0 - 200 nM) was performed. Association- and dissociation was followed for a period of 2 minutes. This was subsequently followed by injection of 100 nM dimeric β2-GPI. For inhibition studies with ApoER2' and the VLDL-R, dimeric β2-GPI (3 µg/mL, 50 µL/well) in the presence of increasing concentrations heparin (0 - 2 mg/mL) or RAP (0 - 200 nM) in PBS/1%BSA was incubated on coated ApoER2' or the VLDL-R (5 µg/mL). Bound protein was detected as described previously.

Analysis of SPR data
Analysis of SPR data was performed using the GraphPad Prism program (GraphPad Prism version 4.0 for Windows, Graph Pad Software, San Diego, CA). Data obtained from SPR analysis was used for the calculation of the steady state constants as follows. Responses at equilibrium (R_{eq}) derived from sensorgrams were plotted against protein concentration. The resulting binding isotherms were subsequently fitted to the following equations;

For a one-site binding model: $$Y = \frac{B_{max} \times X}{K_d + X}$$

For a two-site (heterologous) binding model: $$Y = \frac{B_{max1} \times X}{K_{d1} + X} + \frac{B_{max2} \times X}{K_{d2} + X}$$

Scatchard plots were derived from the saturation curves to verify the use of a one-site- or a two-site (heterologous) ligand-binding model. Results are expressed as mean ± SD (n = 3).

Results

To investigate the effect of dimerization of β2-GPI by an antibody on its interaction with members of the LDL-R homologues, we assessed the binding of plasma β2-GPI in the absence or presence of an anti-β2-GPI antibody (3B7) to LRP, megalin, the LDL-R, ApoER2′ and the VLDL-R. Interaction of β2-GPI with LRP, megalin and the LDL-R was investigated by SPR analysis and binding to ApoER2′ and the VLDL-R was assessed in a qualitative manner using an immunosorbent assay. As shown in *Figure 1,* incubation of plasma β2-GPI in the presence of 3B7 resulted in binding of the immune complex to all receptors tested, except for the LDL-R. Plasma β2-GPI or 3B7 alone had less than 20% binding to the receptors. Thus, β2-GPI binds to LRP, megalin, ApoER2′ and the VLDL-R, however dimerization of β2-GPI is necessary for optimal binding.
Interaction between β2-GPI/antibody complexes and the receptors is complicated to

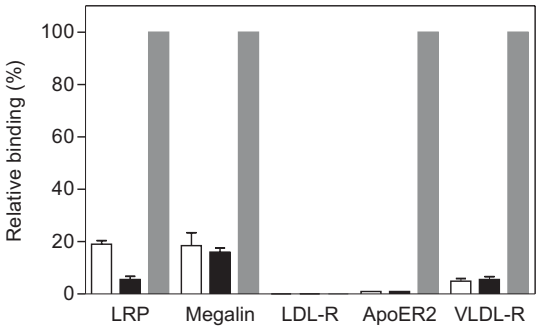


Figure 1. **Binding of a β2-GPI/anti-β2-GPI antibody complex to members of the LDL-receptor family.** Plasma β2-GPI was incubated with an anti-β2-GPI Moab (3B7) at 37°C for 30 minutes. The formed immune complex could interact with LRP, megalin, ApoER2′ and the VLDL-R. Binding of dimeric β2-GPI (grey bars) in the presence of 3B7 to the receptors was set at 100%. Plasma β2-GPI (white bar) and 3B7 (black bar) alone hardly bound to the receptors. Results represent mean ± SD (n = 3).

examine, since the analysis can be influenced by the affinity of the antibody for β2-GPI. Therefore, we studied binding of a stable, recombinant form of dimeric β2-GPI to LRP, megalin, the LDL-R, ApoER2′ and the VLDL-R. In *Figure 2 Panel A*, a representative sensorgram for LRP is shown, obtained from SPR analysis. Plasma β2-GPI, apple4 and the non-dimeric protein apple2-β2-GPI did not bind to LRP. In contrast, dimeric β2-GPI did bind to LRP. Upon replacement of dimeric β2-GPI with buffer (arrow), the response signal declined gradually, indicating that dimeric β2-GPI dissociates from LRP and that binding is reversible.

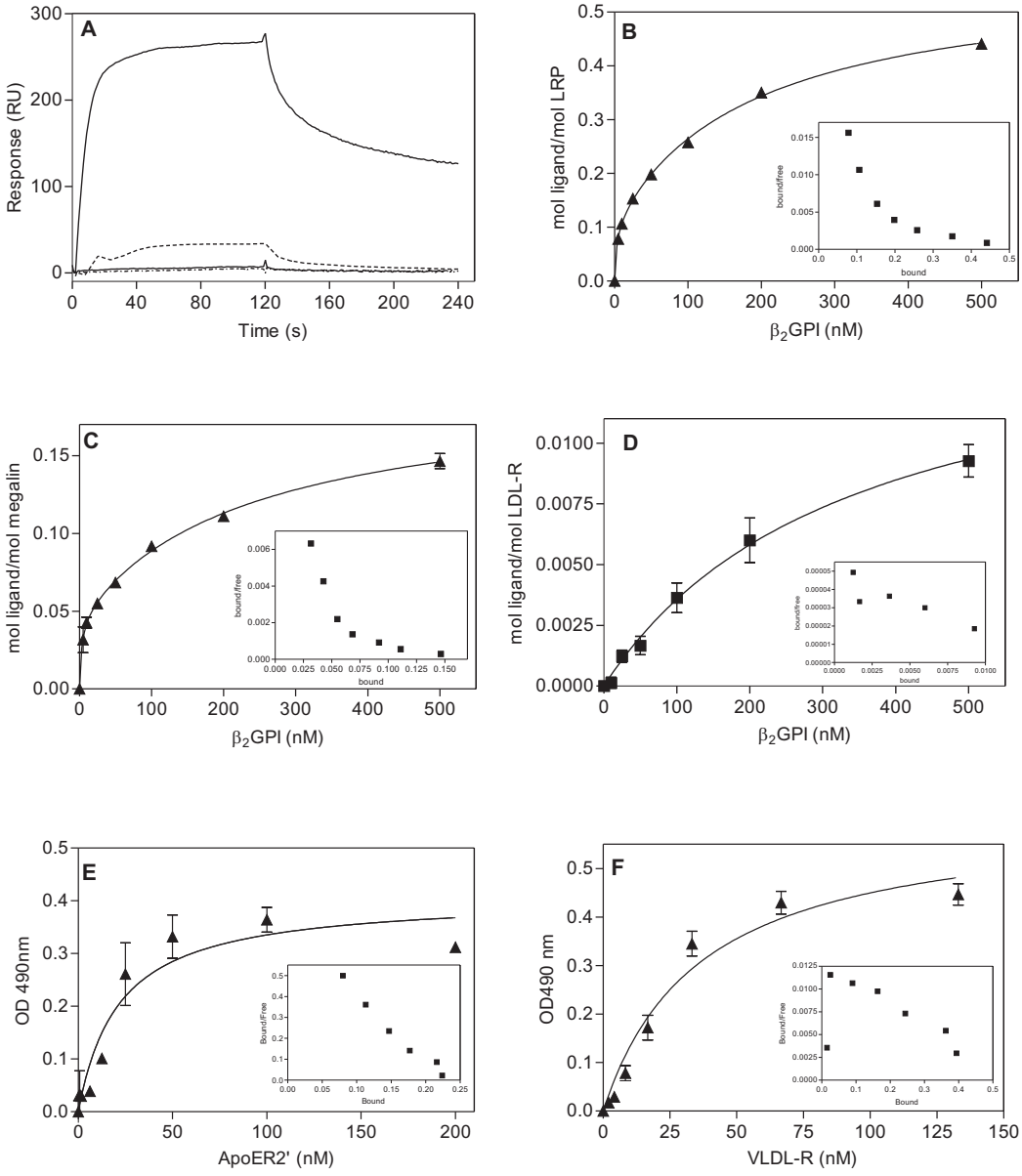


Figure 2. **Binding of β2-GPI/anti-β2-GPI antibody complexes to members of the LDL-receptor family.** Plasma β2-GPI was incubated with an anti-β2-GPI Moab (3B7) at 37°C for 30 minutes. The formed immune complex could interact with LRP, megalin, ApoER2' and the VLDL-R. Binding of dimeric β2-GPI (grey bars) in the presence of 3B7 to the receptors was set at 100%. Plasma β2-GPI (white bar) and 3B7 (black bar) alone hardly bound to the receptors. Results represent mean ± SD (n = 3).

The interaction of dimeric β2-GPI with the LDL-R homologues was studied in more detail by calculating the steady state constants. The binding isotherms of the responses obtained at equilibrium are shown in *Figure 2*. For the interaction between dimeric β2-

GPI and LRP (*Panel B*) and megalin (*Panel C*), Scatchard plot analysis (*inserts*) revealed two classes of binding sites. The correctness of fit (R^2) for the binding isotherms of both LRP and megalin was 0.999 for a two-site binding model and 0.962 and 0.934 for a one-site binding model. Therefore, the interaction between dimeric β2-GPI and LRP and megalin could be adequately described employing a two-site (heterologous) binding model. Consequently, a high (3.1 ± 0.6 nM and 3.1 ± 0.7 nM, respectively) and a low affinity interaction (192.1 ± 13.2 nM and 241.2 ± 30.0, respectively) could be inferred from the data. After Scatchard plot analysis (*inserts*) and careful interpretation of the correctness of fit from the binding isotherms, the interaction between dimeric β2-GPI and the LDL-R (*Panel D*), ApoER2′ (*Panel E*) and the VLDL-R (*Panel F*) showed one class of binding sites (R^2: 0.993, 0.995 and 0.990 respectively). Therefore, the experimental data was best fitted according to a one-site binding model. Compared to the other receptors tested, the LDL-R showed one class of low affinity binding sites for dimeric β2-GPI (K_D = 341 ± 54 nM). Furthermore, the LDL-R has not been described in literature as being involved in signaling processes. Therefore, we decided to continue the experiments with only LRP, megalin, ApoER2′ and the VLDL-R. The results are summarized in *Table I*.

receptor	dimeric β2GPI	delta I	delta II	delta V
LRP	3.1 ± 0.6	5.9 ± 1.6	2.0 ± 0.4	-
	192 ± 13	190 ± 17	244 ± 18	$> 2 \cdot 10^3$
Megalin	3.1 ± 0.7	4.1 ± 0.5	5.4 ± 1.2	-
	241 ± 30	283 ± 24	284 ± 81	212 ± 14
ApoER2′	23 ± 4	16 ± 2	23 ± 6	-
VLDL-R	36 ± 8	26 ± 4	39 ± 6	-
LDL-R	341 ± 54	NT	NT	NT

Table 1. **Steady state constants of domain deletion mutants of β2-GPI.** Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the protein/receptor interaction. When a two-site binding model was used, two steady state constants are calculated (K_D1 and K_D2); for a one-site binding model only one steady state constant (K_D) is calculated. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3). NT = not tested.

The specificity of the interactions was examined by analyzing the interaction between dimeric β2-GPI and individual receptors in the presence of RAP, a universal ligand-inhibitor for this receptor family. For SPR analysis, pre-injections with five different con-

centrations of RAP (0 - 200 nM) were performed prior to injection of β2-GPI. For immunosorbent analysis, dimeric β2-GPI was incubated with RAP and binding to ApoER2' or the VLDL-R was analyzed. As shown in *Figure 3*, binding of dimeric β2-GPI to LRP or megalin was inhibited up to 80% by RAP. In the presence of RAP, binding of dimeric β2-GPI to ApoER2' and the VLDL-R was inhibited up to 75%, and 100%, respectively.

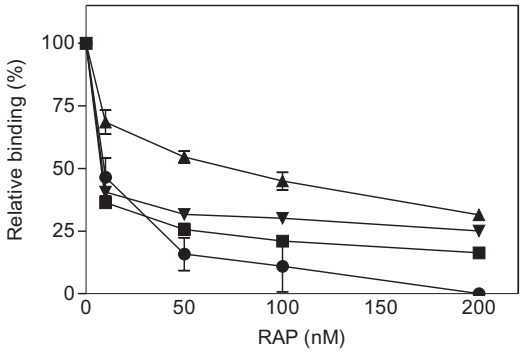


Figure 3. **Inhibition of dimeric β2-GPI–LDL-receptor family interaction by RAP.** For LRP and megalin, SPR analysis was applied to study the effect of RAP. Pre-injections with five different concentrations of RAP were performed for 2 minutes, directly followed by injections of 100 nM dimeric β2-GPI on immobilized LRP (■) and immobilized megalin (▲) for 2 minutes. Interaction of dimeric β2-GPI with coated ApoER2' (▼) or VLDL-R (•) in the presence of RAP was measured using a solid phase immunosorbent assay. Binding of dimeric β2-GPI in the absence of RAP was set at 100 %. Results represent mean ± SD (n = 3).

β2-Glycoprotein I consists of two regions that are enriched in cationic residues. These residues are located on domain I and V, with the largest cationic patch on domain V (~2,000 Å). Since LDL-R homologues are known to recognize such cationic residues, we investigated the ability of heparin (that also recognizes cationic residues) to inhibit the interaction between dimeric β2-GPI and LRP, megalin, ApoER2' and the VLDL-R. As shown in *Figure 4*, heparin inhibited the interaction of dimeric β2-GPI with LRP or megalin according to a bi-phasic inhibition model. An initial rapid inhibition phase up to approximately 50 - 75 % (residual binding of 46.2 ± 0.5 % and 23.2 ± 1.9 %, respectively) with 0.0625 mg/mL heparin and a slow inhibition phase up to >95% with 2.0 mg/mL heparin. In contrast, binding of dimeric β2-GPI to ApoER2' or the VLDL-R was inhibited by heparin in accordance with a mono-phasic pattern. A gradual inhibition was observed up to >95%. Heparin alone did not bind to the receptors (*not shown*). Half-maximal inhibition was observed at 0.05 mg/mL for LRP and 0.03 mg/mL for megalin. In addition, for ApoER2' and the VLDL-R half-maximal inhibition was observed at 0.46 mg/mL and 0.17 mg/mL, respectively.

To further characterize the interaction between dimeric β2-GPI and LDL-receptor family members, we determined the binding characteristics of the domain deletion mutants for LRP, megalin, ApoER2' and the VLDL-R. SPR measurements and the immunosorbent assays were performed under the same conditions as for full-length dimeric β2-GPI. Deletion of domain V of β2-GPI appeared to be crucial for binding to LRP and megalin. A small increase in the response signal was observed with increasing concen-

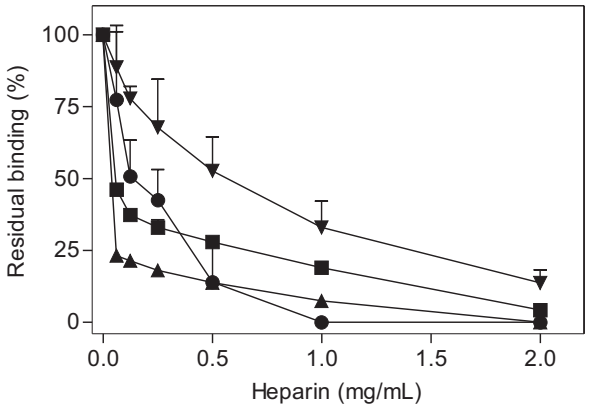
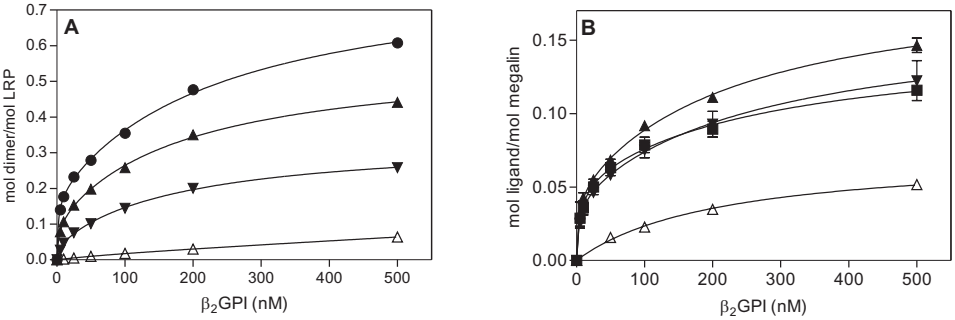


Figure 4. **Effect of heparin on the interaction between dimeric β2-GPI and receptors.** Dimeric β2-GPI (100 nM) was pre-incubated with increasing concentrations of heparin (0 – 2 mg/mL) at 37°C for 30 minutes. Afterwards the effect of heparin on binding of dimeric β2-GPI to immobilized LRP (■) or megalin (▲) was investigated using SPR analysis. The effect of heparin on the interaction between dimeric β2-GPI and ApoER2′ (▼) or the VLDL-R (•) was investigated using a solid phase immunosorbent assay. Percentage of inhibition in the presence of heparin was calculated as a result of binding of proteins to the receptors without heparin. Results represent mean ± SD (n = 3).

trations of delta V, whereas a significant increase in the response signal was observed when delta I and delta II were passed over LRP or megalin (*data not shown*). After replacement with buffer, dissociation of delta V started rapidly and protein was completely gone before the dissociation time (120 sec) was ended, whereas delta I and delta II dissociated gradually from LRP and megalin (similar to full-length dimeric β2-GPI). As shown in *Figure 5 Panel A*, maximal binding to LRP was lower for delta I and higher for delta II compared with full-length dimeric β2-GPI. The results are summarized in Table 1.



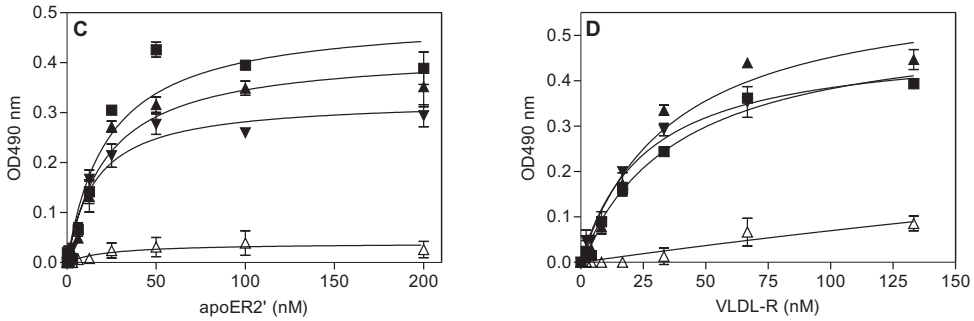


Figure 5. **Binding isotherms of the domain deletion mutants for members of the LDL receptor family.** Binding characteristics for the domain deletion mutants for the LDL-R homologues was investigated using SPR analysis and with a solid phase immunosorbent assay. A, increasing concentrations of full-length dimeric β2-GPI (▲), delta I (❮), delta II (■) or delta V (Δ) (0 – 500 nM) were able to interact with LRP. B, interaction of the domain deletion mutants with megalin. Except for delta V, the experimental data was best fitted with a two-site binding model. Data for LRP and megalin is expressed as mol ligand bound to mol receptor. C and D; increasing concentrations of ApoER2′ and VLDL-R were coated on a hydrophobic 96 wells plate. Full-length dimeric β2-GPI (▲), delta I (❮), delta II (■) and delta V (Δ) (3 μg/mL) were able to interact with the receptors. The data obtained for the interactions was best fitted using a one-site binding model. Steady state constants derived from the binding isotherms are listed in Table 1. Results represent mean ± SD (n = 3).

A model describing the interaction between delta I and delta II and two independent binding sites (two-site binding model) was found to provide the best fit of the experimental data for LRP (K_{D1} 5.9 ± 1.6 nM, K_{D2} 189.6 ± 16.4 nM and K_{D1} 2.0 ± 0.4 nM, K_{D2} 283.4 ± 80.7 nM, respectively). Delta V displayed a more than 600 times decreased affinity ($K_d > 2 \cdot 10^3$ nM) for LRP. Binding characteristics for megalin were similar to LRP; interaction of delta I and delta II with megalin (*Panel B*) displayed no major differences in maximal response compared with full-length dimeric β2-GPI (*Table 4*). Except for delta V (K_D 211.4 ± 13.7 nM), a two-site binding model could be applied for both delta I and delta II (K_{D1} 4.1 ± 0.5 nM, K_{D2} 283.1 ± 24.1 nM and K_{D1} 5.4 ± 1.2 nM, K_{D2} 283.4 ± 80.7 nM, respectively). For ApoER2′ (*Panel C*) and the VLDL-R (*Panel D*) similar binding characteristics were observed; delta V displayed reduced binding to both receptors, whereas delta I and delta II displayed a dose-dependent interaction that was comparable with full-length dimeric β2-GPI.

LRP consists of four clusters enriched in complement-type repeats, of which clusters II and IV encompass the main ligand-binding domains of LRP. The observation that interaction between dimeric β2-GPI and LRP was best characterized with a two-site binding model may suggest heterogeneity of LRP. To identify LRP regions involved in binding dimeric β2-GPI, purified recombinant cluster II and IV were used. When plasma β2-GPI was passed over both clusters II and cluster IV, no significant increase in the resonance signal was observed (*data not shown*). Binding of dimeric β2-GPI displayed a reversible and dose dependent binding to both ligand-binding clusters. The experimental data for each cluster fitted best using a two-site binding model. Therefore, as shown in *Table 2*, a high and a low affinity binding site could be calculated; for cluster II 1.6 ± 0.6 nM (K_{D1}) and 158.5 ± 30.5 nM (K_{D2}) and for cluster IV 1.7 ± 0.4 nM (K_{D1}) and 171.9 ±

receptor	dimeric β2GPI	delta I	delta II	delta V
LRP (RU)	507 ± 30	283 ± 40	608 ± 13	59 ± 22
Megalin (RU)	375 ± 14	343 ± 34	310 ± 20	120 ± 5
Cluster II (RU)	253 ± 9	194 ± 7	209 ± 3	28 ± 3
Cluster IV (RU)	201 ± 8	164 ± 6	180 ± 3	52 ± 2
ApoER2′ (OD490 nm)	0.352 ± 0.06	0.294 ± 0.03	0.389 ± 0.05	0.026 ± 0.03
VLDL-R (OD490 nm)	0.437 ± 0.02	0.394 ± 0.01	0.404 ± 0.01	0.086 ± 0.02

Table 2. **Steady state constants of the domain deletion mutants for cluster II and IV of LRP.** Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the type of interaction. When a two-site binding model was used, two binding constants (KD1 and KD2) are calculated; for a one-site binding model only one KD is calculated. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3).

14.6 nM (K_{D2}). To further characterize the interaction, binding characteristics of the do-

receptor fragment	dimeric β2GPI	delta I	delta II	delta V
Cluster II of LRP	1.6 ± 0.6	2.4 ± 0.8	2.0 ± 0.3	-
	159 ± 30	205 ± 38	157 ± 19	365 ± 32
Cluster IV of LRP	1.7 ± 0.4	1.4 ± 1.2	4.2 ± 0.7	-
	172 ± 15	249 ± 53	186 ± 22	143 ± 7

Table 3. **Steady state constants of the Trp316Ser mutant versus full-length dimeric β2-GPI.** Binding isotherms were fitted accordingly to a one-site or a two-site binding model, dependent on the type of interaction. When a two-site binding model was used, two binding constants (KD1 and KD2) are calculated; for a one-site binding model only one KD is calculated. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3).

main deletion mutants were investigated. Delta V showed reduced binding to both cluster II and IV (K_D 365.4 ± 31.7 nM and 143.1 ± 6.9 nM, respectively). Delta I and delta II showed similar binding characteristics compared with full-length apple4-β2-GPI.
To address the possibility that binding of dimeric β2-GPI to members of the LDL-re-

ceptor family has similar characteristics as binding to anionic phospholipids, we performed binding experiments with the non-phospholipid-binding mutant of dimeric β2-GPI. As shown in *Figure 6*, the mutant displayed dose-dependent association with LRP (*Panel A*), megalin (*Panel B*), ApoER2′ (*Panel C*) and the VLDL-R (*Panel D*). In contrast to full-length dimeric *β2*–GPI, the maximal response of the mutant was lower for LRP and megalin. As shown in *Table 3*, there are no significant differences in the affinity constants (K_d) of the mutant for either the LDL-R homologues or both clusters of LRP.

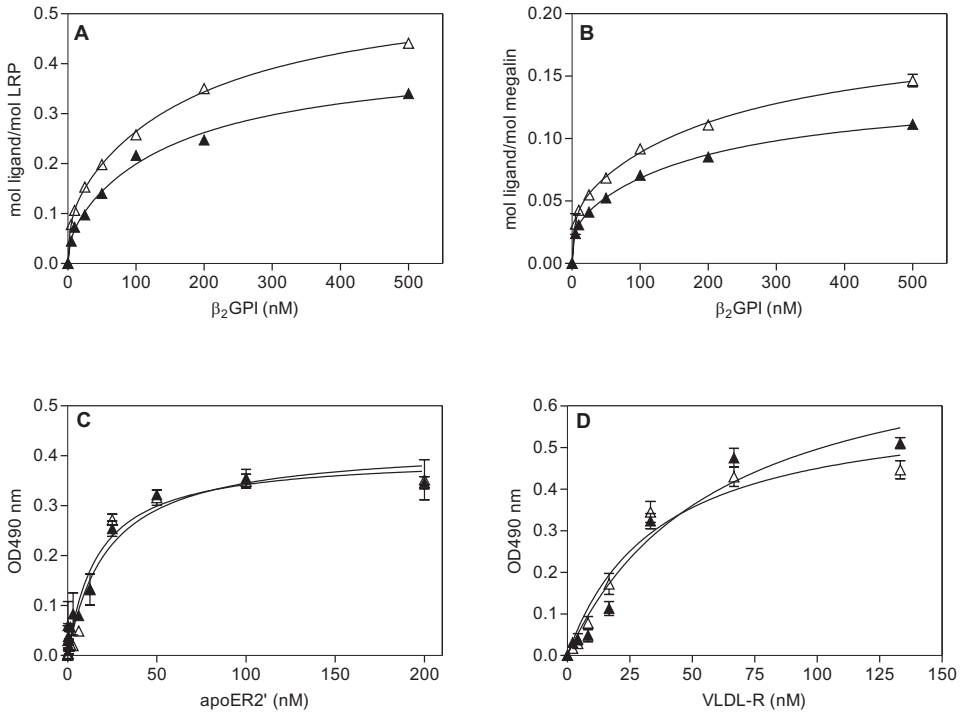


Figure 6. **Effect of a mutation in the phospholipid-binding region in domain V of β2-GPI.** For SPR analysis, increasing concentrations (0 – 500 nM) of dimeric β2-GPI (Δ) and Trp316Ser mutant (▲) were past over A, immobilized LRP or B, megalin. Association and dissociation was allowed for two minutes with a flow of 20 µL/min at 25°C. Afterwards, binding isotherms corresponding to seven different concentrations were fitted accordingly to a one-site- or a two-site binding model. Data is expressed as mol ligand bound to mol receptor. C and D; increasing concentrations of ApoER2′ and VLDL-R (0 – 10 µg/mL) were coated on a hydrophobic 96 wells plate; dimeric β2-GPI (Δ) and Trp316Ser mutant (▲) were able to interact with the receptors. Experimental data were best fitted using a one-site binding model. Results represent mean ± SD (n = 3). Steady state constants derived from the binding isotherms are listed in Table 3.

receptor	dimeric β_2GPI	Trp316Ser
LRP	3.1 ± 0.6	3.7 ± 0.4
	192 ± 13	162 ± 47
Megalin	3.1 ± 0.7	2.6 ± 0.5
	241 ± 30	198 ± 17
Cluster II of LRP	1.6 ± 0.6	1.7 ± 0.19
	158 ± 30	197 ± 10
Cluster IV of LRP	1.7 ± 0.4	1.2 ± 0.3
	172 ± 15	175 ± 13
apoER2′	22 ± 4	34 ± 9
VLDL-R	38 ± 8	60 ± 17

Table 4. **Maximal binding for all constructs of dimeric β2-GPI.** Maximal binding to LRP and megalin is shown in response units (RU) and for ApoER2′ and the VLDL-R the maximal response is expressed in OD280 nm. Data represent mean ± SD (n = 3).

Discussion

In the present study we have investigated interaction between beta2-glycopprotein I (β2-GPI) and five members of the low density lipoprotein receptor (LDL-R) family. Among these are *1.* the low-density lipoprotein receptor related protein (LRP), a promiscuous and ubiquitously expressed receptor involved in several physiological processes, *2.* the Apolipoprotein E receptor 2′ (ApoER2′) which participates in neuronal development but is also expressed on platelets, *3.* the low-density lipoprotein receptor (LDL-R), *4.* the very low-density lipoprotein receptor (VLDL-R) which is expressed for instance on endothelial cells (EC) and *5.* megalin, a multifunctional receptor expressed in various resorptive epithelia (including the proximal renal tubule) pointing to a predominant role in endocytosis and transport. The current report addresses a number of specific questions. In previous experiments we have shown that platelets could be activated by a complex of β2-GPI and an anti-β2-GPI antibody, involving the receptor ApoER2′ [13].

The interaction between β2-GPI and ApoER2′ results in phosphorylation of ApoER2′ followed by p38 MAPK phosphorylation and thromboxane synthesis [4]. This raised the question if other LDL-R homologues also recognize a β2-GPI/anti-β2-GPI antibody complex. Indeed, we found that LRP, megalin and the VLDL-R recognize only a complex of β2-GPI/anti-β2-GPI but not β2-GPI alone (*Figure 1*) and this interaction can be mimicked by our recombinant form of dimeric β2-GPI (*Figure 2*). Two binding models were identified for the interactions; interaction between dimeric β2-GPI, ApoER2′, the LDL-R and the VLDL-R could be described with a one-site binding model, whereas with LRP or megalin a two-site (heterologous) binding model was more suitable. In contrast to the other receptors tested, the LDL-R showed one class of low affinity binding sites (340 nM) for dimeric β2-GPI. The LDL-R is a classical endocytosis receptor, whereas other LDL-R family members have been shown to regulate intracellular signaling processes [24], suggesting the minor role of the LDL-R in β2-GPI-mediated signaling. We could not observe a two-site binding model in the solid-phase binding assay, presumably because the multiple washing steps in this system may amplify association or dissociation defects. SPR analysis measures binding under equilibrium conditions and is therefore not affected by washing steps. In order to confirm whether data obtained from SPR analysis or from solid-phase binding assays are comparable, we analyzed interaction between dimeric β2-GPI and LRP using the solid-phase binding assays (as described for ApoER2′ or the VLDL-R). The resulting saturation curve could be adequately described employing a one-site binding model, representing a high affinity-binding site with half-maximal binding of 2.7 ± 1.0 nM (*data not shown*). This was in concordance with the high-affinity site (3.1 ± 0.6 nM) derived from the SPR data. In this study we have further investigated the receptor/protein interaction by using several recombinant constructs of dimeric β2-GPI. We demonstrated that domain V of β2-GPI is the crucial domain for recognition by the LDL-R homologous tested (*Figure 5*) and that this recognition site is not identical to the phospholipid-binding site within domain V of β2-GPI (*Figure 6*). It has been proposed that positive electrostatic surface potentials, not a primary sequence, in different ligands constitute receptor-recognition domains [25;26]. Our observations from this study suggest that indeed positively charged residues are important (*Figure 4*). We cannot exclude that this observed inhibition is due to steric interference by heparin. However, in a previous study we have shown that interaction between dimeric β2-GPI and ApoER2′ on platelets can be inhibited by a peptide spanning amino acids 282-287 in domain V of β2-GPI [19]. Furthermore, it has been reported that Lys (284), Lys (286) and Lys (287) in domain V are essential for the interaction of β2-GPI with heparin [27]. This implicates that both electrostatic interactions and amino acid sequences are important for the interaction between dimeric β2-GPI and LDL-R homologues. Interaction of dimeric β2-GPI with LRP or megalin was best described with a two-site binding model, suggesting the presence of two interaction sites on the surface of the receptors or on β2-GPI. For this purpose, we analyzed the interaction between β2-GPI and cluster II and IV of LRP. We could still observe a two-site binding model with both clusters, suggesting that LRP recognizes two surface potentials on β2-GPI. Two domains that expose cationic charges were of interest; domain I and domain V of β2-GPI. However, delta I interacted similar with the receptors or both clusters of LRP compared to full-length dimeric β2-GPI. Therefore, it is likely that domain V contains both

regions involved in the receptor-interaction. Site-directed mutagenesis is required to establish the relative importance of cationic residues in domain V for the assembly of the β2-GPI/LDL-R complex. Besides members of the LDL-R family, a number of other cellular receptors have been described for β2-GPI. The contribution of LDL-R family members in β2-GPI-mediated cell-activation in comparison with these other receptors is unknown. One of the candidate receptors, annexin A2 [39,40], does not have a transmembrane and intracellular domain, making it unlikely that annexin A2 is directly involved in signal transduction. It has been reported that anti-β2-GPI antibodies display a signaling cascade in endothelial cells comparable to that induced by members of the toll-like receptor (TLR) family. TLR2 [41] and TLR4 [42] have been implicated in this event. Interesting, also glycoprotein Ib, another possible member of the TLR family, has been shown to bind β2-GPI/anti-β2-GPI antibody complexes [43]. Our next challenge is to understand how cell signaling induced by anti-β2-GPI antibodies is coordinated by all these different receptors. Besides β2-GPI, other proteins have been implicated as possible target for aPL, in particular prothrombin. However, the majority of the performed studies indicate that anti-β2-GPI antibodies correlate best with the observed clinical manifestations [28]. In addition, addition of purified anti-prothrombin antibodies to whole blood did not result in increased platelet deposition (unpublished observations). Therefore, we do not believe that interaction of prothrombin-anti-prothrombin antibody complexes with cells result in cellular activation. An important step in resolving the pathways that explain clinical symptoms in APS is the identification of cellular receptors that can interact with the protein of interest. The classical clinical manifestations observed in APS are thrombosis and pregnancy morbidity [28-30]. However, patients often suffer from other aPL associated clinical manifestations, such as heart valve abnormalities, thrombocytopenia, proteinuria, chorea, neuropathy and livedo reticularis [31-35], which are undoubtedly frequently observed in patients with aPL antibodies in their plasma. Here we have shown that β2-GPI/anti-β2-GPI-antibodies complexes can interact with different LDL-R homologues. It is clear now that these receptors have the potential to induce signaling processes in different cell types [36-38]. Different members of the LDL-R family are expressed on almost all cell types. It is interesting to speculate that β2-GPI/anti-β2-GPI antibody complexes can activate a large number of different cells via LDL-R homologues, resulting in responses specific for that cell type. We speculate that the heterogeneous clinical manifestations observed in APS are due to interaction between β2-GPI/anti-β2-GPI antibody complexes and LDL-R family members on different cell types.

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Platelet adhesion to dimeric β2-glycoprotein-I under conditions of flow is mediated by at least two receptors: Glycoprotein Ibα and ApoER2′.

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Abstract

The major antigen implicated in the antiphospholipid syndrome (APS) is β2–GPI. Dimerized β2–GPI binds to ApoER2′ on platelets and increases platelet adhesion to collagen under conditions of flow. We therefore aimed to investigate whether the interaction between dimerized β2–GPI and platelets is sufficiently strong to resist shear stresses. We studied the interaction of platelets with immobilized dimerized β2–GPI under conditions of flow, further analyzed the interaction using surface plasmon resonance and solid phase immuno-assays. We found that dimerized β2–GPI supports platelet adhesion and aggregate formation under venous flow conditions. Adhesion of platelets to dimerized β2–GPI was completely inhibited by both addition of soluble forms of both ApoER2′ and GPIbα, addition of RAP and removal of GPIbα from the platelet surface. GPIbα co-precipitated with ApoER2′ suggesting the presence of complexes between GPIbα and ApoER2′ on platelet membranes. The interaction between GPIbα and dimeric β2–GPI was of intermediate affinity (Kd 180nM) and Zn^{2+}, but not Ca^{2+} dependent. Deletion of domain V from dimeric β2-GPI strongly reduced its binding to both GPIbα and ApoER2´. Antibodies that inhibit the binding of thrombin to GPIbα inhibited platelet adhesion to dimeric β2–GPI completely, while antibodies blocking the binding of vWF to GPIbα had no effect. Dimeric β2–GPI showed reduced binding to low-sulfated GPIbα compared to the fully sulfated form. In conclusion we show that platelets adhere to dimeric β2-GPI under both arterial and venous shear stresses. Platelets adhere via two receptors, GPIbα and ApoER2′. These receptors are present in a complex on the platelet surface.

Introduction:

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease characterized by the presence of arterial and/or venous thrombotic complications, both early and late pregnancy losses and preeclampsia in combination with the presence of antiphospholipid antibodies in plasma of affected patients [1]. These so called antiphospholipid antibodies do not recognize anionic phospholipids directly but are directed against proteins bound to anionic phospholipids [2;3]. The two proteins that are responsible for binding of the majority of the antiphospholipid antibodies are β2–Glycoprotein I (β2–GPI) and prothrombin [4]. The presence of antibodies directed against β2–GPI that are able to induce lupus anticoagulant (LAC) highly correlates with the presence of clinical symptoms and β2–GPI is therefore considered to be the most important antigen in APS [5]. β2–GPI is a plasma protein present at concentrations of approximately 200 μg/ml. It is mainly synthesized in the liver and consists of 5 homologous complement-binding repeats of which domain V is slightly aberrant. Domain V contains a large positively charged patch with a phospholipid insertion loop, which is responsible for binding to anionic phospholipids [6]. The interaction of plasma β2–GPI with anionic phospholipids is relatively weak (Kd = 330nM) but when β2–GPI is dimerized by antibodies, its affinity for anionic phospholipids increases several hundred fold [7]. We have previously shown that dimerization of β2–GPI with monoclonal antibodies results in increased platelet adhesion to collagen under conditions of flow [8]. Similar results were obtained with a recombinant protein in which β2–GPI was dimerized using the apple4 domain of factor XI [9]. The increase of platelet adhesion to collagen by dimeric β2–GPI could be inhibited with Receptor Associated Protein (RAP) [8], which is a universal inhibitor of ligand binding to members of the LDL receptor family. ApoER2' is the only member of the LDL receptor family known to be expressed by human platelets. It is a truncated splice variant of the ApoER2 receptor. It lacks exon 5 and therefore LDL-binding domains 4, 5 an 6 [10]. To investigate whether the dimeric β2–GPI–platelet interaction is sufficiently strong to support platelet adhesion under flow, we studied the interaction of platelets with immobilized dimeric β2–GPI in an *in-vitro* perfusion system under conditions of shear stress.

Methods:

Reagents:
Monoclonal antibody 6D1 raised against GPIbα was kindly provided by Dr. B.S. Coller (Mount Sinai Hospital, New York, NY). Monoclonal antibody 3B7, raised against β2–GPI, was developed in our laboratory using standard hybridoma cloning procedures (unpublished data). Monoclonal antibody 21B2, raised against β2–GPI, was a kind gift from Prof J. Arnout [11]. RAP was produced as previously described [12]. Thrombin was purchased from Kordia (Leiden, the Netherlands). Monoclonal antibodies 12E4, 6B4, 2D2 and 10H9 were raised against GPIbα as published before [13]. Monoclonal antibody AK2, raised against GPIbα was purchased from Abcam (Cambridge, U.K.). Snake venom NK was a kind gift from Dr. Andrews, Monash University, Melbourne. A1(R543Q) is the binding domain of vWF to GPIbα with a type 2B substitution induc-

ing spontaneous binding to GPIbα and was produced in our own laboratory [14]. Monoclonal antibody directed against TF was purchased from American Diagnostica (American Diagnostica inc. MoAb 4508, Stamford, USA). Rabbit anti ApoER2 (186) was kindly provided by Dr. J. Nimpf, Medical University of Vienna, Vienna, Austria.

Purification of monomeric β2–GPI:
Monomeric β2-GPI was isolated from fresh citrated human plasma as described previously [15]. Purity of the protein was checked on a 4-15% SDS-polyacrylamide gel showing a single band of 47 kD. Concentration of the protein was determined using the BCA protein assay.

Cloning and expression of dimeric β2-GPI and deletion mutants:
Recombinant dimeric β2–GPI was constructed and purified as described previously [9]. Purified constructs were analyzed by SDS-PAGE.

Cloning, expression and purification of soluble ApoER2:
Mature megakaryocytes were cultured from citrated umbilical cord blood as described previously [16]. Soluble ApoER2' (sApoER2') was cloned as previously described [17]. Purified constructs were analyzed by SDS-PAGE. Only 1 band was observed at 45 kD.

Cloning, expression, and purification of soluble GPIbα:
Recombinant human soluble GPIbα (sGPIbα) was cloned and expressed as previously described [14]. Purified protein was analyzed by SDS-PAGE. Only 1 band was observed at 35 kD.

Platelet and red blood cell preparation:
Reconstituted blood and washed platelets were prepared as described by Weeterings et al.[18].

Coating of cover slips:
Glass cover slips (Menzel-Galzer 40x50) were cleaned over-night in 80% ethanol, rinsed with distilled water and dried before coating. Coating was done by incubating cover slips back to back, overnight with 400 μl of either a monoclonal anti β2–GPI antibody (21B2) or monoclonal anti tissue factor antibody (TF), (American Diagnostica inc. MoAb 4508) at 50 μg/ml in a humified chamber. Cover slips were then blocked in 1% BSA and incubated with 25 μg/ml monomeric β2–GPI, 25 μg/ml dimeric β2–GPI or buffer for 1 hour at room temperature.

Perfusion studies:
Perfusion experiments were performed in a single pass triplo perfusion chamber consisting of a silicon sheet gasket which maintained a flow path height of 0.125 mm and width of 2 mm [19;20]. To test shear dependency, reconstituted blood was perfused over 21B2 coated cover slips, pre-incubated with plasma β2–GPI, at different shear forces (100^{-s}, 300^{-s}, 800^{-s}, 1300^{-s} and 1600^{-s}). To test inhibitory capacity of different proteins, pro-

teins were added to platelets and mixed carefully to avoid activation. The RBCs were then added gently to allow them to settle on the bottom of the tube. Reconstituted, unmixed blood was incubated for 5 minutes at 37°C. The reconstituted blood was then mixed gently to homogeneity and directly used for perfusions. RAP, sGPIbα and sApoER2' were added at 35 μg/ml and antibodies were used at a concentration of 50 μg/ml. NK was pre-incubated at 5 μg/ml with platelets for 30 minutes at 37°C with 3 mM Ca^{2+}. Reconstituted blood was perfused over coated cover slips for 5 minutes at a shear rate of 300 s^{-1} using a infusion pump (pump 22, model 2400-004; Harvard, Natrick, MA). Afterward, the cover slips were removed 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 [21]. 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. Perfusions were done with blood with 3 different donors and for every donor three independent flow experiments were performed. Evaluation of platelet adhesion was performed at 20 fields and measured at 5 different positions spaced by 1mm starting at a distance of 5 mm from the blood inlet. Analysis was performed perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion to dimeric β2–GPI. Results are expressed as mean relative coverage (mean ± SD, n = 9). Statistical analysis was performed using the Student-T test.

Immuno-precipitation: Interaction of dimeric β2–GPI with GPIbα:
For immuno-precipitations, 500 μl aliquots of washed platelets (200.000/μL) resuspended in Hepes/Tyrode buffer pH 7.4 and incubated with TBS, monomeric β2–GPI or dimeric β2–GPI at 100 μg/ml for 5 minutes at 37°C. Platelets were then lysed on ice with 1% CHAPS solution (containing 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 50 mM MES (2-(N-Morpholino) ethanesulfonic acid), 3 mM $CaCl_2$ and 150 mM NaCl, pH 7.4). GPIbα was precipitated with 1 μg/ml of a monoclonal anti-GPIbα (6D1) in combination with 50 ml of 10% protein G-Sepharose slurry (Amersham Biosciences) and incubated for 18 h at 4 °C. Sepharose beads were then washed three times with lysis buffer, resuspended in non-reducing Laemmli sample buffer (0.001% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8), and boiled for 5 min. The supernatant was separated on a 10% SDS-polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris buffered saline (25mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween 20 (TBST) containing 2% (w/v) non-fat dry milk powder (MP) (Nutricia, the Netherlands) for 1 h at room temperature. The blot was then incubated with mouse anti β2–GPI antibody (3B7, 3 μg/ml) overnight in TBST with 1% milk powder and washed three times with TBST. After incubation with peroxidase labeled rabbit anti mouse antibodies for 1 h at RT (1:2500; Dako, Glostrup, Denmark) blots were washed again with TBST-1%MP and developed with enhanced chemiluminescence reagent plus (Perkin Elmer life sciences, Boston USA)

Interaction of ApoER2' with GPIbα:
250 µl aliquots of washed platelets (300.000/µL) resuspended in Hepes/Tyrode buffer and treated with Cytochalasin D (30 µM, Sigma Aldrich) for 30 minutes at 37°C. Cytochalasin D disrupts the actin cytoskeleton of the platelets and by pre-treatment, co-precipitation due to the cytoskeleton attachment of both receptors can be excluded. Platelets were then incubated with either, TBS, monomeric β2–PGI or dimeric β2–GPI at 100 µg/ml for 5 minutes at 37°C. Platelets were then lysed by adding 25 ml of 10x RIPA lysis buffer (PBS with 10% Nonidet P40, 5% octylglucoside, 1% SDS, 1.86% EDTA 10 mM $NaVO_3$, and SIGMA protease inhibitor cocktail according to manufacturers protocol). Lysate was then put on ice for 30 minutes and ApoER2' was precipitated with 1 µg/ml of a polyclonal anti-ApoER2 antibody (D-18, Santa Cruz Biotechnology, Santa Cruz, CA) in combination with 50 ml of a 10% protein G-Sepharose slurry (Amersham Biociences). The immunoprecipitations were incubated for 18 h at 4 °C in a head-over-head rotor, washed three times with 1x lysis buffer, resuspended in reducing Laemmli sample buffer (0.001% (w/v) bromphenol blue, 2% (w/v) SDS, 10% (v/v) glycerol, 25mM DTT in 62.5 mM Tris, pH 6.8), and boiled for 5 min. The supernatants were analyzed on a 10% SDS-polyacrylamide gel and electro blotted onto Protran Nitrocellulose membrane. Blots were blocked with TBS with 0.1% (v/v) Tween 20 (TBST) containing 4% (w/v) BSA (Sigma) for 1 h at room temperature. Incubation with monoclonal anti-GPIbα antibody AK-2 (1 µg/ml) was performed overnight in TBST supplemented with 1% BSA. The membranes were washed three times and incubated with RAMPO (1:2500; Dako, Glostrup, Denmark) in the same buffer. Bands on blots were visualized with enhanced chemiluminescence reagent plus (Perkin Elmer life sciences, Boston USA)

Scanning Electron Microscopy:
Reconstituted blood was perfused over 21B2 coated cover slips, pre-incubated with plasma β2–GPI, for 5 minutes. Adhered platelets were fixed in 3% paraformaldehyde/0.25% glutaraldehyde for 30 minutes and washed 3 times in TBS (25mM Tris, 150mM NaCl). Cover slips were blocked with 1% BSA + 1.1% glycine for 15 minutes. Cover slips were then incubated with mouse anti GPIbα (6.30) at 1µg/ml and washed 3 times with TBS. Cover slips were then incubated with protein A-Gold 15 nm (DAKO) 1:100 for 30 minutes and washed 3 times with TBS. First step immunogold labeling was fixed with 3% paraformaldehyde/0.25% glutaraldehyde for 30 minutes to block all free protein A binding sites. Cover slips were washed 3 times with TBS and rabbit anti ApoER2 (186) 1:500 was added for 30 minutes. The cover slips were washed 3 times with TBS. After incubation with protein A-Gold 10 nm (Dako) cover slips were washed 3 times with TBS and fixed again with 3% glutaraldehyde/0.25 % glutaraldehyde. Sequential dehydration was done with resp. 80%, 100% ethanol and Hexamethyldisilazane. Cover slips were sputter coated with platinum (6.5nm) and scanning electron microscopy was performed.

Surface Plasmon Resonance Analysis:
Surface plasmon resonance (SPR) binding assays were performed employing a Biacore 2000 system (Biacore AB, Uppsala, Sweden). For binding experiments, sGPIbα was immobilized on a CM5 sensor chip using the amine-coupling kit as instructed by the sup-

plier (Biacore AB, Uppsala, Sweden). One channel was activated and blocked in the absence of protein and afterwards its signal was used to correct for a-specific binding. Analysis of binding was measured in TBS/0.005% Tween 20, 3 mM Ca^{2+} or 15 μM Zn^{2+} with a flow rate of 10 ml/min at 25°C. All proteins were injected for 2 minutes and regeneration of the surface was performed by application of 0.1 M sodium citrate containing 10 mM EDTA and 1 M NaCl, pH 5.0. Analysis of binding curves was done using Biaevaluation 3.0 and Graph Pad 4.1 software. Data obtained from SPR analysis was used for the calculation of the affinity constants (K_D) as follows. Responses at equilibrium (R_{eq}) derived from sensorgrams were plotted against protein concentration. The resulting binding isotherms were subsequently fitted to the following equations: For a one-site binding model:

GPIbα-dimeric β2–GPI interaction studied in a solid phase binding assay:
Both fully sulfated and low sulfated sGPIbα were coated in TBS on 96 wells Costar hydrophobic ELISA plates (5 μg/ml, 37°C for 1 hr). Wells were then blocked with TBS containing 4% BSA (1 hr, RT). For inhibition studies, 1.25 μg/ml dimeric β2-GPI was pre-incubated with 12.5 μg/ml thrombin or 12.5 μg/ml antibodies for 30 minutes at RT and the mixture was then added to the wells. Wells were then incubated with polyclonal rabbit anti-β2-GPI and bound antibodies were visualized using peroxidase labeled swine anti rabbit (Dako, Glostrup, Denmark) followed by staining with orto-phenylenediamine (OPD). For binding curves of dimeric β2–GPI to non-sulfated and fully sulfated GPIbα, serial dilutions were incubated in GPIbα coated wells and detected as described above. Analysis of binding curves was done using Graph Pad software.

Results:

To investigate whether the interaction between blood platelets and dimeric β2–GPI is sufficiently strong to support platelet adhesion under conditions of flow, perfusions were performed with reconstituted blood over different surfaces. As shown in figure 1a, dimeric β2–GPI supported both platelet adhesion and aggregate formation at venous shear (300 s^{-1}). Comparable results were found when monomeric β2–GPI was incubated on the 21B2 coated cover slips (fig 1e). Maximal adhesion was obtained at a shear of 800^{-s} but only a minimal difference was observed in relative surface coverage when a shear of 300^{-s} was used (fig. 1d). We therefore used 300^{-s} as the standard shear in this study to minimize protein usage in the inhibition experiments performed in this study No adhesion was observed when anti β2–GPI antibody 21B2 coated cover slips were not pre-incubated with β2–GPI (fig 1b) or when a control antibody (monoclonal anti TF antibody) pre-incubated with β2–GPI used (fig.1c). To test whether ApoER2' was involved in platelet adhesion and aggregate formation on a dimeric β2–GPI coated surface under conditions of flow, perfusions were repeated in the presence of RAP or sApoER2'. Addition of RAP and sApoER2' inhibited platelet adhesion and aggregate formation completely (Fig 1f). We furthermore tested whether other receptors play a role in the adhesion to dimeric β2–GPI. Addition of sGPIbα almost completely inhibited platelet deposition to dimeric β2-GPI (Fig 1f).

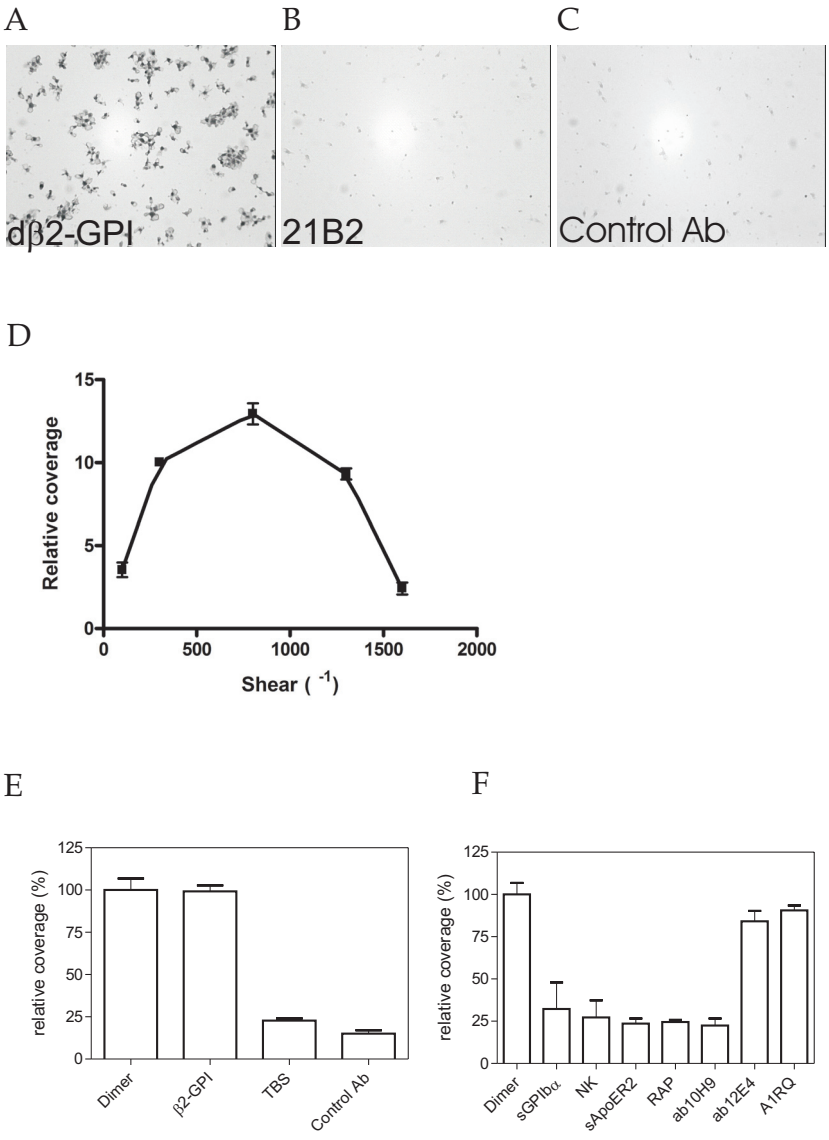


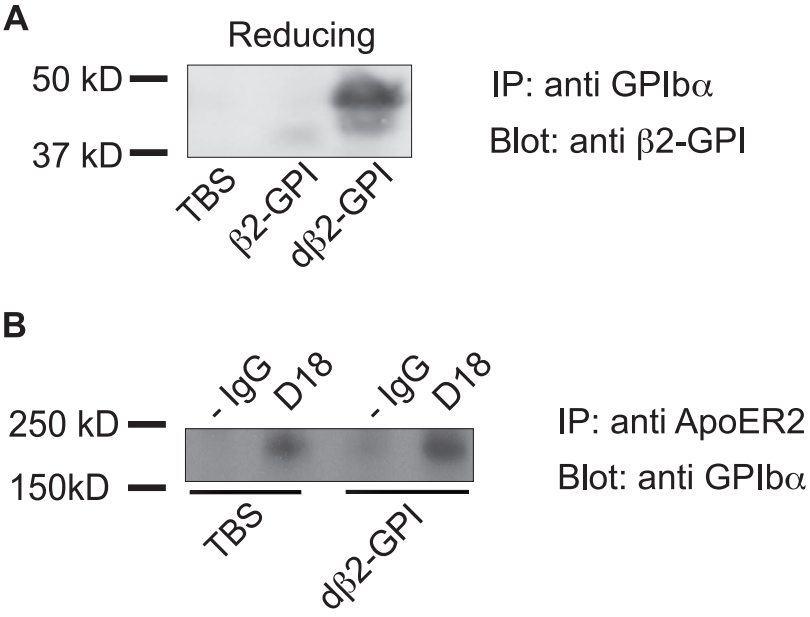
Figure 1. **Dimeric β2-GPI is able to support platelet adhesion and aggregate formation via ApoER2'and GPIbα.** Glass cover slips were coated with either anti β2-GPI or anti Tissue Factor antibodies (control antibody). Cover slips were then blocked and incubated with either monomeric β2-GPI (e) or dimeric β2-GPI (a,c,e,f) or buffer (b). Reconstituted blood was then perfused over 21B2 incubated with dimerized β2-GPI, 21B2 incubated with buffer or a control IgG incubated with dimeric β2-GPI and pictures were taken from the present platelet adhesion (figure 1a, 1b, 1c respectively). Maximal surface coverage was measured at a shear of 800-s (fig. 1d). Experiments were performed at a shear of 300-s. Surface coverage on dimeric β2-GPI was set at 100%. Figure 1e shows relative coverage of platelets to anti β2-GPI incubated with dimeric β2-GPI, anti β2-GPI or control antibody (anti TF) incubated with dimeric β2-GPI relative to the % of the total surface of dβ2-GPI coated cover slips was covered with platelets after 5 minutes. Figure 1f shows the relative coverage after pre-incubation with a panel of potential inhibitors compared to the coverage on dimeric β2-GPI (fig 1f). Data is presented as mean ± SD, n=9

Also, cleavage of GPIbα from the surface of platelets using snake venom NK completely inhibited the adhesion of platelets to dimeric β2–GPI (fig 1f). We subsequently tested a set of well-characterized antibodies against GPIbα for their capacity to inhibit adhesion of platelets to dimeric β2–GPI. Anti GPIbα antibody 10H9, inhibiting thrombin binding to GPIbα, almost completely blocked adhesion of platelets to dimeric β2–GPI whereas an antibody directed against the VWF binding site on GPIbα (12E4) did not affect platelet adhesion and aggregate formation. The addition of VWF domain A1 expressing a type 2B VWD mutation (R543Q) also did not inhibit platelet adhesion to dimeric β2–GPI (fig 1f).

The capability of soluble GPIbα to completely abolish platelet adhesion to dimeric β2–GPI suggests a direct interaction between dimeric β2–GPI and GPIbα. In order to probe such a direct interaction, pull-down experiments were performed using an antibody directed against GPIbα (6D1) in order to detect co-precipitation of dimeric β2–GPI with GPIbα from washed platelets. Dimeric β2–GPI, but not monomeric β2–GPI could be co-precipitated with GPIbα indicating that a direct interaction between GPIbα and dimeric β2–GPI occurs when platelets were incubated with dimeric β2–GPI (fig 2a).
To test whether GPIbα and ApoER2' are present in a complex or that a complex is formed on the surface of platelets upon incubation with dimeric β2–GPI, immuno-precipitations were performed using an antibody directed against ApoER2 (D-18) with a-specific rabbit IgG's as negative control (Goat anti Mouse IgG-PE, B&D technologies). GPIbα was co-precipitated with ApoER2' indicating a complex of GPIbα and ApoER2' present on the membrane of platelets (fig. 2b). However, the complex between GPIbα and ApoER2' already exists on the platelet membrane surface in the absence of dimeric β2–GPI (fig 2b). The interaction between GPIbα and ApoER2' was independent of an intact cytoskeleton since pre-incubation of the platelets with Cytochalasein-D did not disrupt the complex (fig 2b). Immuno-gold labeling for scanning electro-microscopy (SEM) also showed ApoER2'and GPIbα to present in close proximity of each other on the surface of the adhered platelets (fig. 2c). No data was obtained from the SEM experiments in relation to relative distances between both receptors in complex.

To further characterize the interaction between dimeric β2–GPI and GPIbα, soluble GPIbα was coupled to a CM5 SPR chip. Dimeric β2–GPI bound to immobilized sGPIbα (fig. 3) and this interaction was dependent on the presence of Zn^{2+} (fig. 3), whereas the presence or absence of Ca^{2+} did not affect the binding of dimeric β2–GPI to GPIbα (fig. 3 inset). The binding of dimeric β2–GPI to GPIbα increased with increasing concentrations of Zn^{2+} and no saturation was reached when Zn^{2+} concentrations exceed the plasma concentrations (15 mM). No binding to GPIbα was observed with monomeric β2–GPI. To determine which domain of dimeric β2–GPI binds to GPIbα, surface plasmon experiments were performed with domain deletion mutants. The different proteins were injected at different concentrations and saturation curves were plotted (fig. 4). The affinity of dimeric β2–GPI for GPIbα was calculated to be 180±20 nM. No differences in affinity and B_{max} were observed upon deletion of domain I. The curves for dimeric β2–GPI and dimeric β2–GPIΔD1 were best described employing a one-site binding model. When domain II and III were deleted from dimeric β2–GPI, a slight non-significant

decrease in Kd was observed for both deletion mutants (280±80 nM and 280±15 nM resp.). When domain V was deleted from dimeric β2–GPI, the affinity reduced from 180±20 nM to 590±160 nM indicating that the major binding site of dimeric β2–GPI for GPIbα resides in domain V.



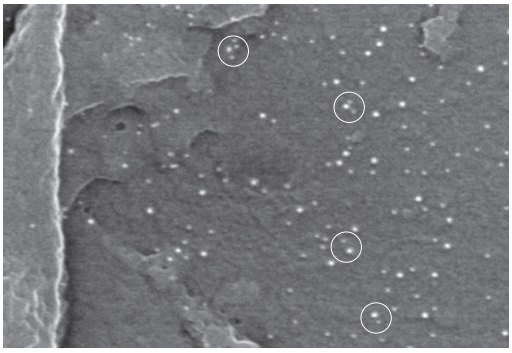


Figure 2. **Dimeric β2-GPI binds to GPIbα present on the platelet surface and a complex is formed with GPIbα and ApoER2'.** Washed platelets were incubated with Cytochallasein-D to disrupt the actin cytoskeleton. Platelets were then incubated with TBS, monomeric β2-GPI or dimeric β2-GPI for 5 minutes. Suspensions were then lysed and incubated with anti GPIbα or anti ApoER2' in combination with protein G-Sepharose beads. Beads were then washed and immuno-precipitations were separated on SDS-PAGE under reducing and non-reducing conditions. Proteins were blotted and incubated with mouse anti-β2-GPI (fig. 2a) or with mouse anti GPIbα (fig. 2b) Blots were incubated with RAM-HRP and proteins were visualized using chemiluminescence ECN. Immuno-scanning electron microscopy was performed showing GPIbα and ApoER2' to be present in close proximity on the platelet surface (fig 2c).

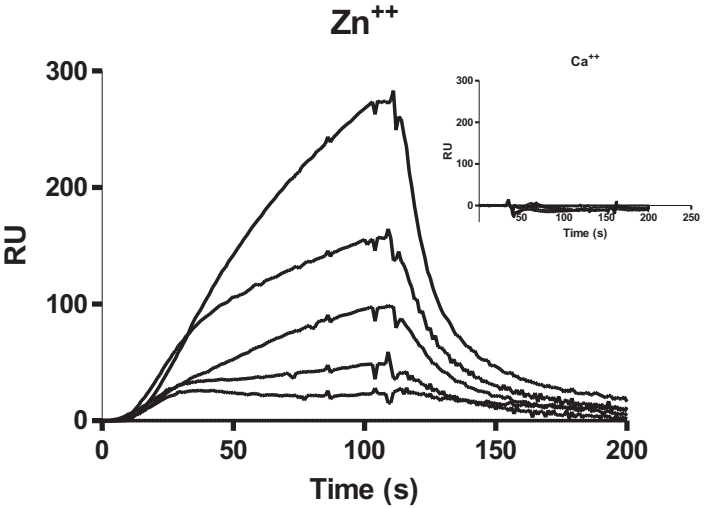


Figure 3. **Binding of dimeric β2-GPI to GPIbα is dependent on the presence of Zn2+.** Soluble GPIbα was immobilized on a CM5 chip using the amine-coupling kit. Dimeric β2-GPI was injected over the surface at 10 µl/min. in the presence of increasing concentrations of either Zn2+ (figure 3) 1=60 µM, 2=30 µM, 3=15µM, 4=7.5 uM, 5 3.75µM or Ca2+ (fig 3 inset) concentrations range from 50 mM to 3.125 mM. (RU means arbitrary Response Units)

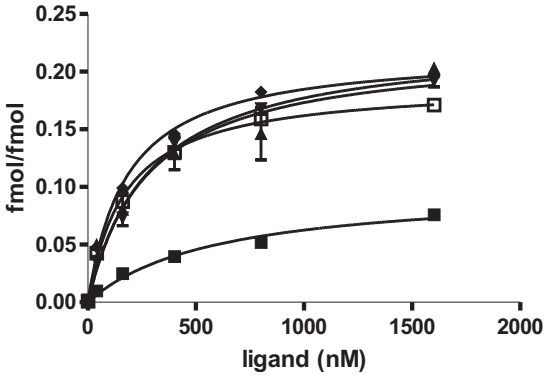


Figure 4. **Dimeric β2-GPI binds to GPIbα via domain V.** Increasing concentrations of dimeric β2-GPI and domain deletion mutants of dimeric β2-GPI were injected over a CM5 chip coupled with GPIbα at 10 µl/min in the presence of Zn2+ (15 µM). □ represents dimeric β2-GPI, ♦ represents dimeric β2-GPI ΔD1,▲ represents dimeric β2-GPI ΔD2, ▼represents dimeric β2-GPI ΔD3 and ■ represents dimeric β2-GPI ΔD5. Saturation curves were plotted and affinities were calculated from these curves. Curves are presented as mean ± SD (n=3)

To determine the binding site for domain V of dimeric β2–GPI located in GPIbα, several antibodies against GPIbα were tested for the capacity to block dimeric β2–GPI binding to GPIbα. GPIbα was coated on an ELISA plate and dimeric β2–GPI was pre-incubated with a 10 fold molar excess of antibodies of which the epitopes haven been previously determined. Monoclonal antibodies that inhibit vWF binding to GPIbα (12E4, 6B4, 6D1) did not inhibit dimeric β2–GPI binding to sGPIbα and even showed a slight increase in

binding of dimeric β2–GPI to GPIbα. Monoclonal antibodies that inhibit thrombin binding to GPIbα (2D2, 10H9) inhibited dimeric β2–GPI binding to sGPIbα by 50%. When purified thrombin was added in a 10 times molar excess, binding was also inhibited by 50% (fig 5a).

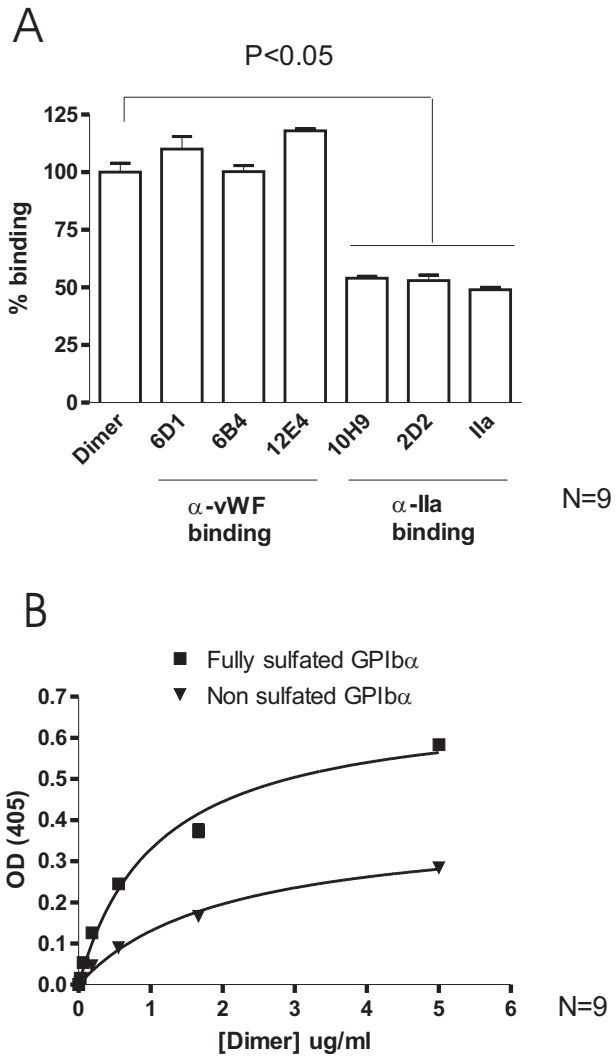


Figure 5. **Dimeric β2-GPI binds in close proximity of the thrombin-binding site in GPIbα.** A: Soluble GPIbα was coated on an ELISA plate and the wells were blocked with BSA . Dimeric β2-GPI was pre-incubated with a panel of GPIbα binding inhibitors in a ratio of 1:10 (μg:μg). This mixture was then incubated in the GPIbα coated wells and binding of dimeric β2-GPI was detected using a monoclonal anti β2-GPI and RAM-HRP. OPD was used for staining. Statistical analysis was done using student-T test. B: High (2 or 3) or low (0 or 1) sulfated sGPIbα was coated on an ELISA plate and wells were then blocked with BSA. Wells were then incubated with an increasing concentration of dimeric β2-GPI and binding was detected using the same system as described above. ■ Represents dimeric β2-GPI binding to high sulfated GPIbα, ▲ represents low sulfated GPIbα.

As the main thrombin-binding site on GPIbα resides in sulfated tyrosines (residues 276-282) we tested the involvement of the sulfated region in GPIbα in the interaction with dimeric β2–GPI. Low-sulfated and fully sulfated GPIbα were coated on an ELISA plate and incubated with dimeric β2–GPI. The binding of dimeric β2–GPI to low-sulfated GPIbα was 50% lower then the binding to fully sulfated GPIbα. The resulting affinity for dimeric β2–GPI was reduced two fold when tyrosine residues 276, 278 and 279 in sGPIbα were low sulfated. (fig 5b)

Discussion

We now accept that not antibodies against anionic phospholipids but rather antibodies against β2–GPI are the important pathological antibodies present in the antiphospholipid syndrome. However, we lack a generally accepted model how these antibodies cause the clinical manifestations observed in this syndrome. It is now evident that these so-called "antiphospholipid" antibodies dimerized β2–GPI, resulting in a strongly increased affinity of β2–GPI for cells such as platelets and endothelial cells. Recently we have shown that ApoER2' present on platelets is involved in the activation of platelets by dimerized β2–GPI. In this study we show that there is a second receptor for dimeric β2–GPI present on platelets, notably GPIbα. The interaction between dimeric β2–GPI and platelets under conditions of flow was inhibited with both soluble ApoER2' and soluble GPIbα. Both the cleavage of GPIbα from the platelet surface and blocking ApoER2' using RAP completely abrogate platelet adhesion and aggregate formation under conditions of flow. Furthermore, antibodies against the thrombin-binding site but not antibodies against the vWF binding site within GPIbα are able to block platelet adhesion and aggregate formation on dimeric β2–GPI. Similar results were found in a solid phase binding assay where binding of dimeric β2–GPI to GPIbα could be substantially inhibited by thrombin itself and by antibodies blocking thrombin binding to GPIbα (e.g., 10H9, 2D2). Antibodies blocking vWF binding to GPIbα (12E4, 6B4, 6D1) did not inhibit dimeric β2–GPI binding to GPIbα. The tyrosine sulfated region of GPIbα, representing the main thrombin binding site on GPIbα seems to be important for optimal interaction of dimeric β2–GPI as highly sulfated GPIbα (2 or 3 sulfated tyrosine residues) binds dimeric β2–GPI much stronger then GPIbα with non or only 1 sulfated tyrosine residue. Dimeric β2–GPI binds in close proximity of the thrombin-binding site present on GPIbα. Just as for ApoER2', the main binding site for GPIbα within dimeric β2–GPI is located in domain V, as was shown by SPR experiments using deletion mutants of dimeric β2–GPI. The interaction between dimeric β2-GPI and GPIbα appeared to be Zn^{2+} and not Ca^{2+} dependent. Whether this represents an effect of Zn^{2+} on β2-GPI or on GPIbα remains unclear and requires further investigation. Domain V of β2–GPI contains a large positively charged patch and one might speculate that the interaction of this patch with the negatively charged sulfate groups on the tyrosines is important for the binding of dimeric β2–GPI to GPIbα. Taken together, these results show that there are two receptors for dimeric β2–GPI on the platelet membrane, ApoER2' and GPIbα. The interaction of dimeric β2–GPI with ApoEr2'and GPIbα not only results in adhesion of platelets but also in activation of platelets as aggregates were observed on

the dimeric β2–GPI coated surfaces. The affinity of dimeric β2–GPI for ApoER2′ is higher (12±1 nM, unpublished data) than the affinity for GPIbα (180±20 nM). On the other hand, the copy number of GPIbα on the platelet surface is much higher. We therefore propose, in analogy to activation of platelets by thrombin [22], a mechanism in which GPIbα serves as a docking site for dimeric β2–GPI after which, through positioning and concentrating dimeric β2–GPI on the platelet surface, binding of dimeric β2–GPI to ApoER2′ and subsequent signaling occurs. In this respect, it is interesting to note that ApoER2′ is present in complex with GPIbα on the platelet membrane, as was demonstrated by pull-down experiments in the presence or absence of dimeric β2–GPI. Recent publications have shown that GPIbα can form receptor complexes with several other platelet receptors such as GPV, GPVI, FcγRIIa and PAR1, resulting in different properties of GPIbα [23-25]. In this study ApoER2′ is added to the list of co-receptors for GPIbα. We speculate that this complex is involved in the pathogenesis of the antiphospholipid syndrome. One of the clinical criteria of the anti-phospholipid syndrome is the occurrence of both arterial and/or venous thrombosis. Shear forces tested in this study represent both arterial and venous flow condition. The role of platelets in the formation of arterial thrombosis is well established but a role for platelets in the development of venous thrombosis is normally not anticipated. We have no information of the patho-physiology of deep venous thrombosis induced by antiphospholipid antibodies. The data presented in this study support the so-called second hit model in which dimeric β2-GPI functions as a sensitizer or pre-activator of blood platelets. This sensitization or pre-activation results in an increased response to a second stimulus such as the exposure of collagen upon vascular damage. We propose a mechanism by which dimeric β2-GPI acts as a "cross-linker" between receptors on platelets resulting in increased adhesion and aggregate formation. Both GPIbα and ApoER2′ are required for this process since the blockade of either one of the receptors completely abolished platelet adhesion and aggregate formation. A possible mechanism in which endothelial cells are involved is conceivable. Since several members of the LDL-receptor family are present on the surface of endothelial cells, activation of endothelial cell by dimeric β2-GPI can be anticipated. Padilla *et al*.[26] showed that activation of endothelial cells results in the secretion of ultra large vWF which sticks to the endothelial surface via P-Selectin. As such platelets adhered to endothelial cells would deliver an ideal pro-coagulant surface for coagulation. This hypothesis is presently under investigation. We did not observe any difference in platelet adhesion and aggregate formation on cover slips incubated with either monomeric β2–GPI or dimeric β2–GPI while only dimeric β2–GPI and not monomeric β2–GPI binds to GPIbα. We have coated monomeric β2–GPI to the glass cover slip via an antibody and we propose that the antibody dimerized β2–GPI making it indistinguishable from dimeric β2–GPI. Direct coating of monomeric β2–GPI or dimeric β2–GPI to the cover slip showed varying adhesion results, probably because β2–GPI binds preferentially with its positively charged domain V to the negatively charged glass cover slips. The data presented in this paper have been presented in part at the XXth Congress of the International Society of Thrombosis and Haemostasis (abstract nr. OR256) and after submitting this article, the group of Krilis [27] also showed an interaction between β2-GPI and GPIbα. In conclusion, we have shown here that there are two receptors present on the surface of platelets for dimeric β2–GPI, ApoER2′and

GPIbα, that are present in a complex. The individual roles of both receptors in adhesion and activation of the platelets need further studies. The possible involvement of GPIbα in the activation of platelets by β2–GPI could open new avenues in the treatment of the antiphospholipid syndrome.

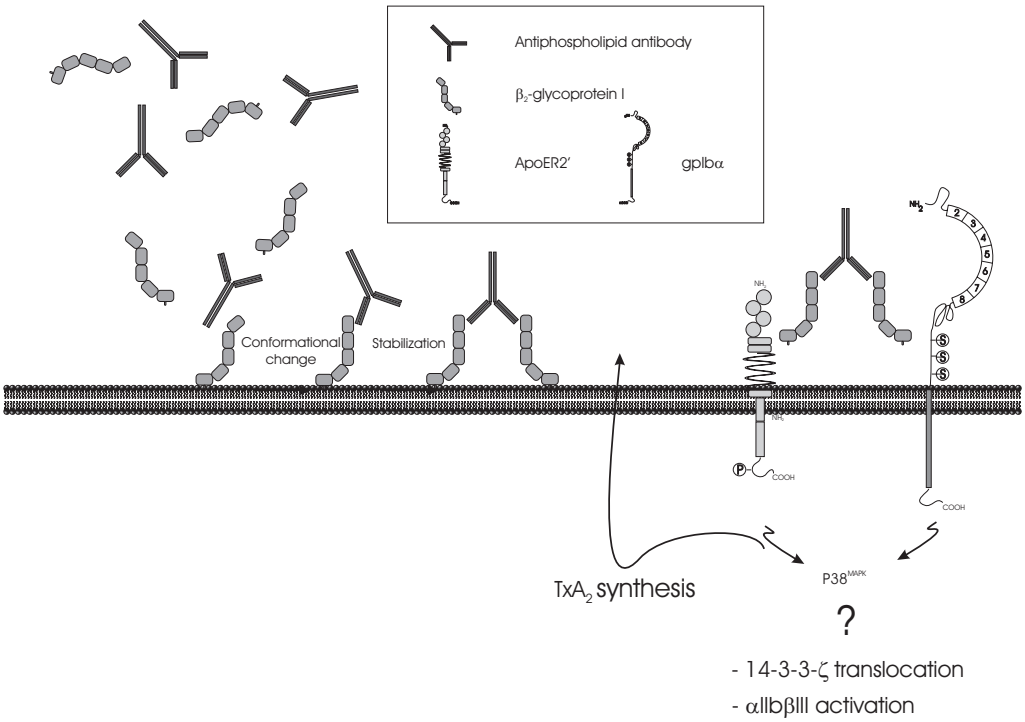


Figure 6. **A schematic model of events occurring on the platelet surface.** Exposure of negatively charged phospholipids facilitates β2-GPI binding to platelets and subsequent formation and stabilization of dimeric β2-GPI. Dimeric β2-GPI then can bind to both GPIbα and ApoER2′ on the platelet surface leading to subsequent increased activation due to a second stimulus. Sensitization by dimeric β2-GPI leads to an enhanced activation. ApoER2′ becomes phosphorylated and adhesion to a collagen surface increases in a TxA2 dependent way [28]. Activation of GPIbα can lead to GPIbα and 14-3-3-ζ translocation to the cytoskeleton, P38MAPK phosphorylation and TxA2 synthesis. TxA2 and P38MAPK phosphorylation have been described to be involved in signaling events downstream of both ApoER2′ and GPIbα [29-31].

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6

Activation of platelets by dimeric β2-glycoprotein I requires independent signaling via both Glycoprotein Ibα and Apolipoprotein E Receptor 2′

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Manuscript in preparation

Abstract

Dimerization of β2-Glycoprotein I (β2-GPI) by auto-antibodies is thought to trigger the clinical manifestations observed in the antiphospholipid syndrome. Previous work has shown that binding of dimeric β2-GPI to the platelet receptors Apolipoprotein E Receptor 2′ (ApoER2′) and Glycoprotein Ibα (GPIbα) mediates increased platelet activation in an in vitro model of the antiphospholipid syndrome. The individual roles of either receptor in mediating platelet activation by dimeric β2-GPI is hitherto unclear. In this study we have determined the roles of GPIbα and ApoER2′ in platelet activation by dimeric β2-GPI. Platelet activation by dimeric β2-GPI was studied under conditions of flow over a fibronectin surface at s shear rate of 300 sec^{-1}. The increase in adhesion caused by dimeric β2-GPI (150% ± 33 compared to a buffer control) was completely blocked by inhibition of the interaction of dimeric β2-GPI with both GPIbα and ApoER2′. Upon platelet stimulation with dimeric β2-GPI, GPIbα translocated to the cytoskeleton via the adapter molecule 14-3-3ζ in a time dependent manner, a process that could be inhibited by competition with soluble GPIbα. Translocation could not be inhibited by competition with the soluble first LDL-binding domain of ApoER2 or addition of RAP, the general inhibitor of the LDL-receptor family. Concomitantly ApoER2′ was phosphorylated and transiently dissociated from the cytoskeleton bound adapter molecule Disabled 1, a process that could be inhibited by competition with the soluble first LDL-binding domain of ApoER2 and not with soluble GPIbα. In conclusion, we show that dimeric β2-glycoprotein I signals via two distinct pathways in platelets, both of which are required for platelet activation. Abrogation of either signal results in loss of activation.

Introduction

The 45 kDa plasma protein β2-glycoprotein I (β2-GPI) is the major antigen in the antiphospholipid syndrome (APS), a non-inflammatory autoimmune disease characterized by the co-occurrence of vascular thrombosis and/or pregnancy morbidity and the presence of antiphospholipid antibodies (aPL) in plasma of affected individuals [1]. Despite its abundance in human plasma, the function of β2-GPI remains unknown. The general consensus is that dimerization of β2-GPI by aPL results in a conformational change in the molecule, greatly increasing its affinity for anionic phospholipids and several cellular receptors, enabling it to interact with cells [2]. Although dimeric β2-GPI has been reported to bind to several receptors on monocytes, endothelial cells and platelets, only the interaction of dimeric β2-GPI with the platelet receptors Apolipoprotein E Receptor 2' (ApoER2') and Glycoprotein Ibα (GPIbα) has been studied in more detail. Previous work from our group has shown that ApoER2', the only member of the LDL-receptor family present on platelets, mediates the increase in platelet adhesion to collagen under conditions of flow seen in the presence of dimeric β2-GPI [3;4]. We and others have also shown the interaction of dimeric β2-GPI with another platelet receptor; GPIbα [5;6]. In perfusion experiments over immobilized dimeric β2-GPI, the role of both GPIbα and ApoER2' was shown to be crucial in sustaining platelet adhesion, as inhibition of the interaction of dimeric β2-GPI with either receptor reduced platelet adhesion to background level [5]. The effect of dimeric β2-GPI on platelet adhesion to collagen suggests the triggering of an intracellular signaling cascade. Ligand binding to both GPIbα and ApoER2' is known to lead to intracellular signaling, although not much is known about signaling downstream from the latter. This study addresses several issues. We have studied the role of GPIbα and ApoER2' in platelet activation by dimeric β2-GPI under conditions of flow. To discern whether platelet activation by dimeric β2-GPI is caused by either GPIbα or ApoER2', we have subsequently studied the signaling processes originating from GPIbα and ApoER2' by studying the interaction of either receptor with 14-3-3ζ and Dab1 respectively. Both signaling molecules have been shown to be of crucial importance in the generation of intracellular signaling upon binding of relevant ligands [7;8]

Methods

Reagents:
Monoclonal anti-ApoER2 antibody MP4-3 was produced in our own laboratory by immunization of mice with the peptide WRCDEDDDCLDHSDED. Fibronectin was purchased from Sigma (Zwijndrecht, the Netherlands). Goat anti-Dab1 antibody was purchased from Santa Cruz (Santa Cruz, California). Mouse anti-phosphotyrosine antibody (4G10) and rabbit anti-14-3-3ζ antibody were purchased from Bio-connect (Huissen, the Netherlands). RAP was produced as described elsewhere [9].

Cloning and expression of dimeric β2-GPI.
Recombinant dimeric β2-GPI was constructed and purified as described previously [10]. In short, the sequence encoding the mature β2-GPI protein was amplified with the

primers β2-GPI-*Xho*I (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and β2-GPI-*Xba*I (GC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG A) by PCR. To construct chimerical fusion proteins of the dimerization domain of factor XI (apple4) and β2-GPI the PCR product was cloned with *Xho*I and *Xba*I into the vector zPL7-apple4-tissue-type plasminogen activator (tPA)-S478A [11] replacing tPA. The dimeric β2-GPI construct was then transfected into BHK cells and stable transfectants were selected in MTX containing medium. Dimeric β2-GPI was affinity purified using monoclonal anti β2-GPI antibody 21B2 and further purified using ion exchange (MonoQ, Amersham).

Cloning, expression and purification of LDL-binding domain 1 of ApoER2':
Two PCR's were performed which were combined by a third PCR afterward. PCR 1: LDL-binding domain 1 was amplified using primer 1: shApoER2'-BD1 *Bam*HI Forward TAA TAG GAT CCG GGC CGG CCA AGG ATT GCG and primer2: shApoER2' ex2 overlap ex7 reverse primer GTT GTG CAG ACA CTC GTT CAG CCT GGG GCA GTC GTC CTC GTC GCG CTG TG. The EGF domains were amplified with PCR 2 using primer3: shApoER2' ex7 overlap ex2 forward CAC AGC GAC GAG GAC GAC TGC CCC AGG CTG AAC GAG TGT CTG CAC AAC and primer4: shApoER2'+EGF *Not*I reverse TAA TAG CGG CCG CCT TGC AGT TCT TGG TCA GTA GGT CC. Both PCR products were then combined with PCR 3 using primers 1 and primer 4 to obtain a construct containing the LDL-binding domain 1 fused to the EGF domains of ApoER2'. Purified protein was analyzed by SDS-PAGE. Only 1 band was observed at 25kD.

Cloning, expression, and purification of soluble GPIbα:
Recombinant human soluble GPIbα (sGPIbα) was cloned and expressed as previously described [12]. Purified protein was analyzed by SDS-PAGE. Only 1 band was observed at 35kD.

Platelet and red blood cell preparation:
Reconstituted blood and washed platelets were prepared as described by Weeterings et al. [13]. In short, blood was centrifuged at 200g for 15 minutes. Platelet rich plasma was acidified and spun again at 500g for 15 minutes. Platelets were washed with Hepes Tyrode buffered saline (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$) with 0.1% glucose pH 6.5 and again spun at 500g for 15 minutes. Platelets were resuspended in 1/10 volume of Hepes Tyrode buffered saline and 0.1% glucose. Platelet count was set at 220.000/μl with Hepes Tyrode buffered saline with 0.1% glucose pH 7.35 for activation and signal transduction experiments. Platelet count was set at 330.000/μl with Hepes Tyrode buffered saline with 0.1% glucose pH=7.35 and 4% human serum albumine (HAS) for perfusion studies with reconstituted blood. Red blood cells were washed three times with physiological salt solution (150 mM NaCl).

Perfusion studies:
Perfusion experiments were performed in a single pass triplo perfusion chamber consisting of a silicon sheet gasket which maintained a flow path height of 0.125 mm and width of 2 mm [14;15]. To test inhibitory capacity of different proteins, proteins were added to platelets and mixed carefully to avoid activation, followed by addition of the red

blood cells. Reconstituted, unmixed blood was incubated for 5 minutes at 37°C. Reconstituted blood was then mixed gently to homogeneity and used directly for perfusions. RAP, sGPIbα and sBD1-ApoER2 were added at 50 μg/ml, 65 μg/ml and 125 μg/ml respectively. Reconstituted blood was perfused over coated cover slips for 5 minutes at a shear rate of 300 s^{-1} using a infusion pump (pump 22, model 2400-004; Harvard, Natrick, MA). Afterward, the cover slips were removed 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 [16]. 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. Perfusions were done with blood with 3 different donors and for every donor three independent flow experiments were performed. Evaluation of platelet adhesion was performed on 20 fields and measured at 5 different positions spaced by 1mm starting at a distance of 5 mm from the blood inlet. Analysis was performed perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion to dimeric β2-GPI. Results are expressed as mean relative coverage (mean ± SEM, n = 3). Statistical analysis was performed using the Mann-Whitney test for non-parametric correlation.

Coating of coverslips:
Glass cover slips (Menzel-Galzer 40x50) were cleaned over-night in 80% ethanol, rinsed with distilled water and dried before coating. Coating was done by incubating cover slips back to back, overnight with 400 μl of Fibronectin (100 μg/ml) o/n at 4^0C. Cover slips were then blocked in 1% HAS at room temperature for 1 hour.

Translocation of GPIbα and ApoER2':
500 μl aliquots of washed platelets (220.000/μL) resuspended in Hepes/Tyrode buffer pH 7.35 were incubated with TBS or dimeric β2-GPI at 50 μg/ml for 0.5, 1, 3, 5 or 10 minutes at 37^0C in the presence or absence of inhibitors. Pre-incubation of TBS or dimeric β2-GPI with inhibitors was done for 5 minutes at 37°C. Soluble GPIbα, RAP and soluble BD1-ApoER2 were used at 65 μg/ml, 50 μg/ml and 125 μg/ml, respectively. Platelets were incubated with premixed TBS or dimeric β2-GPI in the presence or absence of inhibitors for 1 to 5 minutes while stirring and lysed by adding 1/10th volume of 10 times Triton lysis buffer (10% Triton-100, 200mM Tris, 50mM EGTA and EDTA free proteinase inhibitor cocktail tablets according to manufactures protocol (Sigma)). Cytoskeleton fractions was spun down at 13.000g for 30 minutes and washed twice with Triton lysis buffer and supernatant was used as the cytosolic fraction. The cytoskeleton fractions were then resuspended in 500 μl RIPA buffer (PBS with 1% Nonidet P40, 0.5% octylglucoside, 0.1% SDS, 0.186% EDTA 1mM $NaVO_3$ and EDTA free protease inhibitor cocktail according to manufacturers protocol (Sigma)). Polyclonal anti-14-3-3-ζ antibody or polyclonal anti-Dab1 antibody was used at 1μg/ml to immuno-precipitate GPIbα or ApoER2 in combination with 50ml of 10% protein G-Sepharose slurry (Amersham Biosciences) and incubated for 18 h at 4 °C. Sepharose beads were then washed

three times with lysis buffer, resuspended in 40µl reducing Laemmli sample buffer (0.001% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM Tris, pH 6.8 and 0.5% β-mercaptoethanol), and boiled for 5 min. Samples were separated on a 8% SDS-polyacrylamide gel and transferred onto an Immobilon-P polyvinylidene difluoride membrane. Blots were blocked with Tris buffered saline (25mM Tris, 150 mM NaCl) with 0.1% (v/v) Tween 20 (TBST) containing 2% (w/v) BSA (Sigma) for 1 h at room temperature. The blots were then incubated with mouse anti-GPIbα antibody (6.30, 0.5µg/ml), mouse anti-ApoER2 antibody (MP4-3, 1 µg/ml) or mouse anti-phosphotyrosine antibody (4G10, 0.5 µg/ml) overnight in TBST with 1% BSA and washed three times with TBST. After incubation with peroxidase-labeled rabbit anti-mouse antibodies for 1 h at RT (1:2500; Dako, Glostrup, Denmark) blots were washed again with TBST and developed with enhanced chemiluminescence's reagent plus (Perkin Elmer Life Sciences, Boston USA).

Results:

We have studied the role of the receptors GPIbα and ApoER2' on dimeric β2-GPI induced platelet activation under conditions of flow at a shear rate of 300^{-s} for a time period of 90 seconds. As GPIbα is a key player in platelet adhesion to endothelial cell matrix proteins such as vWF and collagen we have chosen fibronectin as a surface, adhesion to which is mostly dependent on the $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins. As vWF, and therefore GPIbα, contributes to platelet adhesion under conditions of flow to a fibronectin surface in whole blood [17], we performed all experiments in the absence of vWF. Stimulation of platelets with dimeric β2-GPI resulted in increased platelet adhesion compared to the buffer control which was set at 100% (relative coverage 152 ± 33%, $p<0.05$) (Figure 1). Blocking the binding site on dimeric β2-GPI for GPIbα by competition with soluble GPIbα completely abrogated the increased adhesion due to the presence of dimeric β2-GPI (relative platelet coverage 75 ± 10%). Likewise, competition with the soluble first LDL-binding domain of ApoER2 (sBD1-ApoER2) also completely inhibited the effect of dimeric β2-GPI (relative platelet coverage 93 ± 15%). Blockage of the binding site on ApoER2' for dimeric β2-GPI by the addition of Receptor Associated Protein (RAP), an inhibitor of ligand binding to members of the LDL-receptor family, completely blocked the effect of dimeric β2-GPI as well (relative coverage 78 ± 8%). Addition of sGPIbα, sBD1-ApoER2 or RAP in the absence of dimeric β2-GPI had no effect on platelet adhesion to fibronectin.

As the experiments mentioned above do not exclude a role for either receptor as a docking site, we have studied the effect of dimeric β2-GPI on intracellular signaling. In neurons, binding of the extracellular matrix protein reelin to ApoER2 leads to signaling via the adapter molecule Dab1, which interacts with the non-phosphorylated intracellular NPxY motif of ApoER2 via its phosphotyrosine binding (PTB) domain [18]. Stimulation of platelets with dimeric β2-GPI leads to a transient dissociation of ApoER2' from cytoskeleton bound Dab1, with maximal dissociation occurring after 30 seconds (Figure 2A). Since Dab1 does not bind to phosphorylated NPxY motifs, these results suggest phosphorylation of ApoER2'. Indeed, immune precipitation experiments with an anti-

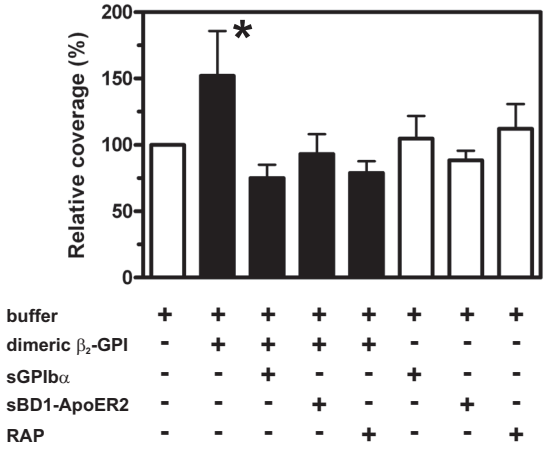

Figure 1. **Both GPIbα and ApoER2' are required for the increase in platelet adhesion to fibronectin caused by dimeric β2-GPI.** Reconstituted blood was perfused over a fibronectin surface at a shear rate of 300-s for 90 seconds. The buffer control was set at 100 %. Data is depicted as mean ± SEM and represents at least three experiments. * $p < 0.05$

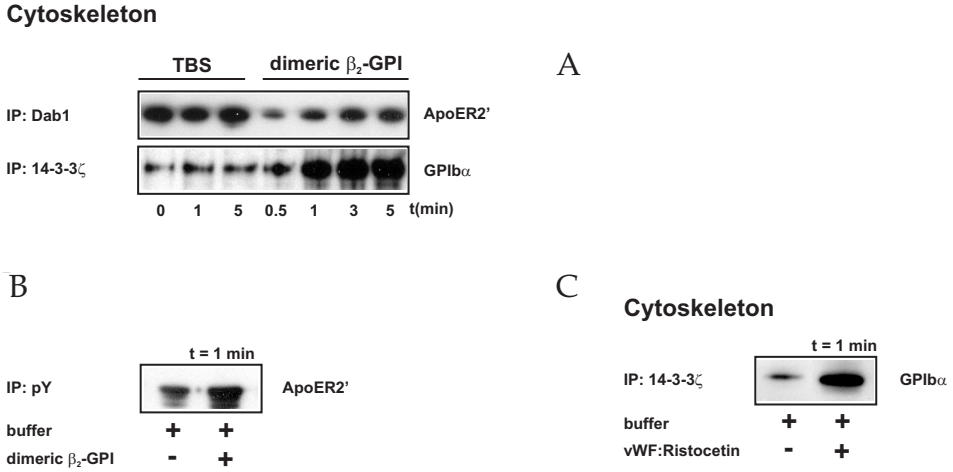

Figure 2. **Dimeric β2-GPI induces signaling via both GPIbα and ApoER2'.** A Upon stimulation with dimeric β2-GPI, ApoER2' dissociates from the cytoskeleton associated adapter molecule Disabled 1 (Dab1) in a time dependent manner due to phosphorylation of ApoER2'. Concomitantly stimulation of platelets with dimeric β2-GPI leads to the association of GPIbα with the cytoskeleton via the scaffold protein 14-3-3ζ in a time dependent manner. B ApoER2' is tyrosine phosphorylated upon stimulation with dimeric β2-GPI. C Stimulation of platelets with ristocetin activated vWF causes association of GPIbα with the cytoskeleton via 14-3-3ζ. Data shown is representative for at least three experiments.

phosphotysine antibody show phosphorylation of ApoER2' upon stimulation with dimeric β2-GPI (Figure 2B). Platelet activation with ristocetin activated vWF has been shown to result in translocation of GPIbα to the cytoskeleton via the adapter molecule 14-3-3ζ (Figure 2C)[7]. In fact, inhibition of the ability of GPIbα to interact with 14-3-3ζ completely abrogates ristocetin-activated vWF induced platelet aggregation [19].

As shown in figure 2A we have observed a time dependent association of GPIbα with the cytoskeleton upon stimulation with dimeric β2-GPI, suggesting a role for GPIbα in signaling by dimeric β2-GPI.

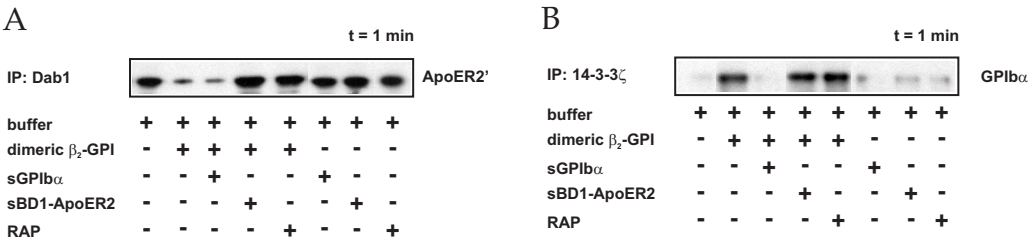

Figure 3. **Dimeric β2-GPI induces two independent signaling pathways in platelets.** A The dissociation of ApoER2' from Dab1 induced by dimeric β2-GPI could be inhibited by competition with either sBD1-ApoER2 or RAP, whereas competition with sGPIbα could not inhibit dissociation. B Likewise, GPIbα association with the cytoskeleton induced by dimeric β2-GPI could be inhibited by competition with sGPIbα, whereas neither sBD1-ApoER2 nor RAP had an effect. Data shown is representative for at least three experiments.

GPIbα and ApoER2'have been reported to be in a complex on the platelet surface [5]. In this context, one could argue that signaling induced by association of dimeric β2-GPI with one receptor could passively induce signaling via the other. To exclude this possibility we have performed inhibition studies on ApoER2'dissociation from Dab1 and GPIbα association with the cytoskeleton (Figure 3). All experiments were performed at t = 1 minute. Upon competition for the binding site on dimeric β2-GPI for GPIbα by pre-incubating dimeric β2-GPI with sGPIbα we could completely block GPIbα translocation to the cytoskeleton (Figure 3B), although it had no effect on the dissociation of ApoER2' from Dab1 (Figure 3A). Likewise, addition of sBD1-ApoER2 fully inhibited dissociation of ApoER2' from Dab1, whereas no effect on GPIbα translocation was observed. Addition of RAP to platelets prior to stimulation with dimeric β2-GPI fully inhibited ApoER2' dissociation from Dab1 and had no effect on GPIbα translocation. Neither sGPIbα, sBD1-ApoER2 nor RAP alone had an effect on GPIbα translocation or ApoER2' dissociation.

Discussion

Dimerization of the plasma protein β2-GPI by patient auto-antibodies is thought to be the trigger for the thrombo-embolic manifestations of the antiphospolipid syndrome. The dimerization of β2-GPI causes a conformational change in the molecule, allowing it to bind to cells and cellular receptors with high affinity. Previous studies have identified the platelet receptors ApoER2' [3] and GPIbα [5;6] as binding sites for dimeric β2-GPI on platelets. The interaction of dimeric β2-GPI with these receptors was shown to be mediated by domain V of β2-GPI [4-6] , which also contains the phospholipid binding site. Although the importance of ApoER2' in platelet activation by dimeric β2-GPI has been elucidated by our group in the past [3], the role of GPIbα remains unclear. We have studied the roles of both GPIbα and ApoER2' in platelet activation induced by dimeric β2-GPI. To be able to distinguish between effects mediated by the association of dimeric β2-GPI with ApoER2' and those through an interaction with GPIbα, we have studied the

GPIbα-independent adhesion of platelets under conditions of flow. As the adhesive receptor GPIbα is a key player in platelet adhesion to collagen and vWF, we have chosen for fibronectin as an adhesive surface, adhesion to which is mostly dependent on the integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ [17]. In this model, dimeric β2-GPI greatly increased the platelet adhesion to fibronectin compared to a buffer control, an effect that was abolished by competing with soluble variants of both GPIbα and ApoER2' as binding sites for dimeric β2-GPI. As has been described for the effect of dimeric β2-GPI on adhesion to collagen [3], the addition of RAP completely blocked the observed increase in platelet adhesion as well. Although these experiments indicated an involvement of both GPIbα and ApoER2' in platelet activation by dimeric β2-GPI, one might speculate that either receptor may play a role as a docking site for dimeric β2-GPI, whereas only one is involved in intracellular signaling. In order to test this hypothesis we have studied the effects of dimeric β2-GPI on signal transduction via both ApoER2' and GPIbα. Vega-Ostertag et al. [20] have reported the phosphorylation of $P38^{MAPK}$ in platelets upon stimulation with anti-β2-GPI antibodies. $P38^{MAPK}$ however, has been reported as a downstream mediator of signaling via both GPIbα [21] and ApoER2' [22]. Shi et al. [6] have described effects of dimerized β2-GPI on the activation of the phoshatidylinositol-3-kinase (PI-3 kinase) pathway in platelets and attribute this effect to an interaction with GPIbα. Activation of the PI-3 kinase pathway however, has also been shown to occur upon the interaction of Reelin with ApoER2 in neurons [23]. It would therefore be feasible to at least partly attribute this effect to the interaction of dimerized β2-GPI with ApoER2'. We have therefore chosen to look at signaling molecules upstream in the signaling cascade, focusing instead on the association of ApoER2' with the adapter molecule Dab1 and of GPIbα with the scaffold protein 14-3-3ζ. Both Dab1 and 14-3-3ζ have been shown to be of crucial importance in the signaling pathways of reelin in neuronal cells [8] and vWF in platelets [7;19], respectively. Stimulation of platelets with dimeric β2-GPI caused a transient dissociation of ApoER2' from Dab1, which coincided with phosphorylation of ApoER2'. As Dab1 has been reported to be unable to bind to phosphorylated NPxY motifs via its PTB domain [24], these results suggest that the interaction between dimeric β2-GPI and ApoER2 results in phosphorylation of the NPxY motif of ApoER2'. Which process is responsible for the phosphorylation of ApoER2' remains to be elucidated, as ApoER2' is not reported to be associated with tyrosine kinases. Concomitant with Dab1 dissociation, GPIbα associated with the cytoskeleton via the scaffold protein 14-3-3ζ, implying signaling via GPIbα as well. As GPIbα and ApoER2' have been reported to be in a complex on the platelet surface [5], one might envision a signaling process similar to the GPVI-FcγRIIa axis, in which binding of collagen to GPVI leads to signaling through the FcR γ chain. To be able to differentiate between direct and indirect effects of dimeric β2-GPI on signaling via either ApoER2' or GPIbα, we have performed competition studies with soluble GPIbα, soluble BD1-ApoER2 and with RAP. Interestingly, signaling via ApoER2' could only be inhibited by competition with sBD1-ApoER2 and RAP, whereas Dab1 dissociation could not be inhibited by the addition of sGPIbα. Similarly, only the addition of sGPIbα could inhibit GPIbα translocation. Neither sBD1-ApoER2 nor Rap had an effect. Thus, both GPIbα and ApoER2' elicit intracellular signaling independent of the interaction of dimeric β2-GPI with the other. Interestingly, as blockage of the interaction between dimeric β2-GPI and ApoER2' with the soluble first LDL-binding domain of

ApoER2 could not prevent the translocation of GPIbα, these results also suggest different binding sites on dimeric β2-GPI for ApoER2′ and GPIbα. We propose a model for platelet activation in the antiphospholipid syndrome in which both GPIbα and ApoER2′ are of equal importance in the transmission of an activation signal (Figure 4). Upon binding of patient antibodies to the first sushi domain of β2-GPI, the molecule is dimerized [25]. Dimerized β2-GPI subsequently binds to both GPIbα and ApoER2′ via two different epitopes on its fifth domain. Signaling ensues via the adapter proteins Dab1 and 14-3-3ζ, associated with ApoER2′ and GPIbα, respectively. As neither process is enough to sensitize platelets for adhesive surfaces such as fibronectin or collagen, it is highly likely both processes join together in a common downstream pathway, lowering the threshold for platelet activation. The effectors of this common pathway remain to be elucidated. In conclusion, we have shown that platelet activation by dimeric β2-GPI is mediated by both GPIbα and ApoER2′. Binding of dimeric β2-GPI to each of these receptors elicits distinct independent signaling pathways, both of which are required for platelet activation. Insight in the mechanism by which dimerized β2-GPI causes a prothrombotic platelet phenotype might provide clues for future anti-thrombotic therapies.

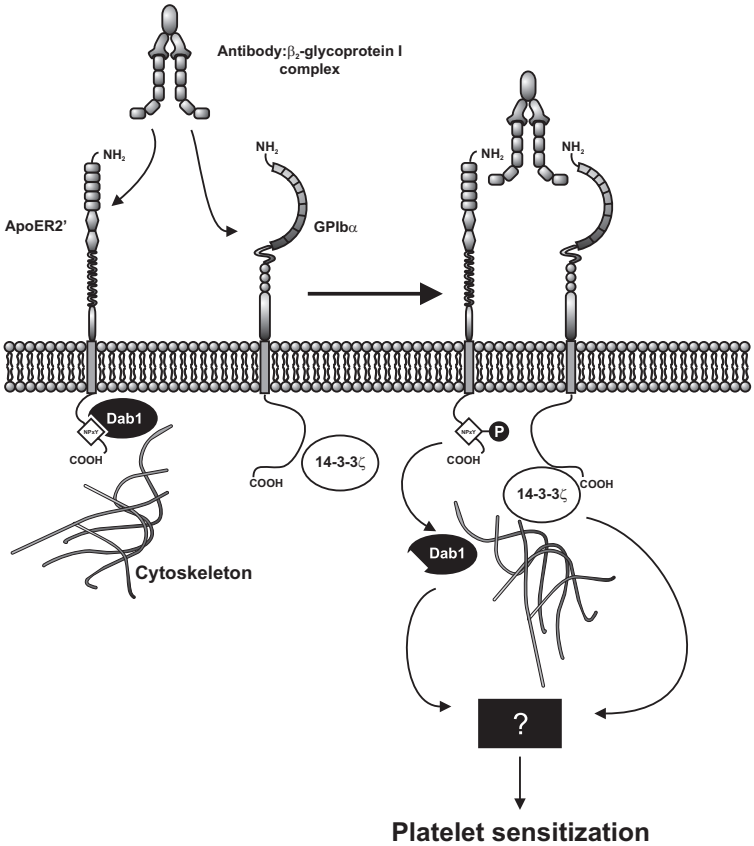

Figure 4. **Schematic representation of the signaling processes in a platelet upon stimulation with dimeric β2-GPI.**

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7

General Discussion

The antiphospholipid syndrome

Classification
The association between the persistent presence of antiphospholipid antibodies in plasma and thrombotic complications and/or specific problems with pregnancy outcome in patients has been termed the antiphospholipid syndrome. The precise criteria defining the antiphospholipid syndrome are described by Miyakis *et al.* [1] and summarized in table 1.

Clinical Criteria (At least one criterion has to be fulfilled)
1: Vascular Thrombosis. One or more confirmed clinical episodes of arterial, venous or small vessel thrombosis in any tissue or organ. No evidence of inflammation should be present.
2: Pregnancy morbidity. i: One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation ii: One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: 1: Eclampsia or severe pre-eclampsia or 2: recognized features of placental insufficiency. iii: Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation without maternal anatomic or hormonal or paternal and maternal chromosomal abnormalities
Laboratory Criteria (At least one criterion has to be fulfilled)
1: Lupus anticoagulant (LAC) present in plasma on two or more occasions at least 12 weeks apart.
2: Anticardiolipin (aCL) antibodies of the IgG and/or IgM isotype in serum or plasma on two or more occasions at least 12 weeks apart.
3: Anti β2-GPI of the IgG and/or IgM isotype in serum or plasma on two or more occasions at least 12 weeks apart.

Table 1: **Classification criteria for the Antiphospholipid Syndrome**

It is now generally accepted that antiphospholipid antibodies are not directed against phospholipids but to phospholipid binding proteins such as β2-GPI and we now accept that antibodies directed against β2-GPI are clinically the most relevant "antiphospholipid antibodies". The precise mechanisms by which anti β2-GPI antibodies are involved in the development of clinical symptoms in patients are still unclear.

Secondary haemostasis: Coagulation
The co-existence of a prolonged clotting time in-vitro and the occurrence of thrombotic events in-vivo defines the first paradox in the antiphospholipid syndrome. In general, the presence of a prolonged clotting time is predictive for a bleeding tendency and not for the occurrence of thrombosis. Auto-antibody binding to β2-GPI results in dimerization of β2-GPI and this greatly increases its affinity for anionic phospholipids [2]. This in-

creased affinity for anionic phospholipids allows dimeric β2-GPI to compete with coagulation factors for binding to catalytic phospholipid surfaces [3]. Competition for binding to a catalytic surface causes a prolongation of the clotting time, which can be corrected by the addition of extra phospholipids. Prolongation of the clotting time that can be corrected by the addition of extra phospholipids is known as lupus anticoagulant (LAC). Although competition for binding to catalytic phospholipid surface with clotting factors can explain the laboratory phenomenon of LAC, it does not explain the occurrence of thrombotic complications in APS patients. In this thesis, a hypothesis has been described that could link the changed properties of β2-GPI with an increased risk of thrombo-embolic complications

Primary haemostasis: Blood platelets
The second paradox in the antiphospholipid syndrome is the occurrence of thrombotic complications in patients, often accompanied by a prolongation of the bleeding time [4]. A prolonged bleeding time is generally considered to be an indication of impaired primary haemostasis and suggests a bleeding tendency rather then predict thrombotic complications. In a population of APS patients studied by Urbanus *et al.* [4], no differences in platelet number and function were observed that could possibly explain the prolongation of the bleeding time. This observation suggests the prolongation of the bleeding time is not due to impaired platelet function but e.a. by increased anti-platelet activity of endothelial cells. Possibly, the prolonged bleeding time is an expression of endothelial cell activition resulting in increased nitric oxide (NO) production, a powerful inhibitor of platelet function.

Primary haemostasis and the formation of arterial thrombosis are generally considered to be platelet driven processes. Furthermore, we have previously shown that anti-β2-GPI antibodies and recombinant dimeric β2-GPI influence platelet function by increasing platelet sensitivity for stimuli such as adhesion to collagen and fibronectin under conditions of flow [5]. Other groups also describe activation of platelets by anti-phospholipid antibodies [6-10]. We therefore assume that platelets do play an important role in the development of thrombotic complications in patients with APS. Furthermore, studies on the interaction of blood platelets with β2-GPI-anti-β2-GPI antibody complexes could be useful as a model to study the influence of antiphospholipid antibodies on cell function in general and thus could provide important insights into the activation of other cell types, such endothelial cells, monocytes and other cells that are thought to be involved in the development of clinical symptoms associated with APS [11-18]. Understanding the role of blood platelets in the development of thrombotic complications in APS patients is the main topic of the research described in this thesis.

The existence of a platelet receptor for dimerized β2-GPI
Previous studies from our group clearly demonstrated that dimerization of β2-GPI by auto antibodies results in a pro-thrombotic phenotype of platelets [5]. Upon dimerization of β2-GPI, its affinity for catalytic anionic phospholipids such as those present on activated blood platelets increases significantly [2;19]. It is however unlikely that simple binding of dimerized β2-GPI to platelet membranes results in activation. We have therefore

postulated the existence of a platelet membrane receptor that can bind β2-GPI only when dimerized. Binding of dimerized β2-GPI to platelet receptors might then leads to activation or sensitization of platelets, resulting in an increased response to second stimuli: The so-called "second hit hypothesis". Two research groups have been able to demonstrate the presence of activated platelets in the circulation of APS patients [20;21].

To support the theory of the existence of a platelet receptor for dimerized β2-GPI, we constructed a recombinant chimeric β2-GPI combining the dimerization domain apple4 of FXI [22] with β2-GPI. Functional testing of this dimeric β2-GPI construct showed that 1: The addition of recombinant dimeric β2-GPI (apple4-β2-GPI) to plasma prolonged the clotting time similar to some anti-β2-GPI antibodies and this prolongation could be corrected by the addition of extra phospholipids [23] and 2: Platelet adhesion to collagen under conditions of flow was increased upon incubation of whole blood with dimeric β2-GPI, similar to the effects seen after of incubation of whole blood with some anti β2-GPI antibodies [5]. These data demonstrated that the effect of recombinant dimeric β2-GPI mimics β2-GPI-anti-β2-GPI-antibody complexes.

Identification of the first platelet receptor for dimeric β2-GPI

The first lead into identification of a platelet receptor for dimeric β2-GPI came when our group found that increased platelet adhesion to collagen in perfusion studies, induced by either anti-β2-GPI antibodies or dimeric β2-GPI, could be reversed by the addition of RAP (Receptor Associated Protein) to blood. RAP is a general inhibitor of ligand binding to members of the LDL-receptor family. Sofar, the only member of the LDL-receptor family shown to be present on blood platelets is a truncated form of apolipoprotein E receptor 2 (ApoER2) called ApoER2' or ApoER2Δ5. To investigate the interaction between dimeric β2-GPI and ApoER2', we cloned and expressed soluble ApoER2Δ5 (shAPoER2Δ5) from four cell types of megakaryocytic origin. Soluble ApoER2Δ5 was used to confirm the direct interaction between dimeric β2-GPI and ApoER2Δ5 observed by Lutters *et al.* [5]. Solid phase binding studies showed the affinity of ApoER2Δ5 for dimeric β2-GPI to be 23 ± 4 nM. Dimeric β2-GPI interacts with shApoER2Δ5 via a specific cationic patch in domain V (KNKEKK, aa282-287). The binding site for ApoERΔ5 did partly overlap with the phospholipid binding site on domain V [24]. ApoER2 binding of dimeric β2-GPI did require the KNKEKK peptide but not the hydrophobic phospholipid insertion loop present in domain V. We furthermore identified two new splice variants of ApoER2 in megakaryocytes, ApoER2Δ4-5 and ApoER2Δ4-5-6. When expression levels of ApoER2 splice variants on circulating platelets were studied, ApoER2Δ4-5 appeared to be the most abundantly expressed splice variant. This expression profile did not confirm data published by the group of Riddell [25]. They described platelets express a splice variant op ApoER2 lacking exon 5 with an apparent molecular weight of approximately 120kD (ApoER2Δ5). In our study we describe a smaller splice variant of ApoER2 (presumably ApoER2Δ4-5) expressed predominantly on platelets. We did, however, detect expression of mRNA coding for ApoER2Δ5 and expression of a splice variant of ApoER2 with an apparent molecular weight of 120kD. This ApoER2Δ5 splice variant was only expressed at low levels. Using domain deletion mutants of ApoER2Δ5, the binding site for dimeric β2-GPI was localized on LDL binding domain 1. This LDL-binding domain 1 is present in all three iso-

lated splice variants and in all three splice variants expressed by platelets. In this study we only analyzed the ligand binding region of ApoER2 splice variants expressed on platelets and megakaryocytes. We did not study differences in splicing variations in the stack, transmembrane domain and the intracellular portion of ApoER2. Therefore, distribution and expression of functional splice variants of ApoER2 remains unclear. For further discussions, functional splice variants of ApoER2 on platelets will be referred to as ApoER2′ unless stated otherwise.

Identification of the second platelet receptors for dimeric β2-GPI

When studying platelet adhesion to dimeric β2-GPI under conditions of flow, we found platelets adhere to dimerized β2-GPI under flow conditions representing both arterial and venous shear stresses. In these adhesion studies we found that platelet adhesion to dimeric β2-GPI under conditions of flow was not only inhibited with soluble ApoER2Δ5 and RAP, but also by adding soluble GPIbα and after proteolytic removal of GPIbα from the platelet surface using the snake venom NK. A direct interaction between GPIbα and dimeric β2-GPI was shown with surface plasmon resonance and immuno-precipitation experiments. The affinity of dimeric β2-GPI for GPIbα was 180 ± 20 nM. Binding of dimeric β2-GPI to GPIbα showed to be Zn^{2+} and not Ca^{2+} dependent. In clotting experiments we observed that addition of extra Zn^{2+} ions to plasma samples with a β2-GPI dependent LAC further prolonged the clotting time (unpublished data). Coagulation using platelet poor plasma is independent of GPIbα and we therefore assume Zn^{2+} ions influence β2-GPI and not GPIbα. Biochemical analysis using solid phase binding assays, surface plasmon resonance and perfusion experiments identified that domain V of β2-GPI can to bind to GPIbα in close proximity of the thrombin binding site on GPIbα. The thrombin binding site on GPIbα is located within a stretch of 10 amino acids (Asp272-Glu282) and contains three sulfated tyrosine residues [26-29]. Dimeric β2-GPI binding to GPIbα did not interfere with the vWF binding site on GPIbα. In conclusion, we have identified two receptors for dimeric β2-GPI present on platelets namely ApoER2′ and GPIbα. Our next question was whether the interaction of dimeric β2-GPI with both ApoER2′ and GPIbα is required for enhanced reactivity of platelets to second stimuli.

Platelet signaling and activation upon incubation with dimeric β2-GPI

Signaling via ApoER2

When expression of ApoER2 splice variants on platelets was studied, we found that ApoER2Δ4-5 was most abundantly expressed. Upon stimulation of platelets with dimeric β2-GPI, ApoER2Δ4-5 also demonstrated to have the most pronounced signaling capacity by dissociation from the cytoskeleton. Dissociation from the cytoskeleton occurred simultaneously with dissociation from the adaptor protein Dab1. Dab1 is a well studied adaptor molecule in neurons with protein scaffolding and signaling properties downstream of ApoER2, VLDL-R, megalin and LRP1 [30;31]. We demonstrate ApoER2′ phosphorylation and subsequent dissociation of Dab-1 from ApoER2′. Phosphorylation of the NPxY motif in the intracellular region of ApoER2′ is known to induce dissociation of Dab-1 due to the inability of Dab-1 to bind to phosphorylated NPxY binding motifs [31]. Phosphorylation of ApoER2′ and Dab-1 and signaling events downstream of ApoER2′ have been investigated further.

Why platelets express three splice variants of ApoER2 remains unclear. It has however been shown that alternative splicing in the binding region of ApoER2 is known to influence ligand binding properties [32] and proteolytic processing [33]. Dimeric β2-GPI binds to ApoER2′ via LDL-binding domain I, which is present in all three splice variants isolated. No differences were observed in dimeric β2-GPI binding to the three recombinant soluble splice variants of ApoER2. We have shown that LDL-binding domain 1 is essential for binding to dimeric β2-GPI, we have not studied the possibility that the presence of other domains influence the affinity of the interaction of ApoER2′ with dimeric β2-GPI. Also, in this study we have not analyzed possible splicing in the protein stack, transmembrane domain and intracellular domain of ApoER2 splice variants and therefore can not exclude differences in these regions.

Signaling via GPIbα

GPIbα signaling induced by binding of "activated" vWF (vWF/ristocetin) or thrombin to GPIbα induces cellular redistribution of GPIbα [34]. When platelets are activated with vWF/ristocetin, the percentage of GPIb± in lipid rafts increased three to six fold [35]. Initial translocation of GPIbα to rafts or glycoprotein enriched micro domains (GEMs) occurs via the adaptor molecule 14-3-3-ζ [36;37]. Lipid rafts are highly organized cholesterol rich lipid micro-domains and are known to localize and concentrate signaling molecules. Disruption of rafts by depletion of cholesterol inhibited vWF/ristocetin induced aggregation and platelet adhesion to vWF under conditions of flow [34]. Rafts thus play an important role in vWF induced GPIbα dependent platelet activation. Translocation of GPIbα to rafts results in co-localization with FcγRIIA and recruitment of several signaling molecules such as Src, Syk and PLCγ [38]. Close association of GPIbα and FcγRIIA in rafts results in FcRγ-chain phosphorylation [35]. When GPIbα signaling induced by dimeric β2-GPI was studied, we found GPIbα translocates to the cytoskeleton similar to GPIbα translocation induced by vWF or thrombin. This translocation was depending on the association of 14-3-3-ζ with both the cytoskeleton and GPIbα.

Both ApoER2′ and GPIbα transmit a signal from outside the platelet across the platelet membrane upon binding of dimeric β2-GPI. We therefore asked ourselves the question whether dimeric β2-GPI induced signaling via ApoER2′ and GPIbα are both required for platelet activation.

Interaction between GPIbα and ApoER2′ signaling

In our studies on the adhesion of platelets to dimeric β2-GPI under conditions of flow we found that blocking of either ApoER2′ or GPIbα results in complete inhibition of platelet adhesion to dimeric β2-GPI. We also studied signaling capabilities of ApoER2′ and GPIbα in the presence of agents that block dimeric β2-GPI binding to either receptor. GPIbα translocation to the cytoskeleton could be inhibited with soluble GPIbα and NK treatment of platelets but not with soluble ApoER2Δ5 or RAP. ApoER2′ dissociation from Dab-1 was inhibited by adding soluble ApoER2Δ5 and RAP but addition of GPIbα and NK treatment did not inhibit ApoER2 translocation. These findings indicate that addition of soluble GPIbα receptors thus does not interfere with dimeric β2-GPI binding to ApoER2′ and soluble ApoER2Δ5 does not interfere with dimeric β2-GPI binding to

GPIbα. It is therefore likely that the binding sites for ApoER2 and GPIbα on dimeric β2-GPI do not overlap. On human blood platelets, the levels of expression of GPIbα and ApoER2′ on human blood platelets differ greatly with GPIbα having a copy number of approximately 30.000 per platelet whereas ApoER2′ is only expressed at approximately 5000 copies per platelet. We observed a major difference in affinity for dimeric β2-GPI by GPIbα and ApoER2′. Despite its relatively low affinity for dimeric β2-GPI, GPIbα was involved in adhesion to dimeric β2-GPI under venous and arterial shear stresses. The large amount of GPIbα expressed on the platelet surface perhaps compensates for the relatively low affinity for dimeric β2-GPI.

Incubation of platelets with dimeric β2-GPI induces independent signaling pathways via GPIbα and ApoER2′. However, adhesion of platelets to dimeric β2-GPI could be inhibited by blocking the interaction of dimeric β2-GPI with only one of these receptors. These observations suggest the existence of a shared pathway downstream of ApoER2′ and GPIbα. Probably, signaling via the two receptors co-operates in the activation of this downstream pathway leading to activation of platelets by dimeric β2-GPI. We therefore conclude that GPIbα and ApoER2′ are functional co-receptors. They both bind dimeric β2-GPI and initiate separate signaling pathways together leading to activation of platelets. To show ApoER2′ and GPIbα are indeed present in a receptor complex on the platelet surface, we performed co-precipitation experiments in which ApoER2′ was immuno-precipitated in the presence or absence of dimeric β2-GPI and cytochalasin D (CytoD). CytoD inhibits actin polymerization and was used to eliminate co-precipitation of GPIbα and ApoER2′ by cytoskeletal association. Samples were analyzed on western blot using an antibody directed against GPIbα. We found GPIbα and ApoER2 are present in a receptor complex independently of the presence of dimeric β2-GPI or an intact cytoskeleton. Co-localization of ApoER2′ and GPIbα was confirmed by scanning immuno-electron microscopy. The role of ApoER2Δ5 in embryonic neuronal development and neuronal regeneration and migration in adult brains is likely to involve cytoskeletal reorganization [39]. A suggested role for ApoER2 signaling in neurons is its involvement in cytoskeletal reorganization by regulating Tau protein phosphorylation[40]. Perhaps, ApoER2′ signaling in platelets by dimeric β2-GPI also results in cytoskeleton re-organization. Preliminary data using confocal microscopy indeed show alterations in the platelet actin cytoskeleton upon incubation with dimeric β2-GPI. In unstimulated platelets, the actin cytoskeleton is mainly present at the edge of the platelet membrane. Stimulation of platelets with dimeric β2-GPI results in an expansion of the actin cytoskeleton towards the centre of the platelets (unpublished data). Whether this reorganization of the platelet actin cytoskeleton is mediated by ApoER2′ signaling requires further investigation. Perhaps cytoskeletal reorganization by ApoER2 signaling facilitates GPIbα/14-3-3-ζ association to the cytoskeleton. In conclusion we have identified GPIbα as a second receptor for dimeric β2-GPI present on platelets by studying platelet adhesion to dimeric β2-GPI under conditions of flow.

Platelet adhesion to surface exposed dimeric β2-GPI

The in-vivo occurrence of a surface exposing dimeric β2-GPI to the blood flow allowing platelets to adhere to is questionable but several possibilities can be thought of. In

this thesis we describe two receptor binding sites for dimeric β2-GPI on platelets. The third binding site for dimerized β2-GPI on platelet is the anionic phospholipid surface which requires platelet activation. Many groups have tried to isolate circulating β2-GPI-anti-β2-GPI antibody immune complexes in APS patients, but such circulating immune complexes have never been demonstrated. This lack of circulating β2-GPI-anti-β2-GPI antibody immune complexes in APS patients led to the assumption that β2-GPI-anti-β2-GPI immune complexes are formed only on phospholipid surfaces. Antibody binding to phospholipid bound β2-GPI furthermore requires the exposure of cryptic epitopes for "pathogenic" anti-β2-GPI antibodies to bind [41-43]. Once a surface bound β2-GPI-anti-β2-GPI antibody immune complex is formed, displacement of this immune complex could lead to binding of dimerized β2-GPI to ApoER2' and GPIbα. The exact sequence of events in the formation of dimerized β2-GPI by auto antibodies and binding to platelets is sofar unknown and can only be speculated on. Two possible mechanisms for a surface bound exposure of dimerized β2-GPI to the blood flow can be thought of. 1: Binding of dimeric β2-GPI to either GPIbα and/or ApoER2' or the catalytic phospholipid surface of adhered platelets results in the exposure of the Fc-tail of the β2-GPI-anti-β2-GPI immune complex to the blood flow. Exposure of the Fc-tail of the immune complex to the blood flow would allow for the interaction with FcγRIIa expressed on platelets in circulation. 2: The β2-GPI-anti-β2-GPI antibody complex formed on the platelet phospholipid surface is transferred to surface exposed FcγRIIA via the Fc-tail of the formed immune complex. Binding to the platelet receptor FcγRIIA via the Fc-tail leaves the two β2-GPI present in the β2-GPI-anti-β2-GPI antibody complex exposed to the blood flow allowing them to interact with either ApoER2' and/or GPIbα expressed on platelets in the circulation. The two mechanisms described above would explain the occurrence of surface exposing dimerized β2-GPI to the blood flow in-vivo involving platelet expressed FcγRIIA in the pathology of APS. A further involvement of the FcγRIIA in APS will be discussed in the next section.

Involvement of the FcγRIIA in platelet activation

As mentioned before, the FcγRIIa is a crucial receptor in transmitting GPIbα signaling after vWF binding. Recent reports also show that ApoER2 localizes in rafts as well as GPIbα and the FcγRIIA [44;45]. This observation localizes 3 compounds that can bind β2-GPI-anti-β2-GPI antibody immune complexes to raft domains namely GPIbα, ApoER2' and FcγRIIA. It is also interesting to note that 1: The clinically relevant antibody correlating with thrombotic complications in APS patient are of the IgG and not IgM isotype and 2: The only receptor for antibodies present on platelets is the FcγRIIA. FcγRIIA is the low affinity receptor for antibodies of the IgG isotype. When antibodies are in complex with antigens or immobilized, the affinity for the FcγIIA increases [46;47]. FcγRIIa expressed by platelets is the largest pools of FcγRIIa in the human body. Polymorphisms in the FcγRIIA on platelet alters the sensitivity for activation by antibodies directed against platelet bound fibrinogen [48]. Studies by Dijstelbloem *et al.* showed the same FcyRIIA-R/R131 polymorphism to prolong the half-life of antibody coated red blood cells in circulation [49]. Although they did not study the FcγRIIA-R/131R polymorphism in APS patients, they found the FcγRIIA-R131R polymorphism to occur more frequently in SLE patients than in healthy controls. In 2003 the group of Karassa studied the FcyRIIA-

R/R131 polymorphism in a APS patients group and found that the homozygous 131R/R polymorphism was enriched in the APS patient group [50]. The role of the FcγRIIa in APS requires further studies in order to elucidate a possible role in adhesion and or signaling events in platelets induced by dimerized β2-GPI.

Increased adhesion to fibronectin by dimeric β2-GPI is mediated by GPIbα and ApoER2'
In order to determine the role of ApoER2' and GPIbα in adhesion to a physiological surface, we performed perfusion experiment over fibronectin using reconstituted blood. Fibronectin was selected since this is an extra cellular matrix (ECM) protein in which GPIbα does not participate in initial adhesion of platelets in reconstituted blood. Increased adhesion to fibronectin after incubation of platelets with dimeric β2-GPI was inhibited by both soluble GPIbα and LDL-binding domain 1 of ApoER2. Addition of RAP and proteolytic cleavage of GPIbα from the platelet surface also fully inhibited the increased adhesion to fibronectin induced by dimeric β2-GPI. This demonstrates that binding of dimeric β2-GPI to both GPIbα and ApoER2' is required to increase fibronectin adhesion.

Preliminary results from our group also show up-regulation of TxA2 synthesis by platelets and increased fibrinogen binding to platelets by stimulation with dimeric β2-GPI. Both TxA2 synthesis and fibrinogen binding are markers of platelet activation and both could be inhibited by the addition of soluble GPIbα and ApoER2' or RAP and proteolytic removal of GPIbα from the platelet surface (unpublished data).

It is not certain that activation of platelet by dimeric β2-GPI is the only cause of thrombotic complications in APS patients. Several other cell types have been studied in relation to APS. Kuwana *et al.* describe the activation of auto-reactive T-cells upon binding of β2-GPI to anionic phospholipids [51]. Other cell types and cellular receptors possibly involved in the development of clinical symptoms associated with APS will be discussed in the next section.

Members of the LDL-receptor family
To investigate the interaction of dimeric β2-GPI and other members of the LDL-receptor family, we tested binding of dimeric β2-GPI to ApoER2Δ5, Megalin (gp330), LRP1, VLDL-receptor and the LDL-receptor. Binding was observed to all members of the LDL-receptor family with LRP1 and megalin having two binding sites having a high affinity (±3 nM) and a low affinity (±200nM) binding site. The affinity of dimeric β2-GPI for ApoER2Δ5 and VLDL-receptor was similar (resp. 23nM and 36nM) and the affinity of dimeric β2-GPI for LDL-receptor was relatively low (341 nM). The LDL-receptor is the only member of the LDL-receptor family without known signaling properties and is considered to be involved in endocytosis only. Due to the lack of signaling capabilities and its relatively low affinity for dimeric β2-GPI, the LDL-receptor is not an important receptor for dimeric β2-GPI. Dimeric β2-GPI bound to all receptors via domain V and no binding was observed with monomeric β2-GPI. Both RAP and heparin were able to inhibit dimeric β2-GPI binding to members of the LDL-receptor family dose dependently. Many other clinical symptoms do correlate with the presence of circulating anti phos-

pholipid antibodies (aPL). These include thrombocytopenia, livedo reticularis, heart valve lesions, hemolytic anemia, epilepsy, myocardial infarction, leg ulcers, and amaurosis fugax [52]. Members of the LDL-receptor family play a major role in metabolic processes such as cholesterol uptake, HDL metabolism but also embryonic neuronal development and haemostatic processes [5;53-59]. One may speculate that the wide spread and diverse distribution of members of the LDL-receptor family in cells and organs might explain the variety of clinical symptoms associated with the presence of aPL antibodies.

For the interaction between dimeric β2-GPI and ApoER2', we identified a specific peptide domain in domain V (aa282-287) of ApoER2' that is responsible for binding of dimeric β2-GPI. Interestingly, Lys(284), Lys(286) and Lys(287) are responsible for heparin binding to β2-GPI [60]. The overlap of the ApoER2Δ5 and heparin binding site indicates that heparin binding to β2-GPI hinders the interaction between dimeric β2-GPI and ApoER2Δ5 by competing for the positively charged KNKEKK region in domain V of β2-GPI.

TLR2 and TLR-4

Endothelial cells are activated upon incubation with anti-β2-GPI antibodies and start the expression of tissue factor (TF) and adhesive molecules such as E-selectin [11;61-63]. Monocytes also start to express TF upon incubation with dimeric β2-GPI [14;15;64]. Several receptors have been implicated in activation of cells. Activation of fibroblast by anti β2-GPI antibodies was mediated by TLR-2 resulting in the upregulation of TF expression and secretion of IL6 [65]. Anti-phospholipid antibody induced activation of endothelial cells possibly is mediated by TLR-4 and Annexin-2 [66-68]. A direct interaction between dimeric β2-GPI and TLR-2 and TLR-4 has not been demonstrated and annexin II is not a transmembrane protein and thus not able to transmit a signal into the cell. Interestingly, both TLR-4 and GPIbα belong to the leucine rich repeat receptor family in which the leucine rich repeats are involved in ligand binding. Besides GPIbα and ApoER2', platelets also express members of the TLR-family [69] but involvement of TLR-4 in APS is mainly described for endothelial cells [68] and not for platelets. We have demonstrated the requirement of the activation of two receptors on platelets for activation. It can be hypothesized that TLR-4 acts as a co-receptors for ApoER2 on endothelial cells, similar to GPIbα on the platelet surface. However, as some reports show GPIbα expression on endothelial cells [70;71], it might be possible that activation of both platelets and endothelial cells occurs via ApoER2 and GPIbα. Sofar, expression of GPIbα on endothelial cells has not been confirmed by our own and other groups and is debatable [72]. Further studies clearly are necessary to obtain more insight into these possibilities.

PSGL-1

Recently, our group identified PSGL-1 on monocytes and polymorphic mononuclear cells (PMN's) as a receptor for dimeric β2-GPI (abstract submitted to ISTH 2007, Geneva, Switserland. Abstract #2365). The receptors PSGL-1 also shows similarities with GPIbα. Both GPIbα and PSGL-1 contain sulfated tyrosine residues which were shown to be important for binding of dimeric β2-GPI to GPIbα. The interaction between PSGL-I and

dimeric β2-GPI was again Zn^{2+} dependent (unpublished data). Furthermore, PSGL-1 is expressed on monocyte derived micro-particles expressing TF. Upon activation, platelets start expressing P-selectin which can bind PSGL-I [73]. Tissue factor bearing micro particles are incorporated in a forming thrombus by the interaction of microparticle PSGL-1 and platelet P-selectin [74]. TF is required for thrombin generation and fibrin formation. A possible additive role for dimeric β2-GPI in capturing PSGL-1 expressing TF bearing microparticle is conceivable. Exposure of dimeric β2-GPI on activated platelets in a forming platelet plug could function as a "receptor" for TF bearing micro-particles. The interaction between dimeric β2-GPI and PSGL-1 could thus contribute to the pro-thrombotic phenotype of platelets mediated by dimeric β2-GPI described in this thesis. Further studies are required to support this possibility.

Interaction of β2-GPI with plasma proteins

Next to the multitude of cellular receptors for dimeric β2-GPI that have been discovered and characterized, an increasing number of "non-receptor" proteins have been described to interact with dimeric β2-GPI or plasma β2-GPI. Some will be discussed with regard to the data presented in this thesis.

vWF
Our laboratory has identified plasma β2-GPI as an inhibitor of vWF mediated platelet aggregation (ristocetin activated vWF). Plasma β2-GPI binds to vWF when the latter molecule is in its GPIbα binding "activated" conformation. Binding of β2-GPI to "active" vWF inhibits binding of vWF to GPIbα. This inhibitor function is lost upon dimerization of β2-GPI by auto-antibodies because dimerized β2-GPI no longer binds to "active" vWF. In the same study, addition of β2-GPI inhibited platelet adhesion to vWF under conditions of flow using reconstituted blood. This inhibition was lost by adding anti-β2-GPI antibodies (unpublished data).

It is interesting to note that the group of Steve Krilis identified the vWF binding domain on GPIbα as the binding site for dimerized β2-GPI [9]. The anti GPIbα antibody (AK2) used in this study to identify the dimeric β2-GPI binding site on GPIbα indeed blocks vWF binding to GPIbα. Binding of dimeric β2-GPI to the vWF binding site on GPIbα then blocks vWF binding to GPIbα. Blocking vWF binding to GPIbα by dimeric β2-GPI more likely induces a bleeding tendency then causing thrombotic complications. Furthermore, Urbanus *et al.* found no differences in vWF induced platelet aggregation by platelets isolated from APS patients [4]. Furthermore, blocking of the vWF binding site on GPIbα by dimeric β2-GPI also contradicts our primary observation that incubation of whole blood with dimeric β2-GPI or anti-β2-GPI antibodies increases platelet adhesion to collagen [5]. Adhesion of platelets to collagen is mainly mediated by collagen bound vWF supporting platelet adhesion via GPIbα.

In this thesis we describe the binding site for dimeric β2-GPI on GPIbα to be in close proximity of the thrombin binding site away from the vWF binding site. We have no explanation for the opposing results found by our group and those by the group of Krilis. Thrombin generation on GPIbα is required for thrombin induced platelet pro-coagu-

lant activity [75]. Dimeric β2-GPI binding to GPIbα possibly inhibits thrombin binding to GPIbα however we speculate that the abundant expression of GPIbα on platelets compensates for this competition and that as a result, thrombin generation is not influenced.

Thrombin

Another plasma protein that has been shown to bind β2-GPI is thrombin [76]. Although our group has not been able to show an interaction between monomeric plasma β2-GPI, recombinant monomeric β2-GPI or dimeric β2-GPI with thrombin, the group of Krilis has described an interaction of plasma β2-GPI with both exosite I and exosite II on thrombin. They found that binding of anti-β2-GPI antibodies to thrombin bound β2-GPI, inhibited FXI activation by thrombin more then 70%. In another study they also describe that binding of β2-GPI to FXI inhibited its activation by thrombin and FXIIa [77]. Interactions described above however, do not contribute to a prothrombotic phenotype found in APS patients. Further investigation into the above interaction and physiological function is required.

oxLDL

The first reports of β2-GPI present on oxidized LDL particles circulating in plasma originate from 1998 and several other groups confirmed or contradicted the existence of oxLDL-β-GPI complexes [78-81]. It is now generally accepted that β2-GPI binds to oxidized LDL-particles and evidence is building that this complex is involved in the development of auto-immune mediated atherosclerosis in APS patients [82-89]. The precise mechanism behind this process in not clear. The general consensus now is that β2-GPI-oxLDL-anti-β2-GPI complexes are more sensitive for uptake by macrophages accelerating the development of foam cells. A recent report contradicts the existence of differences in affinity of aCL antibodies for epitopes on oxidized and non-oxidized molecules [90]. Further studies are required to elucidate the role of anti β2-GPI auto antibodies in the development of atherosclerosis.

Future prospective

This thesis mainly describes the interaction between dimeric β2-GPI and cellular receptors expressed by blood platelets. We identified two receptors that are expressed on platelets and are involved in binding of dimeric β2-GPI. Both GPIbα and ApoER2′ are able to directly interact with dimeric β2-GPI and transmit a signal across the platelet membrane leading to pre-stimulation/activation of platelets. Binding of dimeric β2-GPI induces a sensitized phenotype of platelets with an increased response to second stimuli such as surface exposed collagen, fibronectin or dimeric β2-GPI. Both GPIbα and ApoER2′ transmit a receptor specific signal that could not be inhibited by inhibition of binding of dimeric β2-GPI to the co-receptor. Signaling cascades of GPIbα and ApoER2′ are both required for sensitization/pre-activation of platelets. This demonstrates the requirement of two activating stimuli by dimeric β2-GPI to elicit a pathogenic role. Blocking either one of the receptors reverses the pro-thrombotic phenotype of platelets stimulated by dimeric β2-GPI.

Therapeutic implications

Apart from a better understanding of the role of blood platelets in the development of thrombotic complications in APS patients, this thesis also suggests several alternative therapeutic strategies that could be beneficial for APS patient.

Blocking β2-GPI:
When studying the interaction between dimeric β2-GPI, we identified a positive region (KNKEKK, aa282-287) in domain V of β2-GPI responsible for binding to ApoER2'. We showed in immuno precipitation experiments that addition of this peptide, blocks the interaction between dimeric β2-GPI and ApoER2'. We furthermore showed that blocking of ApoER2' binding of dimeric β2-GPI inhibited the increased platelet adhesion to fibronectin and surface exposed dimeric β2-GPI. The prothrombotic phenotype induced by dimeric β2-GPI can thus be reversed with the KNKEKK peptide suggesting a therapeutic use of drug design following this KNKEKK peptide. We however, do not know the effect of this peptide on functional properties and ligand binding to members of the LDL-receptor on other cell types and tissues.

Blocking ApoER2'
Binding of dimeric β2-GPI to ApoER2 could also be inhibited with LDL-binding domain 1 of ApoER2' and heparin. Heparin would not be a useful long term treatment since heparin induces the development of anti-heparin antibodies. The use of the non-immunogenic low molecular weight heparin (LMWH) as a therapeutic agent seems feasible but further studies are required to characterize the effects of LMWH on dimeric β2-GPI binding to ApoER2'. LMWH also inhibits the formation of thrombin thus having a dual role as both a platelet blocker and as an inhibitor of coagulation.

We identified the LDL-binding domain I of ApoER2' as the binding domain for dimeric β2-GPI. The use of recombinant LDL-binding domain 1 in a therapeutic model would inhibit binding of dimeric β2-GPI to ApoER2' expressed on platelets. The use of recombinant LDL-binding domain I could interfere with binding of critical ligands to members of the LDL receptor family. For instance, reelin signaling via ApoER2' is essential for normal neuronal development and function [91;92]. The effect of heparin on reelin induced ApoER2 signaling is unknown. Interfering in processes such as reelin signaling could have adverse effect and further studies are required to determine the use of recombinant LDL-binding domain I in a therapeutic setting.

Blocking GPIbα
Theoretically, therapeutic use of soluble GPIbα could have adverse effects as it will inhibit primary haemostasis. This could lead to bleeding episodes in patients although studies in baboons have shown no significant risk for bleeding upon blocking of vWF binding to GPIbα [93]. The absence of bleeding complications in baboons treated with anti GPIbα antibodies suggests a possible therapeutic use of soluble GPIbα or antibodies directed against GPIbα in APS.

Signaling of GPIbα plays a role only in vWF adhesion and aggregation. In-vivo, the interaction between vWF and platelets does not occur as a single event. Primary haemostasis is induced by collagen and collagen bound vWF. Platelets have also at least two receptors for collagen, able to induce platelets activation. In the presence of collagen, GPIbα signaling is thought not to play a major role in platelet activation. Blocking of GPIbα signaling in APS patients could offer a therapeutic window blocking platelet activation by dimerized β2-GPI. Theoretically, the use of a peptide that blocks GPIbα signaling could also prove to be a useful therapeutic agent but again further research is required. A peptide that blocks GPIbα signaling has been described [94].

Future challenges in APS research

The main problem in the field of APS research is the inconsistency in assays and readouts used worldwide in laboratories to detect antiphospholipid antibodies. Multi-centre studies are required to select the most specific and sensitive assays for standardization of the diagnosis. Also, to better understand the patho-physiology of the antiphospholipid syndrome, a "true" patient population has to be defined. Since the pathology of APS is not fully understood, defining a "true" patient population has proven to be a major hurdle especially since a number of scientists and medical doctors do not agree with the existing definition of APS.

One of the other main future challenges in APS research is to identify the relevant interactions between dimeric β2-GPI and proteins involved in the development of thrombotic complications. As described above, a rapidly increasing number of receptors and plasma proteins have been identified that interact with dimeric β2-GPI. It is therefore becoming increasingly difficult to identify which interactions do participate in the pathology of the syndrome and which are just bystander effects. APS animal models using knock-out mice probably will be the main tools in identifying the relevant interactions and processes involved in the pathology of APS. Therefore, a good animal model to induce APS is essential.

As with all research, my studies described in this thesis raise more questions then answered. More studies are required in order to fully understand the mechanism behind the clinical symptoms presented by patients suffering from the antiphospholipid syndrome.

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8

Nederlandse samenvatting

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β2-Glycoprotein I (β2-GPI) is een eiwit dat circuleert in plasma (100-300 μg/ml) in een ongebonden vorm en in een lipoproteïne gebonden vorm. β2-GPI bestaat uit 5 zogenaamde sushi domeinen of "complement control repeats". Domeinen I, II, III en IV omvatten ongeveer 60 aminozuren met elk twee disulfide bruggen. Domein III en IV zijn geglycosyleerd en deze suikergroepen bepalen voor ongeveer 20% het molecuulgewicht van β2-GPI (Mw: β2-GPI=45 kD). Domein V is een afwijkend domein en bestaat uit 82 aminozuren met daarin 3 disulfide bruggen. Domein V bevat een positief geladen gebied en een fosfolipiden insertieloop die betrokken is bij de binding van β2-GPI aan negatief geladen fosfolipiden. De binding van β2-GPI aan negatief geladen fosfolipiden is relatief zwak. De functie van β2-GPI is onbekend maar β2-GPI lijkt betrokken te zijn bij meerdere fysiologische processen zoals stolling en lipoproteïne metabolisme.

Het antifosfolipiden syndroom (APS) is een non-inflammatoire auto-immuunziekte die klinisch wordt gekarakteriseerd door de aanwezigheid van zowel arteriële en/of veneuze trombose en/of specifieke zwangerschapsproblemen. Verder hebben patiënten over een langere periode, zogenaamde "antifosfolipiden antistoffen" in hun plasma. Deze antifosfolipiden antistoffen zijn niet gericht tegen fosfolipiden maar tegen eiwitten die aan fosfolipiden binden zoals β2-GPI, protrombine en proteïne S. Aangezien aanwezigheid van antistoffen tegen β2-GPI in plasma van patiënten, het best correleert met klinische complicaties, is het nu algemeen geaccepteerd dat β2-GPI het belangrijkste antigen is in het antifosfolipiden syndroom. Het mechanisme achter het ontstaan van klinische symptomen in APS is tot dusver onbekend. Dit proefschrift richt zich vooral op de rol van bloedplaatjes in het ontstaan van trombotische complicaties in APS patiënten.

In het verleden is aangetoond dat dimerizatie van β2-GPI door autoantistoffen of monoklonale antistoffen leidt tot een 10 maal verhoogde bindingsaffiniteit van β2-GPI aan negatief geladen fosfolipiden en bloedplaatjes. Naar aanleiding van deze observatie is een recombinant dimeer β2-GPI construct gemaakt waarin β2-GPI gefuseerd is aan het dimerizatiedomein van stollingsfactor XI (apple4). Dit recombinant dimeer β2-GPI (dimeer β2-GPI) gedroeg zich in alle validatie-assays hetzelfde als door antistoffen gedimerizeerd β2-GPI. In een in-vivo perfusiemodel waarbij bloedplaatjesadhesie aan een collageenoppervlak werd gemeten, bleek dat dimeer β2-GPI de adhesie van bloedplaatjes aan collageen verhoogde. Deze door dimeer β2-GPI veroorzaakte toename in adhesie kon worden geremd door de toevoeging van RAP (receptor associated protein). Aangezien RAP een universele remmer is van binding van liganden met receptoren van de Low Density Lipoprotein receptor familie (LDL-receptor familie), suggereerde dit dat de toename van bloedplaatjesadhesie werd gemedieerd door een receptor van de LDL-receptor familie. De enige receptor van de LDL-receptor familie die tot dusver op bloedplaatjes is aangetoond is apolipoproteïne receptor 2' (ApoER2'). ApoER2' is een korte splice variant van ApoER2 waaruit, door alternatieve splicing, exon V niet meer aanwezig is. ApoER2' mist hierdoor LDL-bindingsdomeinen 4, 5 en 6. Immunoprecipitatie experimenten toonden aan dat dimeer β2-GPI en niet monomeer plasma β2-GPI

inderdaad bindt aan ApoER2'. Er is dus een specifieke receptor voor dimeer β2-GPI aanwezig op plaatjes, die mogelijk betrokken is bij het ontstaan van trombose in APS patiënten.

Om de interactie tussen dimeer β2-GPI en ApoER2' verder te bestuderen, zijn domeindeletiemutanten gemaakt van dimeer β2-GPI waaruit domein I, domein II of domein V is verwijderd. Zowel Dimeer β2-GPI als domeindeletiemutanten werden getest op binding aan geïmmobiliseerd recombinant ApoER2'. Deletie van domein I en II had geen effect op de bindingscapaciteit en affiniteit van dimeer β2-GPI aan ApoER2'. Deletie van domein V resulteerde in een sterke afname van binding en affiniteit. Domein V van β2-GPI is dus de belangrijkste bindingsplaats voor ApoER2'. Immunoprecipitaties met verse bloedplaatjes bevestigden deze observatie. Dimeer β2-GPI zonder domein V bleek niet meer in staat te binden aan ApoER2' op het bloedplaatjesoppervlak. Competitiestudies met specifieke en door elkaar gehusselde peptiden toonden aan dat een specifiek gebied in domein V (Cys281-Cys288) verantwoordelijk is voor de binding van dimeer β2-GPI aan ApoER2'. Deze stretch van aminozuren is, samen met de al eerder genoemde fosfolipiden insertieloop, mede verantwoordelijk voor de binding van β2-GPI met negatief geladen fosfolipiden.

Voor verdere studie van de interactie van dimeer β2-GPI met ApoER2' was het nodig een niet membraangebonden vorm van ApoER2 te kloneren. Omdat bloedplaatjes geen kern en eigen eiwitsynthese hebben, hebben we hierbij gebruik gemaakt van voorlopercellen van plaatjes uit beenmerg (megakaryocyten). Tijdens het kloneren van ApoER2' uit deze voorlopercellen van bloedplaatjes zijn drie splicevarianten van ApoER2 geïsoleerd. Eén van de geïsoleerde splicevarianten van ApoER2 is de bloedplaatjes splicevariant die al beschreven is in de literatuur (ApoER2' of ApoER2-850). De twee andere splicevarianten van ApoER2 missen steeds een LDL bindingsdomein extra. ApoER2-750 bezit alleen LDL-bindingsdomeinen 1, 2 en 7 en de kortste splicevariant (ApoER2-650) heeft alleen LDL-bindingsdomeinen 1 en 2. Ook zijn twee domeindeletiemutanten zijn gemaakt van ApoER2-650 om de bindingsplaats voor dimeer β2-GPI te identificeren. Uit deze bindingsstudies bleek dat dimeer β2-GPI alleen aan ApoER2 bindt als LDL bindingsdomein I aanwezig was. LDL bindingsdomein I is dus de belangrijkste bindingsplaats voor dimeer β2-GPI.

ApoER2-750 was de meest voorkomende splicevariant van ApoER2 op bloedplaatjes en niet, de in de literatuur beschreven ApoER2' (ApoER2-850). Bij het bestuderen van de signaleringscapaciteit van de verschillende splicevarianten van ApoER2, bleek ApoER2-750 ook het meest aktief bij signalering. Dit is gemeten door het meten van de dissociatie van ApoER2 splicevarianten van het cytoskelet na stimulatie van bloedplaatjes met dimeer β2-GPI. Om aan te tonen dat bloedplaatjes ook daadwerkelijk verschillende splicevarianten van ApoER2 tot expressie brengen, hebben we monoklonale antistoffen geproduceerd tegen LDL-bindingsdomein 1 van ApoER2. In tegenstelling tot wat tot op heden werd aangenomen, brengen bloedplaatjes niet 1 maar inderdaad drie splicevarianten van ApoER2 op hun membraan tot expressie. Deze splicevarianten hebben allemaal LDL-bindingsdomein 1 en zijn dus alle drie in staat dimeer β2-GPI te binden.

In de klinische criteria die het antifosfolipiden syndroom definiëren zijn alleen trombotische complicaties en specifieke zwangerschapsproblemen opgenomen. Echter, een groot aantal andere klinische symptomen correleren met APS en de aanwezigheid van antifosfolipiden antistoffen. Gezien de wijde verspreiding en diversiteit van expressie van leden van de LDL-receptor familie receptoren op verschillende celtypes en organen, zou de interactie van dimeer β2-GPI met andere leden van de familie mogelijk een rol kunnen spelen in het ontstaan van deze, met APS geassocieerde klinische verschijnselen. Door deze observaties zijn we de interactie van dimeer β2-GPI met andere leden van de LDL-receptor familie gaan bestuderen. Voor deze studies zijn recombinant Megalin (Gp330), LDL-Receptor Related Protein (LRP), LDL-receptor (LDL-R) en Very Low Density Lipoprotein Receptor (VLDL-R) gebruikt. Dimeer β2-GPI bindt aan alle geteste leden van de receptor familie, behalve aan LDL-R. Dit is tevens de enige receptor van de LDL-receptorfamilie zonder signaleringscapaciteiten. LRP en Megalin bleken twee bindingsplaatsen voor dimeer β2-GPI te hebben (affiniteit respectievelijk 3nM en 200nm). VLDL-R en ApoER2 hebben maar 1 bindingsplaats (affiniteit 25nM) voor dimeer β2-GPI. Toevoeging van zowel RAP als heparine blokkeert de binding van dimeer β2-GPI aan alle LDL-receptorfamilieleden. Deletie van domein V van β2-GPI resulteerde in het verlies van de bindingsplaats met de hoogste affiniteit op LRP en Megalin terwijl de bindingsplaats met lage affiniteit onveranderd bleef. De bindingsplaats van dimeer β2-GPI met een lage affiniteit bevindt zich dus in een ander domain van β2-GPI. Deletie van domein V uit dimeer β2-GPI resulteerde in het verlies van binding van dimeer β2-GPI aan de LDL-receptor, de VLDL receptor en aan ApoER2'. Bij het nader bestuderen van de interactie van dimeer β2-GPI met LRP bleek dat afzonderlijk clusters van LRP (cluster II en cluster IV) ook twee bindingsplaatsen hadden voor dimeer β2-GPI. Dit suggereert de aanwezigheid van meer dan twee bindingsplaatsen voor dimeer β2-GPI op "full-length" LRP. Een mutatie in de fosfolipideninsertieloop van dimeer β2-GPI had geen effect op binding van dimeer β2-GPI aan receptoren van de LDL-receptorfamilie. Dimeer β2-GPI bindt dus aan de meeste leden van de LDL-receptorfamilie. Het is dus mogelijk dat binding van dimeer β2-GPI aan leden van de LDL-receptorfamilie op andere celtypes en organen, een rol speelt in de ontwikkeling van APS gerelateerde klinische symptomen.

Tijdens het onderzoek naar de binding van dimeer β2-GPI aan ApoER2' stelden we ons de vraag of de interactie tussen dimeer β2-GPI en ApoER2' sterk genoeg zou zijn om bloedplaatjesadhesie in stromend bloed te kunnen ondersteunen. In een perfusiemodel bleek dat bloedplaatjes "plakken" aan een oppervlak met dimeer β2-GPI. Adhesie van bloedplaatjes aan dimeer β2-GPI was meetbaar bij zowel arteriële als veneuze stroomweerstanden. Gebruik makende van algemene remmers van bloedplaatjesadhesie bleek verder dat de adhesie niet alleen afhankelijk was van de interactie van dimeer β2-GPI met ApoER2', maar ook van de interactie van dimeer β2-GPI met Glycoproteine Ibα (GPIbα). GPIbα is een bloedplaatjesreceptor die betrokken is bij de primaire adhesie van bloedplaatjes aan een beschadigde vaatwand via het plasma-eiwit vWF en het extracellulaire matrixeiwit collageen. Blokkeren van zowel ApoER2 als GPIbα remde de adhesie van bloedplaatjes aan dimeer β2-GPI volledig. Binding van dimeer β2-GPI aan beide receptoren was nodig voor de adhesie van bloedplaatjes. Bio-

chemische analyse liet zien dat dimeer β2-GPI aan GPIbα bindt in de nabijheid van de trombine bindingsplaats op GPIbα. Deze binding was Zn2+ afhankelijk. Binding van dimeer β2-GPI interfereerde niet met de vWF bindingsplaats op GPIbα.

Immuno-coprecipitaties en scanning elektronen microscopie (SEM) lieten verder zien dat ApoER2 en GPIbα aanwezig zijn als co-receptoren op het membraan van bloedplaatjes. Deze co-localisatie van GPIbα en ApoER2′ op bloedplaatjes is onafhankelijk van dimeer β2-GPI en de associatie van beide receptoren met het cytoskelet van bloedplaatjes.

Binding van bloedplaatjes aan dimeer β2-GPI aan beide receptoren is dus vereist voor adhesie van bloedplaatjes. Blokkeren van dimeer β2-GPI binding aan ApoER2′ met RAP of "soluble" ApoER2′ blokkeerde signalering van ApoER2 splice varianten. Toevoeging van RAP of "soluble" ApoER2′ inhibeerde de transiënte dissociatie van ApoER2′ van het adapter molecuul Dab-1. Proteolytisch verwijderen van GPIbα of toevoeging van "soluble" GPIbα had geen effect op deze ApoER2′ specifieke signalering.

Proteolytisch verwijderen van GPIbα en de toevoeging van "soluble" GPIbα inhibeerden de signalering van GPIbα. Beide inhibeerden de translocatie van GPIbα naar het cytoskelet via het adaptor eiwit 14-3-3-ζ volledig. Toevoeging van RAP en "soluble" ApoER2′ hadden geen effect op de GPIbα translocatie.

Omdat dimeer β2-GPI geen geaccepteerd fysiologisch oppervlak is voor bloedplaatjes, hebben we de persfusie-experimenten uitgevoerd over het plasma-eiwit fibronectine. Incubatie van gereconstitueerd bloed met dimeer β2-GPI induceerde ook op een oppervlak met fibronectine in een toename van bloedplaatjesadhesie. Deze toename in adhesie kon ook geremd worden door binding van dimeer β2-GPI aan of ApoER2′ of GPIbα te blokkeren. In deze persfusie-experimenten was binding van dimeer β2-GPI aan beide receptoren wederom vereist voor de toename in adhesie van bloedplaatjes.

Blokkeren van een van beide receptoren remt adhesie van bloedplaatjes aan verschillende oppervlaktes, maar het remmen van de interactie tussen dimeer β2-GPI en ApoER2′ of GPIbα verstoort signalering van de co-receptor niet. Deze twee observaties suggereren de aanwezigheid van een gedeelde signaleringroute onder ApoER2′ en GPIbα die het signaal van de afzonderlijke receptoren versterkt. Deze hypothese is op dit moment onderwerp van onderzoek.

Dit proefschrift heeft de moleculaire interactie tussen dimeer β2-GPI en twee bloedplaatjesreceptoren en de daaropvolgende intracellulaire gevolgen beschreven. Door de studies beschreven in dit proefschrift zijn nieuwe inzichten ontstaan met mogelijke implicaties voor nieuwe en verbeterde therapieën voor APS patiënten. Men zou hierbij kunnen denken aan specifieke inhibitie van binding van dimeer β2-GPI aan ApoER2′ met behulp van recombinant LDL bindingsdomein 1, maar ook aan het gebruik van het ontstollingsmiddel low molecular weight heparin (LMWH). LMWH wordt meestal gebruikt in de behandeling van veneuze trombose en is een derivaat van normaal he-

parine. Naar alle waarschijnlijkheid zal LMWH, evenals heparine, de interactie tussen dimeer β2-GPI en ApoER2' remmen. Mocht dat zo zijn, dan zou LMWH ook gebruikt kunnen worden ter preventie voor arteriële complicaties in APS patiënten.

Ook het remmen van GPIbα signalering is een mogelijk interessante optie voor therapeutisch gebruik. Beide therapeutische strategieën vereisen verdere ex-vivo maar ook in-vivo studies om toepasbaarheid te testen.

In de literatuur worden steeds meer plasma-eiwitten en membraan receptoren beschreven waaraan dimeer β2-GPI kan binden. Onderzoeken welke interacties wel en niet klinisch relevant zijn, zal een uitdaging zijn voor onderzoek in de toekomst. "Knock-out" muizen zijn hiervoor een ideaal systeem, maar ook zal gekeken moeten worden in dubbele "knock-outs" om additieve en compenserende effecten van interacties te kunnen analyseren. Hiervoor zal de ontwikkeling van een goed in-vivo model een eerste prioriteit hebben. Het ontwikkelen en valideren van een in-vivo is een van de uitdaging voor toekomstig onderzoek naar de pathologie van het antifosfolipidensyndroom.

Zoals bij alle onderzoek komen er meer vragen bij dan dat er uiteindelijk beantwoord zijn. Ik wens de onderzoekers van dit moment en de toekomst heel veel succes met de gevolgen van het onderzoek, beschreven in dit proefschrift.

9

Appendices

List of publications

Full papers:

BN Fry, V Korolik, JA ten Brinke, MTT Pennings, R Zalm, BJ Teunis, PJ Coloe and BA van der Zeijst.The lipopolysaccharide biosynthesis locus of Campylobacter jejuni 81116**.** Microbiology; 44, 2049-2061. 1998

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Interaction of β2-glycoprotein I with members of the Low Density Lipoprotein receptor family. Journal of Thrombosis and Heamostasis. Aug;4(8):1680-90. 2006

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MTT Pennings, RHWM Derksen, M van Lummel, WL Tekelenburg, J Adelmeijer, K van-Hoorelbeke, RT Urbanus, T Lisman and PhG de Groot. Platelet adhesion to dimeric β2-glycoprotein-I under conditions of flow is mediated by at least two receptors: Glycoprotein Ibα and ApoER2'. J. Thromb. and Heamost. 2007;5:369-377

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Other publications

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MTT Pennings, HB de Laat, RHW Derksen, GM Iverson and PhG de Groot. IgG's isolated from patient plasma of APS patients bind to domain I of β2-Glycoprotein I. Oral presentation. APS conference 2004, Sydney, Australia

MTT Pennings, RHWM Derksen, M van Lummel, J Adelmeijer, T Lisman1, PhG de Groot.
Platelet adhesion to dimeric β2-glycoprotein-I under conditions of flow is mediated by glycoprotein Ibα. Oral presentation. ISTH 2005, Sydney, Australia. Young investigators travel grant from NVTH

MTT Pennings, RHWM Derksen, M van Lummel, WL Tekelenburg, J Adelmeijer, K van-Hoorelbeke, RT Urbanus, T Lisman and PhG de Groot. Platelet adhesion to dimeric β2-glycoprotein-I under conditions of flow is mediated by glycoprotein Ibα and ApoER2'. Poster presentation. BSH/BSTH 2006, Edinburgh, Scotland

Dankwoord

Zo, dat was het dan.

Wat te doen met een dankwoord?

Een proefschrift bij elkaar pipetteren is net als tuinieren. Je zet een grove indeling uit als raamwerk. Vanuit dit raamwerk komt het feitelijk neer op invullen van de gecreëerde ruimtes. Het postuleren van een aantal hypotheses. Hier een rijtje wortels met daarnaast een rij venkel. Dat houdt hopelijk glaswormpjes weg.

Zaai je te dicht op elkaar dan is uitdunnen vaak een noodzakelijk kwaad. Door uitdunnen creëer je ruimte voor planten die anders nooit zullen volgroeien. Ook tijdens een AOI periode moet men snoeien in de eindeloze lijst aan goede ideeën die ontstaan door samenwerking en koffieautomaatdiscussies. Toch is het geen slecht idee om zo nu en dan een kiemplantje te laten staan en je te laten verrassen door het eindresultaat. Soms wordt het kiemplantje een smakelijk kruid en soms ook niet.

Tijdens het seizoen is het voornamelijk bijhouden, snoeien en schoffelen. Onderhouden van het gezaaide en groeiende, ofwel het praktisch uitvoeren van experimenten die leiden tot het bewijzen of afwijzen van een hypothese.

Daarna komt de oogsttijd waarbij je uit moet kijken dat je niet te haastig oogst waardoor schade kan ontstaan aan de gewassen. Ga dus niet roekeloos te werk maar ben zeker ook niet te voorzichtig. Denk goed na over de resultaten, conclusies en discussies die je op papier gaat zetten. Dan is de oogst rijk en een beloning voor het harde werken.

Gedurende het hele seizoen doet men er goed aan te luisteren naar wijze adviezen van ervaren tuinierders om je heen. Dit verhoogt het tuinierplezier en de oogst.

Of het nou gaat om tuinieren of promotie onderzoek, je oogst wat je zaait. Hulp van de weergoden of collega's is onontbeerlijk voor een succesvolle afloop. Ik heb tijdens mijn periode als AIO op de afdeling hematologie (later Laboratorium Klinische Chemie en Hematologie) met veel mensen samengewerkt en die mensen wil ik daarvoor hartelijk danken.

Iedereen die op welke manier dan ook, betrokken is geweest bij de totstandkoming van dit proefschrift wil ik van harte bedanken voor de inzet en hulpvaardigheid die ik heb ervaren.

Lieve collega's en vrienden,
Bedankt voor alles!
Maarten

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 12 juni 1970 te 's-Hertogenbosch. Na het afronden van de middelbare opleiding heeft hij een MBO diploma behaald met als specialisatie biochemie. Daarna heeft hij een HBO diploma behaald met als specialisatie biotechnologie. Tijdens zijn studie aan de Hogeschool Utrecht heeft hij in het stage jaar een "Honours Degree" behaald aan de Royal Melbourne Institute of Technology in Australië onder begeleiding van Dr. V. Kororlik. Na het afronden van het HBO heeft hij twee jaar gewerkt als analist op de afdeling immunologie aan het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. T. Logtenberg. In 1999 werd hij voor 3 jaar aangesteld als junior onderzoeker bij Crucell.

Van 1 november 2002 tot 1 maart 2007 was hij werkzaam als assistent in opleiding bij de afdeling Klinische Chemie en Hematologie aan het Universitair Medisch Centrum Utrecht. Het in deze periode uitgevoerde onderzoek is in dit proefschrift beschreven en werd uitgevoerd onder begeleiding van Prof. Ph. G. de Groot, Prof. J. W. J. Bijlsma en Dr. R. H. W. M. Derksen. In mei 2007 is hij begonnen als post-doc op de afdeling Dermatologie en Allergologie aan het Universitair Medisch Centrum Utrecht.