The role of the Low-Density Lipoprotein Receptor Family in the pathology of the Antiphospholipid Syndrome

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The role of the Low-Density Lipoprotein Receptor Family in the pathology of the Antiphospholipid Syndrome

Rol van Low-Density Lipoproteine Receptoren in de pathologie van het Antifosfolipiden Syndroom

(met een samenvatting in het Nederlands)

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Contents

Abbreviations

α_2AP	alpha$_2$-antiplasmin
β_2GPI	β_2-glycoprotein I
aCL	anti-cardiolipin antibodies
AD	Alzheimer disease
ADP	adenosine diphosphate
APC	Activated Protein C
aPL	antiphospholipid antibodies
apoB	apolipoprotein B
apoE	apolipoprotein E
apoER2'	apolipoprotein E receptor 2'
APS	Antiphospholipid syndrome
APTT	activated partial thromboplastin time
AT	antithrombin
ATP	adenosine triphosphate
BCA	bicinchoninic acid protein assay
BHK	baby hamster kidney
BSA	bovine serum albumin
CCP	complement control protein
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid
CK-MB	creatine kinase myoglobin class A repeats
CR	complement type repeat
Dab1	Disabled 1
DEA	diethynolamine
DM	domain deletion mutants
dRVVT	dilute Russell's viper venom time
DTS	dense tubular system
DVT	deep venous thrombosis
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FII	prothrombin
FV	factor V
FVa	activated factor V
FVII	factor VII
FVIIa	activated factor VII
FVIII	factor VIII
FVIIIa	activated factor VIII
FIX	factor IX
FIXa	activated factor IX
FX	factor X
FXa	activated factor X
FXI	factor XI
FXIa	activated factor XI
FXII	factor XII
FXIIa	activated factor XII
FXIII	factor XIII
FXIIIa	activated factor XIII
FDP	fibrin degradation products
FH	familial hypercholesterolemia
FPLC	fast performance liquid chromatography
GP	glycoprotein
HMWK	high molecular weight kininogen
IE/ml	international units per milliliter
ISTH	International Society of Thrombosis and Haemostasis
KCT	kaolin clotting time
kDa	kiloDalton
K_D	equilibrium dissociation constant
LA	LDL-R type A
LAC	lupus anticoagulants
LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
Leu	leucine
LLD	lupus-like disease
LMWH	low molecular weight heparin
LRP	LDL-R related protein
MegBP	megalin binding protein
MES	2-(N-Morpholino) ethanesulfonic acid
µg	microgram
µm	micro molar
ml	milliLiter

mM	milliMolar
MTS	microtubular system
nM	nanoMolar
NO	nitric oxide
NPP	normal pooled plasma
OCS	open canalicular system
OPD	orthophenylenediamine
oxLDL	oxidized LDL
PAI-1	plasminogen activator inhibitor-1
PC	phosphatidylcholine
PE	phosphotidylethanolamine
PEG	polyethylene glycol
PF4	platelet factor 4
PGI_2	prostaglandin I_2
PL	phospholipids
PnPP	para-nitrophenyl phosphate
PS	phosphatidylserine
PT	prothrombin time
PTC	proximal tubule cells
RAMPO	rabbit anti-mouse antibody peroxidase
RAP	receptor associated protein
Req	Response at equilibrium
SCR	short consensus repeats
SDS-PAGE	sodium dodecyl sulfate-polyacryl amide gel electrophoresis
Ser	serine
SLE	systemic lupus erythematosus
SMC	smooth muscle cells
SorLA	sorting protein-related receptor containing LDL-R
SPR	surface plasmon resonance
SSC	Scientific and standardization Committee
SWARPO	swine anti-rabbit antibody peroxidase
TAFI	thrombin-activatable fibrinolysis inhibitor
TBST	TBS Tween 20
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIA	transient ischemic attacks
TLR	toll-like receptor
TM	transmembrane region
tPA	tissue-type plasminogen activator
Trp	tryptophan
TX	thromboxane
TxA_2	thromboxane A_2
Tyr	tyrosine
U	Units
UFH	unfractionated heparin
uPA	urokinase-type plasminogen activator
VKA	vitamin K antagonists
VLDL	very low-density lipoprotein
VLDL-R	very low-density lipoprotein receptor
vWF	von Willebrand factor

GENERAL INTRODUCTION 1

The Antiphospholipid Syndrome

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease that is defined by both clinical and laboratory criteria. The clinical conditions are characterized by the presence of venous and/or arterial thrombosis and/or pregnancy complications [1;2]. Venous or arterial thrombosis includes deep venous thrombosis (DVT), transient ischemic attacks (TIA) or myocardial ischemia or infarction [3;4]. The syndrome is diagnosed when one of the clinical criteria is present in combination with the presence of antiphospholipid antibodies (aPL) (Table 1) [1;5,220]. aPL are also associated with other clinical features including heart valve abnormalities, livedo reticularis, chorea, nephropathy, thrombocytopenia and haemolytic anaemia [6]. These clinical manifestations, which are not part of the inclusion criteria, are frequently found in patients with APS.

aPL is a general term that describes a variety of closely related antibodies; lupus anticoagulants (LAC), anti-cardiolipin antibodies (aCL) and anti-β_2-glycoprotein I antibodies. The presence of aPL in plasma are detected by a phospholipid-dependent prolongation of a clotting assay (LAC) or by solid phase immune assays, the anticardiolipin- and the anti-β_2-glycoprotein I enzyme linked immunosorbent assay (ELISA). Autoantibodies that cause LAC and aCL are closely related, but not per se identical autoantibodies. This raises the question which antibody is the most relevant. Several studies show that antibodies inducing a LAC correlate best with a history of thrombotic complications [7;8].

The paradoxical association between *in vitro* prolongation of clotting assays (LAC) and *in vivo* observed thrombosis, stimulated scientists to search for the real antigen to which the antibodies are directed. Until the early nineties, it was thought that aPL are directed against anionic phospholipids directly. In 1990, three groups independently discovered a phospholipid-binding plasma protein to which the antibodies are directed; β_2-glycoprotein I (β_2GPI) [9-11]. Since 1991, a second cofactor was discovered in APS, prothrombin, another plasma protein with affinity for anionic phospholipids that could also be a target for aPL [12]. From that moment on a search for other phospholipid binding plasma proteins started.

At present, several proteins have been identified that may serve as antigen for aPL, including β_2GPI, Protein S, Protein C, Protein Z, annexin A5, thrombomodulin, high molecular weight kininogen (HMWK), tissue factor pathway inhibitor (TFPI) and prothrombin [13]. Despite the presence of these proteins as possible target of aPL, β_2GPI is the main cofactor with clinical significance in APS [7;8;14]. For example, LAC positive anti-β_2GPI antibodies correlate best with thromboembolic complications observed in APS patients. Interaction of autoantibodies with β_2GPI seems very important in the pathophysiology of APS.

Table 1. Revised classification criteria for the antiphospholipid syndrome

Clinical criteria	1. One or more clinical episodes of objectively verified arterial, venous or small vessel thrombosis. Thrombosis must be present without indications of inflammation. 2. Pregnancy morbidity: - One or more unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation - One or more premature births of a morphologically normal neonate before the 34th week of gestation - Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation
Laboratory criteria	1. Positive test for anti-β_2GPI dependent anticardiolipin antibodies on two or more occasions at least 6 weeks apart, measured by ELISA 2. Prolongation of at least one phospholipid dependent coagulation assay measured in plasma, on two or more occasions at least 6 weeks apart.

A patient is diagnosed with the antiphospholipid syndrome when the patient meets one or more of the clinical criteria in the combination with one or more of the serological criteria.

Antiphospholipid Antibodies

aPL are classified according to their in vitro method of detection; ELISA's measure anti-β_2GPI antibodies and clotting assays detect LAC. LAC are immunoglobulins of the class IgG or IgM that interfere with *in vitro* phospholipid-depending coagulation assays, such as the prothrombin time (PT), activated partial thromboplastin time (APTT), kaolin clotting time (KCT) or dilute Russell's viper venom time (dRVVT). The APTT and the KCT measure coagulation activated via the intrinsic pathway (Figure 1). This pathway starts with the activation of factor XII. The PT measures coagulation activated via factor VII. In the dRVVT, coagulation starts via the direct activation of factor X. These LAC assays must be performed in accordance with the recommendation of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis (SSC of ISTH) [15]. The laboratory criteria for APS require that aPL (anti-β_2GPI antibodies and/or aCL) tests are positive on at least two occasions with blood samples taken more than 12 weeks apart, to avoid

positive aPL associated with acute infectious diseases. β_2GPI and prothrombin are the main targets for aPL causing LAC. Antiprothrombin antibodies are responsible for prothrombin-dependent LAC and anti-β_2GPI antibodies for β_2GPI-dependent LAC. Specific neutralization with cardiolipin enables differentiation between anti-β_2GPI antibodies or antiprothrombin antibodies dependent LAC [16]. The addition of cardiolipin neutralizes β_2GPI-dependent LAC but not prothrombin-dependent LAC in an APTT based assay. aPL directed against a specific epitope in domain I of β_2GPI correlates best with thrombosis (Gly40-Arg43) [17]. This epitope is shield off when β_2GPI is circulating free in plasma. Only after a conformational change, such as that induced by binding of β_2GPI to phospholipids, this epitope is exposed.

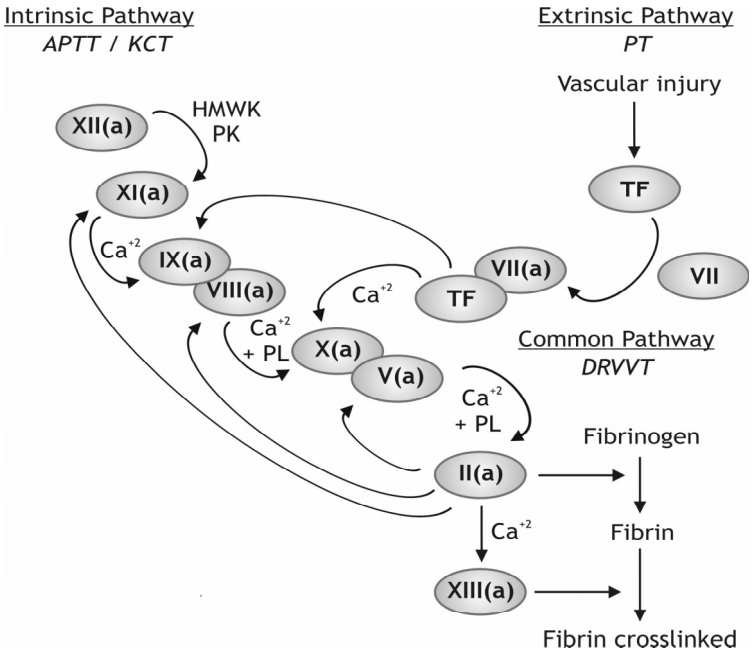

Figure 1. Coagulation cascade and fibrin formation by the intrinsic and extrinsic pathways.
The initiation of the coagulation occurs following vascular injury and the exposure of TF to the blood. This triggers the extrinsic pathway. The intrinsic pathway can be triggered when thrombin is generated, leading to the activation of factor XI. The two pathways converge by the formation of factor Xa. PL refers to phospholipid.

The Haemostatic Process

Normal vascular endothelial cells maintain blood fluidity by inhibiting coagulation and platelet aggregation. These cells also prevent platelets and coagulation factors from exposure to reactive and pro-thrombotic subendothelium. The subendothelium

contains components like collagen, fibronectin and von Willebrand factor (vWF), which promote platelet adhesion. In addition, the subendothelium also exposes a membrane protein called tissue factor (TF) that triggers blood coagulation. After injury of the endothelial layer, these pro-thrombotic components are exposed to the flowing blood. This induces the formation of the haemostatic plug by promoting platelet adhesion and aggregation and by activating coagulation. Haemostasis is composed of four major events that occur in a sequence following the loss of vascular integrity [18]. The completion of each step activates another coagulation factor in a chain reaction that leads to the conversion of fibrinogen to fibrin:

I. The initial phase of the process is vascular constriction in order to limit the blood flow to the site of injury.
II. Next, platelets adhere to the exposed subendothelium followed by platelet aggregation at the site of injury, forming a temporary, soft platelet plug. Fibrinogen and vWF are primarily responsible for stimulating platelet clumping. Upon activation, platelets release among others ADP and thromboxane (TX) A2 (which activate additional platelets), serotonin, factor V and other proteins important for the coagulation cascade. In addition, activated platelets expose anionic phospholipids on their cellular surface to accommodate the formation of the plug.
III. To stabilize the initially loose platelet plug, the coagulation cascade converts fibrinogen to fibrin that replaces the platelet plug.
IV. The clot is dissolved in time to enable tissue repair by a process called fibrinolysis.

Although the haemostatic process is presented here as a sequence of distinct events, the actual process involves a very complex interplay between these subprocesses.

Platelet Biochemistry

In 1882, Bizzozzero described the identification of platelets as a class of blood corpuscles, whereas in 1888 Eberth and Schimmelbusch first reported the importance of platelets for the formation of a haemostatic plug. Another highlight in platelet research was placed by Aschoff in 1925, who's opinion still provides two keys to the understanding of thrombogenesis: *i.* '...aggregations of platelets as they are present in a thrombus can only be sedimented as long as the blood is flowing' and

ii. '...formation of fibrin is not a primary event in thrombosis, but is preceded by important changes of the corpuscular elements of the blood'. To understand the mechanism of arterial thrombosis the latter changes have to be understood.
Platelets are the smallest corpuscular components of human blood (diameter 2 – 4 µm) - the physiological number varies from 150.000 to 450.000/µL blood. Platelets are no complete cells, as they do not have a nucleus. The origin of platelets is the bone marrow, where megakaryocytes (diameter 40 – 50 µm) liberate platelets as the endproduct of protrusion of their membranes [19]. The typical shape of resting platelets is discoid. Upon activation, they undergo a shape change to a globular form with pseudopodia (up to 5 µm long) (Figure 2). Platelets maintain their disc shape by a circumferential coil of microtubules and by actin and myosin [20]. Actin and myosin assemble into microfilaments upon platelet activation, providing the contractile force necessary for shape change and pseudopod formation. A second two-dimensional network of shorter actin fibres serves as a membrane skeleton, responsible for the discoid shape of the resting platelet. In the periphery, close to the microtubular system (MTS) a membrane system is located called the dense tubular system (DTS). The DTS serves as a pool for platelet calcium and is the major compartment of arachidonate accumulation and thromboxane synthesis [21]. The open canalicular system (OCS) surrounds the organelle zone and offers additional membrane capacity during platelet activation. The majority of actin and cytoskeletal-associated proteins and filaments are distributed throughout the platelet cytoplasm. These cytoskeleton proteins may be involved in traffic of organelles, proteins and intracellular signaling molecules [22;23].

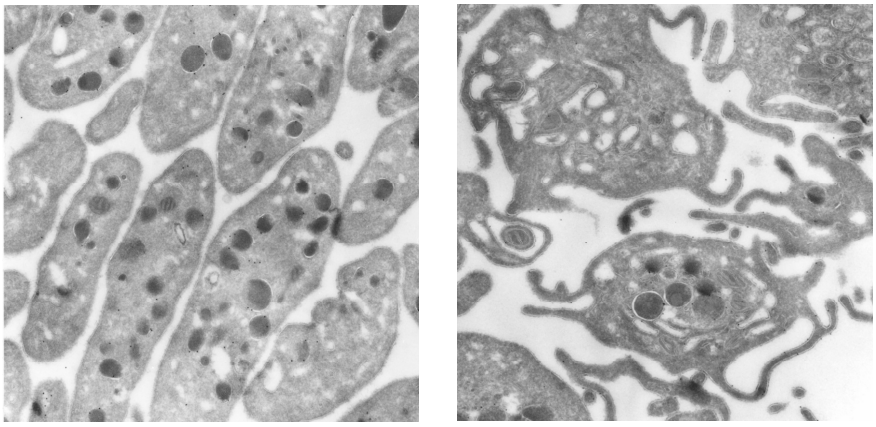

Figure 2. Platelet shape after activation. *Resting platelets (Panel A) are normally discoid shaped and undergo a shape change to a globular form with pseudopodia upon activation (Panel B). Dr. Harry Heynen, the Netherlands, kindly provided these figures.*

Platelets and Haemostasis

Platelets play a critical role in haemostasis. The reactions involved include platelet adhesion to the injured vessel wall, spreading, secretion of stored platelet constituents and formation of platelet aggregates (Figure 3) [18]. Under physiological conditions, platelets do not adhere to the vascular endothelium. Synthesis and secretion of nitric oxide (NO) and prostaglandin I_2 (PGI_2) by endothelial cells support the non-thrombotic nature of the endothelium [24;25]. After damage of the endothelium, platelets adhere to the exposed subendothelium. This occurs in two steps; binding of subendothelial von Willebrand factor (vWF) to glycoprotein (GP) Ib on the platelet surface is required to slow down platelets followed by the binding of platelet receptors $\alpha_2\beta_1$ and GPVI to the exposed collagen. GPVI and $\alpha_2\beta_1$ play important roles to activate platelets in the early stage and they cooperate with GPIb to fully activate platelets to form thrombi [26]. A critical event in primary haemostasis is a change in the conformation of $GPII_b/III_a$, a platelet surface receptor that binds to fibrinogen, as well vWF, fibronectin and vitronectin [27]. Fibrinogen is a symmetric protein with two identical sites. It enables platelet-platelet interaction by binding to two $GPII_b/III_a$ on to two different platelets. In addition, thrombospondin, a plasma glycoprotein that potentiates platelet activation and stabilizes platelet aggregates, can also interact with glycoproteins on the platelet surface [28]. Platelets contain several classes of granules in which intracellular constituents are stored, including dense bodies (containing serotonin, ADP, ATP, pyrophosphate and calcium), α-granules (containing fibrinogen, vWF, coagulation factor V, PF4 and platelet-derived growth factor (PF4)) and lysosomes (containing acid hydrolases) [29]. These constituents, released after platelet activation, are involved in haemostasis and further platelet activation.

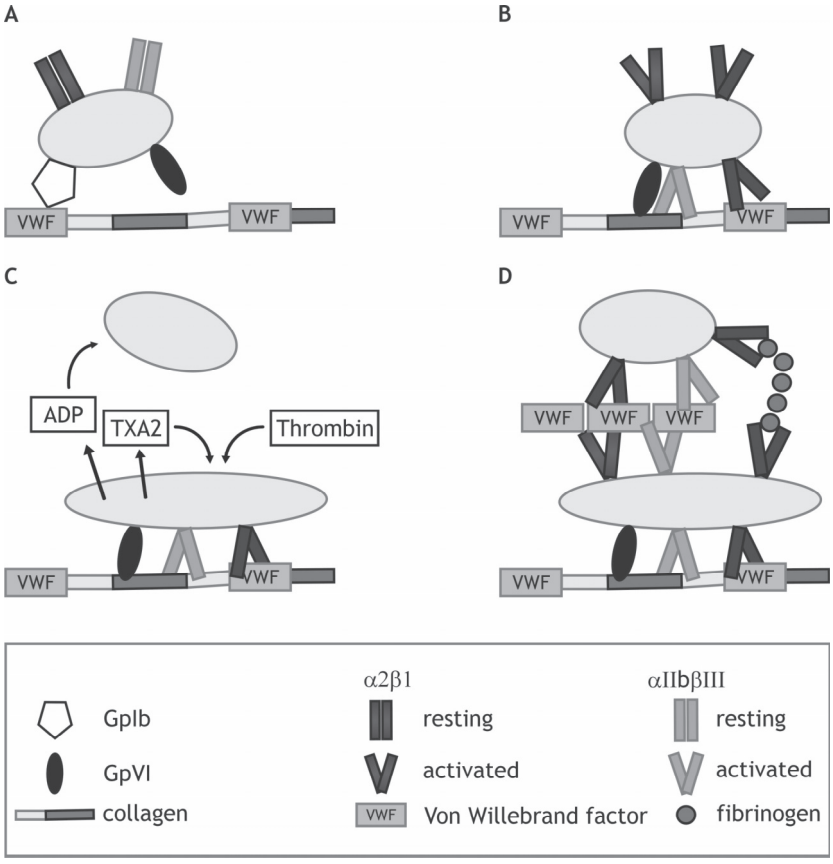

Figure 3. Schematic representation platelet adhesion and subsequent thrombus formation.
A) Rolling of platelets over von Willebrand factor and collagen mediated by glycoprotein (GP) Ib on the platelet surface. B) Platelet arrest mediated by binding of $\alpha_2\beta_1$ and GPVI to collagen, and by binding of $\alpha_{IIb}\beta_3$ to collagen-bound vWF. C) Platelet activation, spreading and secretion. D) Platelet-platelet interaction mediated by binding of $\alpha_2\beta_1$ to vWF, and by binding of $\alpha_{IIb}\beta_3$ to fibrinogen.

Coagulation

The coagulation cascade is the process of fibrin formation and encompasses the intrinsic- and extrinsic pathway. These pathways involve components from both the blood stream and a procoagulant surface expressing anionic phospholipids. The extrinsic pathway is the principal initiation pathway of *in vivo* blood coagulation [30]. This pathway is initiated at the site of injury in response to the exposure of TF, a cofactor in the factor VIIa-catalyzed activation of factor X (Figure 1). The formation of a complex between factor VIIa and TF is the first step in the overall clotting

cascade. Monocytes and endothelial cells, exposed to endotoxins or cytokines, synthesize TF [31-33]. In addition, a cryptic form of TF can circulate in the vasculature under normal conditions [34;35]. The intrinsic pathway might become important in pathological conditions like sepsis [36;37].

The first plasma component of the extrinsic pathway is factor VII, a vitamin-K dependent serine protease that is activated into factor VIIa by proteolytic cleavage. Factor Xa is the physiological activator for factor VII bound to TF during injury [38;39]. Vitamin K-dependent proteases contain Gla-residues that require vitamin K for proper synthesis. This modification is crucial for calcium binding, a necessity for vitamin-K dependent coagulation factors to bind to phospholipids [40-42]. Factor VII circulates in the blood in both an inactive and an active form; approximately 1% of total factor VII is in its active form [43]. Factor VIIa does not have any relevant enzymatic activity by itself. Only in complex with TF and in the presence of phospholipids (PL), factor VIIa can activate its substrates. Formation of this TF/factor VIIa complex increases the catalytic efficiency of the enzyme by four orders of magnitude when compared with factor VIIa alone. The assembly of complexes between cofactors, enzymes and substrates is a common theme in coagulation, as it increases efficiency and speed of the reactions. When bound to TF, further activation of factor VII proceeds.

The TF/FVIIa complex cleaves factor IX and factor X to their activated forms, which remain attached to the membrane surface. The required cofactor for factor IXa to convert factor X into factor Xa is the nonenzymatic factor VIII. The cofactor for factor Xa to convert prothrombin into thrombin is the nonenzymatic factor V. A part of factor V that participates in the prothrombinase complex is the result of release from the platelets α-granula [44]. The concentration of TF directs the efficiency of activation of factor IX and factor X; low concentrations of TF activate factor IX, whereas high TF concentrations predominantly activate factor X [45-47].

The intrinsic pathway leads to the activation of factor IX, a vitamin K-dependent serine protease, by factor XIa. Activation of factor XI requires the factor XIIa/activated height-molecular weight kininogen (HMWK) complex. The physiological role of the intrinsic pathway is uncertain; only a deficiency in factor XI is associated with hemorrhagic abnormalities [48]. Moreover, factor XI deficiency results in only a mild disorder of haemostasis in half of the affected individuals. Instead, these coagulation factors contribute in the initiation of inflammatory responses (sepsis) [37;49], complement activation [50] and fibrinolysis [51]. Currently, it is now thought that thrombin is the physiological activator of factor XI [52].

After conversion of factor X to factor Xa by either the extrinsic- or the intrinsic

pathway, factor Xa converts prothrombin (factor II) to thrombin (factor IIa). After cleavage of prothrombin by factor Xa, the N-terminal Gla-domain is removed and thrombin dissociates from the phospholipid surface. By activating factor V (increasing tenase activity), factor VIII (increasing prothrombinase activity) and factor XI, thrombin amplifies its own generation. This results in an increase of thrombin activity and fibrin formation. A relative small amount is necessary to cleave fibrinogen into fibrin. A larger amount of thrombin activates factor XIII and TAFI, which induce fibrin cross-linking, and inhibition of fibrinolysis, respectively.

Inhibition of coagulation

Generation of thrombin is under control of a number of inhibitory mechanisms, including proteolytic feedback by thrombin [53], inhibition by protease activity (C1-inhibitor, antithrombin (AT)), tissue factor pathway inhibitor (TFPI)) [54], α_1-antitrypsin [55], α_2-macroglobulin [56] and the Activated Protein C (APC) pathway [57].
The protein C pathway provides important control on coagulation by regulating the activities of the tenase- and prothrombinase complex [57;58]. Thrombin can bind to the endothelial cell surface receptor thrombomodulin thereby converting Protein C to Protein Ca. Together with the cofactor Protein S, protein Ca degrades factors Va and VIIIa, thereby limiting the activity of these two factors in the coagulation cascade. TFPI is a Kunitz-type plasma proteinase inhibitor that regulates tissue factor-induced coagulation [59]. TFPI directly inhibits activated factor X and, in a factor Xa-dependent fashion, produces feedback inhibition of the factor VIIa/tissue factor catalytic complex. AT inhibits a large number of coagulation factors including thrombin and factors IXa, Xa, XIa and XIIa [60]. Therefore AT plays an important role in the inhibition of coagulation.

Fibrinolysis

A fibrin clot must be removed in order to allow new tissue growth to establish the integrity of the blood flow. The process involved is called fibrinolysis. Fibrinolysis is initiated by the conversion of the plasma protein plasminogen into plasmin (Figure 4). The major *in vivo* activator of plasminogen is tissue-type plasminogen activator (tPA), a serine protease produced by endothelial cells [61]. Urokinase or urinary type plasminogen activator (uPA) can also activate plasminogen. The importance of uPA is predominantly in tissues where it plays a role in the degradation of the extracellular matrix. This facilitates the migration of cells, a process that is important in wound healing and in tumor invasion and metastasis [62].
A small amount of fibrin bound plasminogen is required to activate fibrinolysis.

Activation of fibrinolysis is a balanced process as α_2-antiplasmin, crosslinked to fibrin by factor XIII, inhibits the rate of fibrinolysis. Thrombin activates thrombin-activatable fibrinolysis inhibitor (TAFI) thus modulating fibrinolysis [63]. Its activity leads to removal of C-terminal lysines from partially degraded fibrin. This leads to an impairment of plasminogen activation, thereby reducing the rate of the fibrinolytic process. Activation of fibrinolysis leads to the degradation of fibrin into fibrin degradation products (FDP). Once the fibrin clot is degraded, formation of normal endothelium takes place maintaining normal vessel wall integrity.

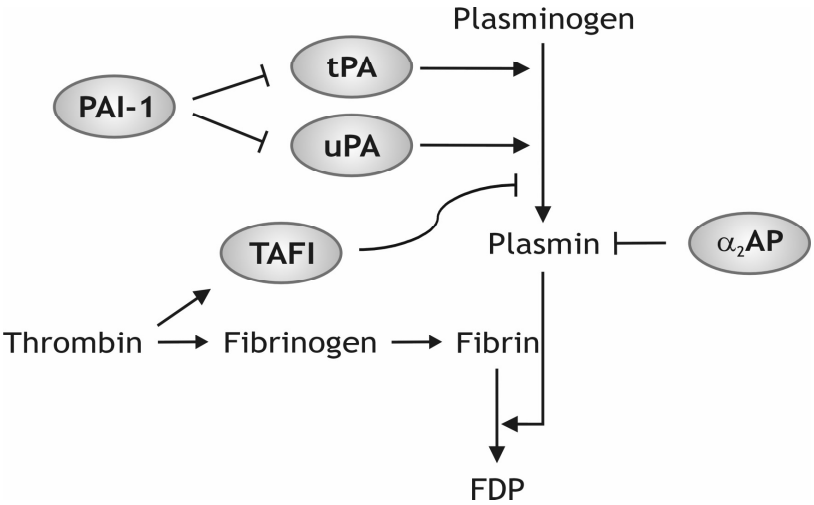

Figure 4. Fibrinolytic pathway.
Schematic representation of fibrinolysis. Arrows indicate activation pathways and blunt ended arrows indicate inhibitory pathways. PAI-1: plasminogen activator inhibitor, tPA: tissue-type plasminogen activator, uPA: Urokinase-type PA, TAFI: thrombin activatable fibrinolysis inhibitor, β_2AP: alpha2-antiplasmin, FDP: fibrin degradation products.

β2-Glycoprotein I

Protein Chemistry

The main protein involved in APS, β_2GPI is a monomeric, soluble glycoprotein present in plasma at concentrations of 10 — 200 μg/mL (0.25 — 5 μM) of which 4 — 13% is associated with lipoproteins [64]. The molecule was first classified as an apolipoprotein and originally termed apolipoprotein H [65]. Messenger RNA of β_2GPI is found in endothelial cells (EC) [66], placenta [67], central nervous system [66], astrocytes [68] and hepatocytes [69;70], but its major source of synthesis is the liver. The gene

consists of eight exons [71]. Four alleles are located on a single locus on chromosome 17q23 [72]. A wide variation in plasma β_2GPI levels in different ethnic populations indicates that expression of β_2GPI might be under genetic regulation [73].

β_2GPI is synthesized as a single chain polypeptide consisting of 326 amino acids with a calculated molecular mass of 36.3 kDa. It contains four potential N-glycosylation motifs that account for approximately 20% (w/w) of its total molecular weight of 45 kDa determined by SDS-PAGE [74-76]. The mature sequence of β_2GPI consists of five consecutive homologous segments referred to as short consensus repeats (SCR), sushi domains or complement control protein (CCR) repeats [77]. The first four domains structurally resemble each other and consist of 60 amino acids residues and four cysteines each. The fifth domain is aberrant and consists of 82 amino acids with two additional cysteines that are involved in intramolecular disulfide bonds.

A 2000 Å^2 large positively charged area of 14 cationic amino acids in domain V is implicated in membrane binding (Figure 5). It includes four lysines from the region defined by Cys^{281}-Cys^{288} in domain V and lysines 308 and 324 that are important in phospholipid binding [77;78]. A flexible loop Ser^{311}-Lys^{317} in the middle of this charged region is essential for phospholipid binding as it serves as a membrane insertion loop [79;80]. In addition to the phospholipid-binding region in domain V, a secondary interaction between domain I and phospholipids may occur at low ionic strength [81]. Therefore, β_2GPI binding to anionic phospholipids could result either from the combined interaction of the lysine-rich region with the hydrophobic loop or from a two-step process involving domain I and domain V.

In summary, the structure of β_2GPI suggests a membrane insertion loop in domain V. Domain III and domain IV are protected from proteolysis by the presence of potential glycosylation sites. Two domains (domain I and II) point away from the membrane surface, thus are able to interact with other proteins, antibodies or cellular receptors. Both hydrophobic as electrostatic interactions are important for the binding of β_2GPI to anionic phospholipids. However, β_2GPI does not bind to nonpolar phospholipids, such as phosphatidylcholine, emphasizing the importance of electrostatic interactions.

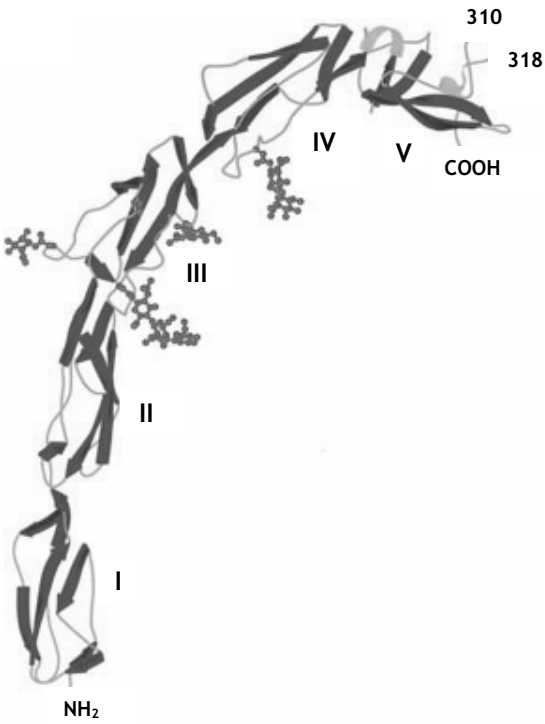

Figure 5. Structural representation of human β_2-glycoprotein I.
Ribbon drawing of β_2GPI showing the five consecutive domains labeled I to V. The N-linked glycans are shown in blue as ball-and-stick model. α-Strands are displayed in green and α-helices in red. The phospholipid insertion loop in domain V is labeled 310-318. Adapted from Bouma et al. EMBO J, 1999.

Physiological function of β2GPI

The physiological function of β_2GPI is not known. Studies in mice show that deficiency of β_2GPI does not result in clinical manifestations [82;83]. In addition, persons deficient of β_2GPI seems to be completely healthy. Based on *in vitro* experiments, the protein is implicated in a variety of physiological processes, including haemostasis [84;85], atherosclerosis [86;87], autoimmune diseases, platelet-depending thrombosis [88-90] and apoptosis [91-93]. However, the *in vivo* data in humans and mice do not support the claimed *in vitro* functions of β_2GPI.

The role of β_2GPI in haemostasis *in vitro* is difficult to understand, because the protein can exert both pro- and anticoagulant properties *in vitro*. Several hypotheses are put forward by which β_2GPI can influence haemostasis. One of the hypotheses is that binding of β_2GPI to anionic phospholipids results in competition between β_2GPI and coagulation factors for phospholipid surfaces [94]. The affinity

of β_2GPI for anionic phospholipids is rather low and therefore the observed *in vitro* activities cannot merely be explained by its affinity for phospholipids. The affinity of β_2GPI for anionic phospholipids increases in the presence of aPL (more then thousand times increased affinity) [95], suggesting that the observed activities of β_2GPI is the result of formation of bivalent complexes. Procoagulant activities of β_2GPI/anti-β_2GPI antibody complexes also include the prevention of factor Va degradation by activated protein C (APC) [96].

Anticoagulant activities of β_2GPI include the inhibition of ADP mediated platelet aggregation [97] and impairment of thrombin generation [84]. β_2GPI inhibits activation of factor XII in the presence of anionic phospholipids, which in turn inhibits the activation of the intrinsic pathway [98;99]. Furthermore, β_2GPI can bind to factor XI thereby preventing the formation of factor XIa by thrombin and FXIIa [100].

β_2GPI not only interacts *in vitro* with factors of the coagulation cascade and platelets, but it also participates in the regulation of fibrinolysis. Monoclonal aCL, established from a patient with APS inhibit the fibrinolytic activity by an elevation in PAI-I activity [101]. Furthermore, plasmin cleaves β_2GPI in domain V (nicked β_2GPI) [100]. Nicked β_2GPI binds to plasminogen and suppresses plasmin formation in the presence of fibrin or tissue plasminogen activator (tPA) [102]. The conversion of plasminogen to plasmin by tPA is a key event in fibrinolysis.

β_2GPI and cellular activation

The pathophysiological mechanisms explaining the clinical manifestations induced by aPL are not understood. Several hypotheses have been proposed. aPL can bind to and activate endothelial cells and platelets [103;104], interfere with haemostasis, disrupt annexin V binding to anionic phospholipids [72] and interfere with the fibrinolytic system [105]. Still, the physiological relevance of each of these mechanisms is not proven. A selection of the mechanism is discussed below.

aPL activate a variety of cells. After binding of aPL to the cellular surface in the presence of β_2GPI, endothelial cells upregulate several adhesion molecules (ICAM-1, VCAM-1) and secrete cytokines thereby promoting prothrombotic properties [106;107]. After infusion of aPL, ICAM-1 knockout mice are unable to produce thrombus formation seen in their wild type counterparts suggesting that the expression of adhesion molecules is essential for aPL to create a hypercoagulable condition. In addition, IgG aPL (isolated from APS patients positive for anti-β_2GPI antibodies) are able to induce TF due to activation of endothelial cells via a p38 MAPK dependent mechanism [108]. These findings advocate a pleiotropic effect of aPL on endothelial cell functions, responsible for the eventual procoagulant endothelial phenotype in

APS. As mentioned before, tissue factor (TF), formerly known as thromboplastin, is the key initiator of the coagulation cascade. Isolated IgG form APS patients induce TF expression on isolated peripheral blood monocytes in a p38 and ERK-1/2 MAPK dependent manner [109]. Increased tissue factor activity might be a significant contributor towards the hypercoagulability associated with APS [110].

F(ab')2 fragments of purified IgG from patients effectively increased the phosphorylation of platelet p38 MAPK and calcium-dependent cytosolic phospholipase A2 [111]. Furthermore, aPL treated platelets demonstrate increased phosphorylation of p38 MAPK and production of thromboxane B2 [112]. Platelets, sensitized by β_2GPI/anti-β_2GPI complexes show an increase in deposition on a collagen-containing surface [113]. In this study, interaction between recombinant dimeric β_2GPI and apolipoprotein E receptor 2' (apoER2') on platelets was reported.

Binding of oxidized LDL (oxLDL) to macrophages is a key role in early atherogenesis. This results in foam cell formation with subsequent fatty streak formation. aPL can recognize oxLDL, suggesting their involvement in the pathogenesis of atherosclerosis [114]. In addition, complexes of oxLDL and β_2GPI are detected in plasma of APS patients [115]. Antibodies directed against oxLDL/β_2GPI complexes are present in these APS patients. These data clearly show that aPL activate cells involved in coagulation and atherosclerosis leading to subsequent intracellular signaling. These findings are important to understand the pathophysiology of APS.

Cellular activation is not the result of β_2GPI binding to anionic phospholipids, but the result of binding to specific cellular receptors. Several receptors are suggested, among others apolipoprotein E receptor 2' (apoER2'), a member of the low-density lipoprotein receptor (LDL-R) family, on the platelet surface [113], annexin A2 (also known as annexin II) on monocytes [116], toll like receptors (TLR) on endothelial cells [117] and the scavenger receptor on macrophages [118]. Binding of β_2GPI/anti-β_2GPI antibody complexes to apoER2' is not unique for this receptor. Involvement of the low-density lipoprotein receptor (LDL-R) in the pathogenesis of APS is described in animal models [119]. Thus, other members of the LDL-R family might be involved, including megalin, the low-density lipoprotein receptor (LDL-R), the very low-density lipoprotein receptor (VLDL-R) and the LDL-R related protein (LRP). A variety of cells and tissues involved in the clinical aspects of APS express these receptors, such as platelets, monocytes, endothelial cells, neuronal cells, Kupffer cells, placenta and kidney. Further studies should elucidate the specific role of different receptors in the pathogenesis of APS.

Multiligand Receptors

After ligand binding, cell surface receptors can transduce intracellular signals to the nucleus. The general principle 'one lock-one key' for the interaction between receptors and their ligands was assumed for a long time. However, due to the discovery of the so-called multiligand receptors this original idea was adjusted. Some receptors are capable to bind a range of dissimilar ligands. The multiligand receptor family is a family of membrane-bound receptors that mediate opsonization and endocytosis of ligands. One of these multiligand-binding receptors is the low-density lipoprotein receptor (LDL-R) family. Originally, these receptors were considered to be involved only in the uptake of lipoproteins. However, it is now clear that members of the LDL-R family are involved in binding a variety of ligands followed by induction of intracellular signaling cascades [120]. Therefore, some members of the LDL-R family (LRP, megalin) are designated as scavenger receptors.

The Low-Density Lipoprotein Receptor Family

Traditionally, all members of the LDL-R family are cell surface receptors involved in endocytosis. They function in delivering ligands from the extracellular space to lysosomes for degradation. At present, twelve members are identified in mammals; LDL-R [132], LRP (also known as LRP1) [133], megalin (also known as gp330 or LRP2) [134], LRP1B [135], LRP3 [136], LRP5 [137], LRP6 [138], apoER2 (also known as LRP8) [139], VLDL-R or LR8 [140], a multiple epidermal growth factor (EGF) repeat containing protein, MEGF7 [130], sorting protein-related receptor containing LDL-R class A repeats (SorLA)/LDL-R relative (LR11) [141;142] and LRP12 (formerly known as ST7) [143]. All these members consist of the same structural motifs that resemble the structure of the LDL-R and are shown in Figure 6.

The general vision has been that endocytic receptors mainly regulate the concentration of extracellular ligands and provide these ligands to cells in need of these metabolites. Recent evidence suggests that members of the LDL-R gene family are also involved in signal transduction. The VLDL-R and apoER2' are involved in neuronal development [121], the cytoplasmic tail of LRP serves as a docking site for several adapter proteins involved in signaling [122] and the intracellular domain of megalin interacts with adapter proteins involved in the regulation of endocytosis (Dab2, ARH and megalin binding protein; MegBP) [123;124]. The LDL-R family is classified as a multiligand receptor family as one member can bind several structurally and functionally unrelated ligands. The receptors can bind to proteases [125], protease

inhibitors [126], signaling molecules [127], heat-shock proteins [128], steroid carriers [129], toxins and antibiotics [130] and lipoproteins [131]. Another interesting feature of this receptor family is the interaction with other cell-surface proteins, such as seven transmembrane-spanning receptors, ion-channels, glycosylphos-phatidylinositol (GPI)-anchored proteins or adhesion molecules. Therefore, members of the LDL-R family obtain functions unusual for endocytic receptors. In addition, members of LDL-R family share many functional properties, such as clustering in so-called clathrin-coated pits mediated by adapter-proteins, a pH-sensitive ligand-uncoupling mechanism and recycling of the receptors back to the cell surface after ligand-dissociation. In this section, five members of the LDL-R family are discussed in detail. Table 2 summarizes cell expression and examples of ligands with respect to several members of the LDL-R family.

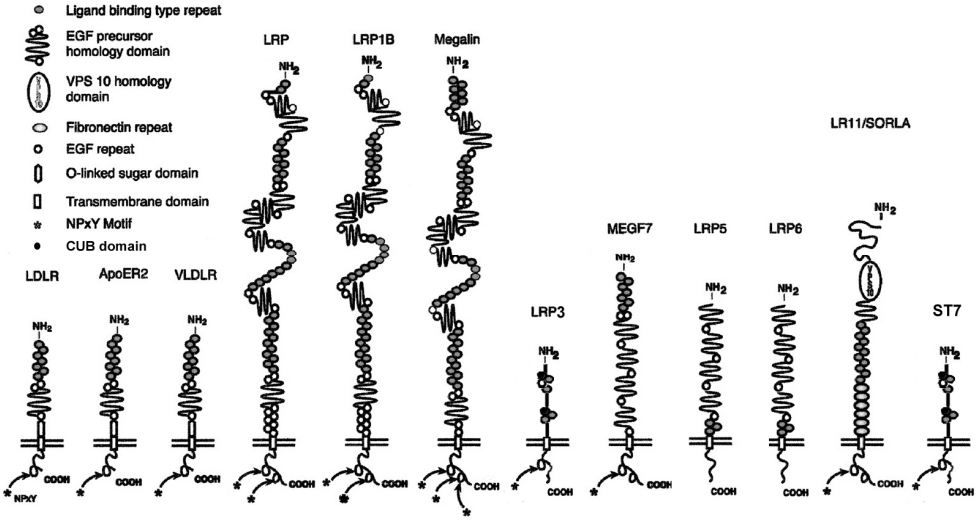

Figure 6. Structural representation of members of the LDL receptor family.
Members of the LDL-R family share common structural motifs, including a single membrane anchor, complement-type repeats that constitute the ligand-binding domains, and epidermal growth factor (EGF) precursor homology domains required for ligand release into endosomes. NPxY designates the four amino-acid motif, Asn-Pro-X-Tyr, that mediates clustering of the receptors in coated pits. O-linked glycans are found in some of the receptors.

Table 2. Expression and ligands of the LDL-R family members

Receptor	Expression	Ligands	Biological function	References
LDL receptor	brain, lung, liver, heart, skeletal muscle, kidney, megakaryocytes	RAP, apoB/E- containing lipoproteins	Cholesterol homeostasis	158-160, 188, 218
VLDL receptor	brain, liver, heart, muscle, adipose tissue	RAP, apoE-containing lipoproteins, pro-uPA, uPA/PAI-1 complex, TFPI, Reelin	Neuronal migration, synaptic transmission	167;168, 188, 218
LRP	brain, lung, liver, adipose tissue, placenta, smooth muscle cells, monocytes, fibroblasts	RAP, apoE-containing lipoproteins, lipases, tPA/PAI-1, TAT, factor VII, lactoferrin	Endocytosis, embryonic development, cell signaling	174;175, 188, 218;219
Megalin	kidney, ileum	RAP, apoB/E-containing lipoproteins, β_2GPI, vitamin D-BP, LDL, VLDL, β_2-macroglobulin	vitamin/nutrient absorption by intestine and kidney, transport across blood-brain barrier	175, 188;189, 217;218
ApoER2	brain, platelets, megakaryocytes, testis	RAP, apoE-containing lipoproteins, lipases, Reelin, LDL, VLDL, β_2-macroglobulin	neuronal migration, synaptic transmission, male fertility	139, 188, 212-215, 218

Common structure of the LDL-R family

The LDL-R family is a family of transmembrane receptors that reside on the cellular surface. The members all consist of the same structural motifs that make up the LDL-R. The modular structures of the extracellular domains of the individual receptors are very similar and remarkably conserved throughout evolution. There are a number of characteristic features of the LDL-R family which include the following [144]: *I*, extracellular ligand-binding domain consisting of complement-type repeats (requirement of calcium for ligand-binding), *II*, epidermal growth factor (EGF) precursor homology domain containing YWTD (Tyr-Trp-Thr-Asp) repeats required for pH dependent ligand release in endosomes [145], *III*, single membrane-spanning anchor. O-linked sugars are present in some of the receptors [146]. The complement-type repeats of approximately 40 amino acids containing six cysteines residues per repeat constitute a ligand-binding motif. These cysteines contribute to the formation of a stable, functional motif. Clusters of repeats constitute a ligand-binding domain and differential clustering of these repeats within a ligand-

binding domain can reveal specificity with regard to ligand binding. In several of the receptors, O-linked sugars separate the ligand-binding domains and the EGF precursor homology domains. The O-linked glycosylation domains keep the ligand away from the cellular surface. All members include a single transmembrane domain consisting of hydrophobic residues that anchors the receptors in the membrane. In contrast to the large extracellular part of the receptors, the shorter intracellular part share very little sequence similarity, with the exception of a short amino acid motif characterized by the consensus sequence NPxY (Asn-Pro-X-Tyr where the X is any amino-acid). This motif mediates clustering of the LDL-R in coated pit before endocytosis [147].

LDL Receptor

After the discovery in 1974 of a cellular pathway for trafficking plasma LDL into cells, the LDL-R was identified in 1986 [132]. This receptor, known as the apoB/E receptor, regulates cholesterol homeostasis by receptor-mediated endocytosis of lipoprotein particles (apolipoprotein E (apoE) and apoB). The LDL-R is also termed the apoB/E receptor referring to its ability to bind both apoB- and apoE containing lipoproteins [131;148;149]. The gene encoding for the LDL-R is located on chromosome 19p13 [150]. Mutations in the gene encoding for the LDL-R in humans, rabbits or mice result in increased plasma LDL particles (familial hypercholesterolemia; FH) and the development of atherosclerotic lesions at an early age [151-153]. The mature LDL-R is a cell-surface, transmembrane receptor consisting of 839 amino acids (160 kDa) [154]. The receptor contains three types of extracellular protein modules. A stretch of 292 amino acids at the N-terminal part of the ligand-binding domain is characterized by seven cysteine-rich, complement type repeats (each consisting of 40 residues) which vary in some amino acids. These modules are known as LDL-R type A (LA). Proper folding of the LA modules requires the presence of calcium ions [155;156]. Complement type repeats 1 and 2 and EGF precursor repeat B are not required for binding of apoB/E to the receptor, whereas complement type repeats 3-7 and EGF precursor repeat A are required for optimal binding of apoB [144]. Complement type repeat 5 is crucial for apoE binding. The negative charges at the carboxy terminus of each of the seven repeats participate in the binding of lipoprotein(s) via cationic residues on apoB/E [157]. The receptor also contains a 400-residue region immediately after the LA modules. This part of the receptor consists of two endothelial growth factor (EGF) precursor homology domains, followed by an YWTD motif and a third O-linked glycosylated EGF precursor homology. This is the least conserved domain of the receptor and is not involved in receptor function.

The transmembrane segment is followed by a cytoplasmic tail of 50 amino acids that also functions in the sorting of the receptor to the basolateral membrane in polarized cells. The LDL-R is expressed in most of the tissues in the human body, with the highest expression on liver cells (Kupffer cells and parenchymal cells) [158;159] and cells of the adrenal gland [160]. In addition, LDL uptake by the LDL-R is also observed in the kidney, spleen and heart [160]. Human platelets do not express the LDL-R since *i*. binding of LDL to platelets is independent of divalent cations [161] and *ii*. an antibody directed against the ligand-binding domain of the LDL-R does not influence binding of LDL to platelets [162]. In contrast, promegakaryoblasts do express the LDL-R [163]. Apart from regulating the plasma concentrations of apoB/E it is proposed that the LDL-R acts in concert with the LDL-R related protein (LRP), another member of the LDL-R family, in regulating plasma levels of factor VIII *in vivo* [164]. This represents a previously unrecognized link between LDL-R and haemostasis.

VLDL Receptor

In 1992, Takahashi *et al*. first cloned the VLDL-R by low stringency hybridization from rabbit heart VLDL-R-subtracted cDNA library by [165]. This receptor is remarkably similar to the LDL-R. Not only the cDNA but also the gene structures are very homologous. In 1994, the same group cloned human VLDL-R cDNA from the THP-1 cDNA library [166]. The gene encoding for the VLDL-R is located on chromosome 9p24. The human VLDL-R contains 19 exons spanning approximately 40 kb. A significant difference is the addition of an extra exon that encodes an additional complement-type repeat in the ligand-binding domain of the VLDL-R. The VLDL-R therefore contains an 8-fold repeat, whereas the LDL-R includes a 7-fold repeat. Two variants of the receptor exist [166]. The polymorphisms result from the presence/absence of an 84 bp region coding for the serine/threonine-rich *O*-linked glycosylation motif. This difference only affects the size of the receptor and not its function. VLDL-R mRNA tissue distribution is rather different from the distribution of LDL-R mRNA; highly abundant in extrahepatic tissues, e.g. the heart (myocardium), muscle, adipose tissue and developing adult brain, but barely detectable in the liver [167]. Platelets do not express the VLDL-R. In human and rabbit atherosclerotic lesions the VLDL-R is expressed in macrophages and smooth muscle cells (SMC) [168]. Thus, the VLDL-R may function in the uptake of lipoproteins in tissues that are active in the metabolism of fatty acids. However, VLDL-R knockout mice did not show any lipoprotein abnormality although these mice showed a reduction in adipose tissue mass [169]. The VLDL-R might be involved in neuronal development. The VLDL-R can

bind Reelin, a large extracellular glycoprotein on their extracellular domains that subsequently induces phosphorylation of Disabled 1 (Dab1) [170].

Low-density lipoprotein Receptor-related Protein

The first identified LDLR-related protein was LRP1. It was cloned in 1988 and identified as a receptor for α_2-macroglobulin [133]. LRP1 is a very large hetero-dimeric cell surface protein synthesized as a single polypeptide precursor protein of 4525 amino acids, consisting of 515- and 85 kDa subunits [171]. Both subunits serve different functions [172]. The precursor protein is cleaved in the *trans*-Golgi-apparatus by a furin-like protease into a α-chain (3925 amino acids) and a β-chain (600 amino acids). The α-chain contains several ligand-binding sites and is entirely extracellular. The β-chain contains an extracellular domain, the transmembrane domain, and a cytoplasmic domain containing the NPXY motifs. The ligand-binding sites in the α-chain are similar to those found in the LDL-R and are clustered as 2, 8, 10 and 11 complement-type repeats in four domains and also includes O-linked glycosylation sites. Ligand binding to the extracellular domain activates LRP due to dimerization [173]. In this process, the cytoplasmic domain performs a catalytic function. LRP is abundantly present in various tissues such as liver, placenta, lung, brain and embryonic tissues, and is expressed in an array of cell types, including parenchymal cells, Kupffer cells, neurons, astrocytes, smooth muscle cells, monocytes, adipocytes, and fibroblasts [174;175]. Ligand binding, as for the LDL-R, depends on the presence of calcium ions [133;176]. The receptor is involved in the catabolism of several unrelated proteins, proteinase-inhibitor complexes [125;177;178], Kunitz-type inhibitors [179] and matrix proteins [180;181]. This extensive range of ligands and properties suggests a role for the receptor in many physiological- and pathophysiological processes ranging from lipoprotein metabolism [182], fibrinolysis [182;183], cell growth and migration [184] to atherosclerosis [185]. Furthermore, LRP has a function in haemostasis, as coagulation factor VIII binds to the receptor [186]. Furthermore, plasminogen activator inhibitor-1 (PAI-1) promotes the clearance of thrombin by LRP at sites of endothelial injury [187].

Megalin

Megalin is an endocytic receptor on the apical plasma membranes of proximal tubule cells (PTC) in the kidney [188;189]. The receptor is involved in the reabsorption and metabolism of various proteins filtered by glomeruli. In addition, the thyroid and parathyroid gland [190;191], visceral yolk sac [192] and the neuro-ectoderm [193] express megalin. Megalin was first identified as a target antigen for antibodies in Heymann

nephritis [194]. Like LRP, megalin is, for its ability to bind a variety of ligands, a scavenger receptor. The protein is expressed as a single polypeptide of 4660 amino acids (600 kDa) containing 36 cysteine-rich ligand-binding domains, 16 EGF precursor homology domains and 40 YWTD repeats in the extracellular domain. Megalin is cloned from rat [134] and human [195] cDNA, where it is located on chromosome 2. Like LRP, there are four clusters of ligand-binding domains consisting of 7, 8, 10 and 11 complement-type repeats that recognize several groups of ligands. In addition, the extracellular domain interacts with the large glycoprotein cubulin that is important for the megalin-cubulin dependent uptake of vitamin B12 and vitamin D by the proximal tubule cells [196-198]. The intracellular domain of megalin contains three NPXY motifs, which can interact with signaling adapter molecules known to be involved in endocytosis (including Dab2, ARH and MegBP) [199-201]. The overall sequence of the intracellular domain is different from that of other members of the LDL-R family. It contains Src-homology binding sites, casein-kinase sites and protein kinase phosphorylation sites, indicating that megalin may be involved in signaling [120;202]. One of the best-characterized functions of megalin is the proximal-tubular reuptake of low-molecular weight proteins, such as vitamin D-binding protein from the glomerular filtrate. Megalin knockout mice develop severe proteinuria and vitamin-D deficiency, demonstrating an *in vivo* role for megalin in the uptake of proteins by the kidney. Megalin is also involved in drug transport [203]. Megalin is also implicated in binding β_2GPI [204].

Apolipoprotein E Receptor 2

The gene coding for the apolipoprotein E receptor 2 (apoER2) was cloned from cDNA library of human placenta in 1995 [139]. The amino acid sequence of the cDNA revealed that human apoER2 consists of five functional domains resembling the LDL- and VLDL receptor. The gene encodes a protein of 963 amino acids with a molecular weight of 105 kDa and is located on chromosome 1p34 [205]. The N-terminal 294 amino acid sequence of human apoER2 is composed of a 40-amino acid sequence repeated 7 times, with characteristics similar to those of the complement-type, cysteine-rich repeats in the ligand-binding domains of the LDL-R and VLDL-R. Approximately 50% of the amino acids in each repeat in the ligand-binding domain of human apoER2 are identical to that in the human LDL-R and VLDL-R. Despite that human apoER2 and the LDL-R contain the same number of complement-type repeats, the ligand-binding domain of apoER2 is closer related to that of the VLDL-R than the LDL-R, regardless of an extra repeat in the VLDL-R. The EGF precursor homology domain consists of three growth factor repeats and

55% of the amino acids are identical to those in the LDL-R and VLDL-R. The amino-acid sequence in the O-linked sugar domain in apoER2' is for approximately 21% and 27% identical with the corresponding domains in the VLDL-R and LDL-R. The amino acid sequence in the transmembrane domain of apoER2 is poorly conserved compared to other members of the LDL-R family. Approximately 35% of the amino acids in this domain are similar to those found in the LDL-R and the VLDL-R. The cytoplasmic domain of apoER2 includes 115 amino acids, which is about 2-fold more than the amount of amino acids in the cytoplasmic domain of the LDL- and VLDL-R (50 and 54 amino acids, respectively). A unique insertion of 59 amino acids in the cytoplasmic domain of apoER2 may comprise a signal for specific localization of the receptor in cholesterol-rich microdomains in the neural cell membrane [206]. The amino-terminus of apoER2 is very similar to the amino acid sequence surrounding the coated pit signal of the LDL-R and the VLDL-R. This may imply clustering of apoER2 in coated pits thereby mediating its ligand internalization [207].

Several studies show the existence of tissue- and species-specific splice variants of apoER2. In chicken, two splice variants of the ligand-binding domain with seven or eight LA repeats are identified [208]. However, in mice three splice variants of which none contains repeats 4 — 6 exist [209]. In addition, two human splice variants are reported; one containing only repeat 1 — 3 and a second splice variant which lacks repeat 4 — 6, but contains an additional LA repeat (that comprise the ligand-binding domains) and a unique cysteine-rich domain present [210;211]. The human splice variant of apoER2 lacking repeat 4 — 6 is also identified on human platelets and is designated as apoER2' [212].

Ligand degradation by apoER2 is low, compared to the other LDL-R family members. This suggests a different function than endocytosis. Indeed, apoER2 has an important function in neuronal development, as Reelin can bind apoER2 expressed by neurons [213-215]. Consequently, intracellular Dab1, which binds to the cytoplasmic domains of apoER2, becomes tyrosine phosphorylated. In addition, deletion of the gene encoding apoER2 results in cortical layering defects. Binding of Reelin to apoER2 clusters apoER2 on the cell surface, subsequently followed by Dab1 dimerization/oligomerization [207]. This process is sufficient to transmit the signal and does not appear to require any co-receptor. Brain and placenta mainly express apoER2, but also vascular cells and platelets express the receptor. This abundant expression in the brain supports the role of apoER2 in brain development. Studies using experimental animal models do not report abnormalities with respect to thrombosis or atherosclerosis. The contribution to lipid metabolism is probably limited [216].

Outline of this thesis

The pathological mechanisms responsible for the heterogeneous clinical manifestations observed in the antiphospholipid syndrome (APS) are largely unknown. We hypothesize that the low-density lipoprotein receptor (LDL-R) family is involved in the pathology of APS. The platelet receptor apolipoprotein E receptor 2' (apoER2') can bind to our recombinant form of dimeric β_2GPI. Binding of this recombinant protein to apoER2' results in platelet activation and downstream signaling, involving p38 MAPK phosphorylation and thromboxane synthesis. To understand the pathophysiology of APS, it is of importance to understand the molecular interaction between cellular receptors and their ligand(s).
The aim of this thesis is to investigate the interaction between recombinant dimeric β_2GPI and members of the LDL-R family on a molecular level. This objective will be addressed following a review of the interaction between β_2GPI and LDL-R family members and their role in the antiphospholipid syndrome (*Chapter 2*). The following questions are addressed:

1. As apoER2' is the only member of the LDL-R family present on platelets, our interest is focussed on this receptor (Chapter 3).
   Is dimerization of β_2GPI by anti-β_2GPI antibodies a prerequisite for binding of β_2GPI to apoER2'?
   Which domain of dimeric β_2GPI is recognized by apoER2'?

2. Are other members of the low-density lipoprotein receptor family able to bind dimeric β_2GPI (*Chapter 4*)?
   Is binding of dimeric β_2GPI to the LDL-R family members characterized by differences in affinity?

3. Which epitope in domain V of β_2GPI is recognized by LDL-R homologues (*Chapter 5*)?
   Does this epitope coincide with the phospholipid binding site in domain V of β_2GPI?

4. Is a conformational change within β_2GPI necessary to induce binding of β_2GPI to a surface (*Chapter 6*)?

Finally, the findings of these investigations are discussed in relation to published data on the LDL-R family in order to propose a mechanism by which β_2GPI/anti-β_2GPI antibody complexes cause the clinical manifestations in the antiphospholipid syndrome (*Chapter 7*).

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BETA2-GLYCOPROTEIN I AND LDL-RECEPTOR FAMILY MEMBERS

2

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Introduction

The antiphospholipid syndrome (APS) is a non-inflammatory disease characterized by the presence of antiphospholipid antibodies (aPL) in the plasma of patients with venous and/or arterial thrombosis or pregnancy morbidity [1;2]. Despite more than 20 years of active research, we do not understand the causal connection between the presence of antiphospholipid antibodies and increased thrombogenicity. A large number of possible pathophysiological explanations have been suggested during these years, however, none of them survived when large cohorts of patient samples were tested [3]. Since the discovery of aPL it is recognized that the term aPL comprises a heterogeneous population of antibodies. It took us, however, rather long to recognize that not all aPL are pathological. There is now firm evidence that only subpopulations of aPL are relevant for the clinical complications that go along with APS. Most studies performed till now on the clinical relevance of possible pathological mechanisms of aPL were polluted by including in the evaluation non-pathological anti-phospholipid antibodies, which might explain the disappointing results of most of these studies. New studies on the correlation between clinical manifestations and the presence of certain subpopulations of aPL suggested that specially anti-β_2GPI antibodies that were able to prolong clotting times are clinical relevant [4]. By selection of only plasma samples with aPL that are relevant, by using new tools and by following new leads how these antibodies might function, we now finally seem to come to the bottom of the mysterious correlation between the presence of aPL and thrombosis. This review will discuss the progress we have made on the pathophysiology of the syndrome so far.

The relevant antibodies

The presence of aPL in plasma of patients can be detected by either prolongation of phospholipid dependent coagulation tests (lupus anticoagulants, LAC), or by solid phase immune assays. Originally, it was thought that aPL were directed against negatively charged phospholipids and therefore cardiolipin was the antigen of choice in the ELISAs. We now know that plasma proteins, in particular β_2-Glycoprotein I (β_2GPI) and prothrombin, are the antigens to which aPL antibodies are directed [5-8]. Antiphospholipid antibodies that cause LAC activity and those resulting in a positive anticardiolipin ELISA are closely related but not identical antibodies. LAC can be caused by anti-prothrombin or anti-β_2GPI antibodies, while detection of antibodies with the anticardiolipin ELISA should be directed against β_2GPI. Meta analyses on the predictive value of the different types of aPL antibodies showed that antibodies that induce LAC activity correlate best with a history of thrombo-embolic complication [9]. Apparently, the antibodies that inhibit a biological activity are more relevant than antibodies that only recognize the protein. In a recent study we have shown that patients with a β_2GPI-dependent LAC activity have a very high correlation with a history of thrombo-embolic complications whereas for patients with a LAC not dependent on β_2GPI hardly any correlation was found [4]. Thus, anti-β_2GPI antibodies that cause LAC activity are the best candidates to explain the increased thrombogenicity of patients with the antiphospholipid syndrome. The intriguing question, however, is why this particular set of antibodies is the thrombogenetic one.

A long puzzle

In a LAC assay, the presence of aPL is detected by a prolongation of a clotting times, an assay normally used to detect a bleeding tendency. It is difficult to envision how these antibodies that are detected with assays for a bleeding tendency can cause thrombosis. Is there a link between the way the antibodies induce a prolongation of an in vitro clotting assay and the pathophysiology seen in patients with these antibodies circulating in their blood? Now we do know why the antibodies induce a prolongation of clotting tests. The antibodies dimerize β_2GPI or prothrombin, thereby increasing their affinity for negatively charged phospholipids [10;11]. Negatively charged phospholipids are essential cofactors in normal coagulation [12]. Dimerization of β_2GPI or prothrombin increases the affinity for phospholipids to such an extent that binding competes with that of clotting factors, and coagulation is inhibited. An important observation was that β_2GPI, dimerized after binding of antibodies, not only has an increased affinity for phospholipids but also for

cellular surfaces. The increased affinity of the complex results in binding of β_2GPI to cells. In particular increased binding of β_2GPI to endothelial cells, monocytes and platelets have been shown after interaction with anti-β_2GPI antibodies [13;14]. Important progress in unravelling the apparent contradiction between in vitro testing and in vivo clinical observations was the notion that binding of β_2GPI to cells resulted in activation of these cells [15;16]. Activation of endothelial cells, monocytes or platelets results in a shift towards a more thrombotic phenotype. Anti-prothrombin antibodies also induce dimerization of prothrombin and an increased affinity to negatively charged phospholipids and cells. However, in contrast to β_2GPI, binding of prothrombin-antibody complexes to platelets does not result in activation of platelets (*unpublished results*). Thus, binding of antibody complexes to platelet surfaces is not enough to activate them. There must be an additional interaction of β_2GPI–antibody complexes at the platelet membrane to explain why these complexes can activate platelets

Platelet activation

The central importance of platelets in the development of arterial thrombosis and cardiovascular disease is well established [17]. An increased tendency of platelets to aggregate is an important risk factor for the development of cardiovascular disease and inhibition of platelet function is the therapeutic option in the management of high-risk patient populations. The measurement of possible platelet activation in vivo is very difficult due to the fact that collecting of blood by itself can already activate platelets. A reliable assay to measure in vivo platelet activation is the measurement of thromboxane metabolites present in urine [18]. Forastiero *et al*. showed that patients with the antiphospholipid syndrome have increased levels of 2,3 dinorthromboxane B2 in their urine, an important indication that activated platelets do circulate in this patient group [19]. There are also several in vivo studies that show that antiphospholipid antibodies can activate platelets [20-22].

It is difficult to envision that mere binding of β_2GPI to negatively charged phospholipids on the surface of cells activates these cells. Other proteins with high affinity for negatively charged phospholipids such as annexin A5 and prothrombin have been shown to bind to platelets without activating them. However, we and others have clearly shown that binding of β_2GPI/anti-β_2GPI-antibody complexes to platelets resulted in activation of these cells [13;22]. In general, cells are activated after binding of a ligand to a receptor. Therefore, we started a search for platelet membrane receptor proteins that can bind β_2GPI or β_2GPI-antibody complexes. Major progress was made when we found that RAP (receptor associated protein),

a universal inhibitor protein that prevents the binding of all ligands to every member of the LDL-receptor family, was able to inhibit the platelet activation induced by β_2GPI-antibody complexes. As apo-ER2' is the only member of the LDL-receptor family on platelets, our interest was focussed on this receptor protein [23]. We have now shown that β_2GPI-antibody complexes activate platelets and induce thromboxane synthesis in an apo-ER2' dependent way. We have proposed a mechanism by which the antibody complexes pre-activate platelets to such an extent that they become more sensitive for other agonists [15] (see below). Apart from apoER2', two other proteins have been suggested as possible receptors for β_2GPI, annexin A2 on monocytes and a Toll-like receptor on endothelial cells [16;24]. Further studies should elucidate the respective roles of the different receptors. However, there is evidence that the binding of β_2GPI antibody complexes to LDL-receptor family members is not unique for apoER2. It is now recognised that β_2GPI-antibody complexes and for some members also β_2GPI alone can bind to all of the members of the LDL-receptor family tested so far (Megalin, apo-ER2', LRP-1 and VLDL). These other family members are expressed on a number of other cells (e.g. endothelial cells, monocytes, neuronal cells, smooth muscle cells) that are thought to be involved in various clinical aspects of the antiphospholipid syndrome. Inhibition of binding of β_2GPI-antibody complexes to endothelial cells by RAP also inhibits the induction of tissue factor expression.

LDL-receptor family

Cell surface receptors can transduce signals from the extracellular environment to the nucleus of cells, so that the cells can adapt themselves to a changing environment [25]. It has been assumed that this process was very specific and a 'one ligand-one receptor' notion was a general rule. However, this concept is due to revision with the discovery of so-called multiliga family members. The first identified member was the low-density lipoprotein receptor (LDLR). The family members contain a characteristic set of structural domains that include ligand binding repeats composed of cysteine rich, small complement type (CR) domains, EGF-like domains, six-bladed β-propellor domains with multiple copies of a YWTD consensus sequence, a single membrane spanning domain and a cytoplasmic domain that harbours NPxY motifs. The presence of repetitive negative charges in the CR domains is responsible for the binding of the ligands. Two CR domains are sufficient for high affinity binding. Some ligands bind to identical or partially overlapping sites, while other ligands have unique binding sites. The binding domain(s) for β_2GPI is/are unknown. Not all family members have the same number of CR domains and

it has been shown that besides the CR domains, the other domains can participate in the binding of ligands.
A number of different natural occurring variants of the apoER2' have been described and some of them seem to be cell and tissue specific [27]. ApoER2 present in plates lacks binding repeats 4-6 (apoER2delta4-6) but contains the full-length cytoplasmic tail. It has been shown that apoER2 in platelets is involved in cellular signalling and induction of the synthesis of NO. Interestingly, in the first publication on the role of apoER2 in platelets, it was proposed that this receptor might be involved in inhibition of platelet function [23]. In the two publications that followed shortly, apoER2 was suggested to be responsible for enhanced platelet aggregability [15;28].

A model of platelet activation in the presence of anti-ß$_2$GPI antibodies

The binding of β_2GPI-antibody complexes to the phospholipids of the cellular membrane is a prerequisite before physiological concentrations of β_2GPI can bind to apoER2'. Thermodynamically this can be understood because binding to a membrane reduces the entropy of the receptor-ligand interaction, allowing also low affinity interactions. Alternatively, due to mass action effects the local concentration of β_2GPI is high enough for this interaction. A third explanation is that binding of the antibodies to β_2GPI induces a conformational change into β_2GPI, exposing a neo-epitope that is involved in the interaction with the receptor [29]. Thus, the binding of β_2GPI-antibody complexes to negative charges on cellular surfaces positions β_2GPI optimally for a subsequent interaction with apoER2'. Of importance, the negative charges on a cellular surface do not necessarily be provided by phospholipids as heparan sulphates might also provide negative charge [30]. The role of the anti-β_2GPI antibodies is that it supplies β_2GPI with enough affinity to bind to cells.
After binding to the cellular surface, β_2GPI interacts with apoER2' (*Figure 1*). Both domains I and domain V of β_2GPI are able to interact with members of the LDL-receptor family. Although the affinity of domain V for LDL family receptors is much higher than the affinity for domain I, a monoclonal antibody directed against domain I inhibits platelet activation completely, pointing to an important role of domain I in this interaction. The interaction of β_2GPI to apoER2' resulted in phosphorylation of apoER2' followed by phosphorylation of p38 MAP kinase and synthesis of thromboxane A2. Thromboxane A2 mediates further activation of the platelets.
The activation of cells by antiphospholipid antibodies is normally weak and not enough to fully activate the cell. Antiphospholipid antibodies make the cells more sensible for other activators. Alternatively, it enables that other activators can

activate cells at lower concentration. A second hit always seems necessary. This explains why although the antibodies are permanently present in the plasma of patients, the patients do not suffer continuously from thrombotic complications. Only the risk to develop thrombosis is increased.

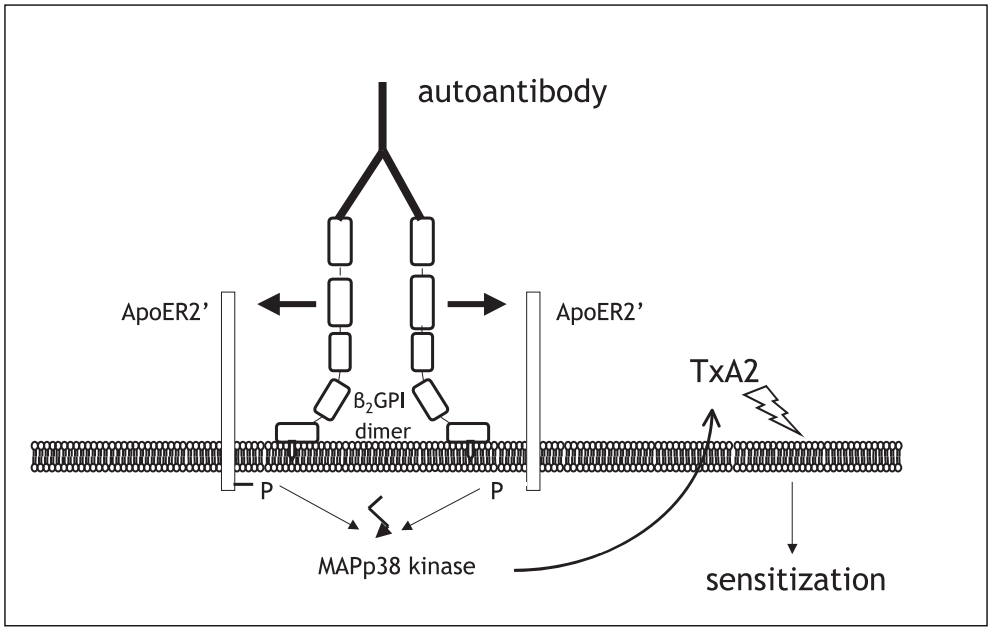

Figure 1. A model describing how anti-ß2GPI antibodies could sensitise platelets.

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THE BINDING SITE IN BETA2-GLYCOPROTEIN I FOR APOER2' ON PLATELETS IS LOCATED IN DOMAIN V

3

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Abstract

The antiphospholipid syndrome (APS) is caused by auto-antibodies directed against β_2-glycoprotein I (β_2GPI). Dimerization of β_2GPI results in an increased platelet deposition to collagen. We found that apolipoprotein E receptor 2' (apoER2'), a member of the LDL receptor family, is involved in activation of platelets by dimeric β_2GPI. To identify which domain of dimeric β_2GPI interacts with apoER2', we have constructed domain deletion mutants of dimeric β_2GPI, lacking domain I (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 β_2GPI; delta V had no effect on the clotting time. Secondly, delta I and delta II bound to anionic PL, comparable to full length dimeric β_2GPI. Delta V and the Trp316Ser mutant bound with decreased affinity to anionic PL. Platelet adhesion to collagen increased significantly when full length dimeric β_2GPI, delta I or delta II (mean increase 150%) was added to whole blood. No increase was found with plasma β_2GPI, delta V or the Trp316Ser mutant. Immunoprecipitation indicated that full length dimeric β_2GPI, 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 β_2GPI to anionic PL and in interaction with apoER2' and subsequent activation of platelets. The binding site in β_2GPI for interaction with apoER2' does not overlap with the hydrophobic insertion loop in domain V.

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 2 3 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 (β_2GPI 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 β_2GPI has been found in a variety of cells such as trophoblasts, placental cells, endothelial cells and neurons [7 8 9]. The mature sequence of human β_2GPI consists of 326 (44 kDa) amino acids (aa) with four N-linked glycosylation sites. It is composed of five repeating units that belong to the complement control protein (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 11 12 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 β_2GPI harbors another cationic region. Involvement of this region in binding to PL has also been described [16]. Apolipoprotein E receptor 2 (also known as apoER2 or LRP8) is a member of the low density lipoprotein (LDL) receptor family. It has been identified by Kim *et al*. in 1996 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 21 22 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 β_2GPI can interact with apoER2'on platelets [25 22]. Until now, little is known about the interaction between (dimeric) β_2GPI and platelets. As for phospholipid binding, cationic patches might play an important role, because ligand-binding to apoER2' is dependent on electrostatic interactions [26]. 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 β_2GPI to apoER2'on platelets. To understand the mechanism of the interaction between apoER2' and dimeric β_2GPI it is essential to know; *i*. which domain(s) and *ii*. what structures in these domains are involved in interaction with apoER2'.

Experimental procedures

Construction of dimeric constructs of β_2GPI

The dimer apple 4 – β_2GPI and the apple 2 – β_2GPI, which is not able to form dimers, were constructed as described previously [27]. To exclude the possibility that apple 4 – β_2GPI binds via the dimerization domain of factor XI (apple 4), dimer apple 4 was constructed. The sequence of dimeric apple 4 was amplified from the vector apple4 – tissue – type plasminogen activator (tPA)-S478A with the primers apple 4-*Bgl* II (GCC AGA TCT TTC TGC CAT TCT TCA) and apple 4-*Xba* I (GGT CTA GAC TCG AGT CCC TCC TTT GAT GCG TG). The PCR product was subcloned into the vector pCR®2.1-TOPO® (Invitrogen, Breda, The Netherlands), and cloned into the vector apple 4 – tPA – S478A with *Bgl* II and *Xba* I (underlined in apple 4-*Bgl* II and apple 4-*Xba* I, respectively). The starting point for the construction of the domain deletion mutants (DM) was the full-length cDNA of apple 4 – C321S – β_2GPI (in short apple 4 – β_2GPI) cloned into the vector apple 4 – C321S – tPA – S478A. The domain I deletion was constructed with the primers domain II β_2GPI-*Xho* I forward (CC CTC GAG AT CCC AGA GTA TGT CCT TTT GCT G) and β_2GPI-*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 β_2GPI-*Xho* I forward (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and domain I β_2GPI reverse (TGT ACA TTT CAG AGT GTT GAT G) and for domain III-V amplification the primers domain III β_2GPI forward (ACT CTG AAA TGT ACA CCC ATC ATC TGC CCT CCA CCA) and β_2GPI-*Xba* I reverse. These two products served as a template in a second PCR to amplify the full-length domain I deletion using β_2GPI-*Xho* I forward and β_2GPI-*Xba* I reverse. The domain V deletion was constructed with the primers β_2GPI-*Xho* I forward and domain IV β_2GPI-*Xba* I reverse (TCT AGA TCA TTT ACA ACT TGG CAT GGC AGA CCA). To construct fusion proteins of apple 4 and the domain deletion mutants of β_2GPI, the PCR product was cloned with *Xho* I and *Xba* I into the vector apple 4 – C321S – tPA – S478A. In this way DM of apple 4 – β_2GPI 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-SRß-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 β_2GPI 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 β_2GPI-ELISA. Domain deletion mutants of apple 4 – β_2GPI fusion proteins were purified from cell culture medium with a monoclonal antibody against β_2GPI (21B2) coupled to a CNBr-activated Sepharose column (Amersham 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 apple 4 – β_2GPI were pooled, concentrated with polyethylene glycol (PEG) and dialysed against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Apple 4 was purified using monoclonal antibody XI-1 (generous gift of Dr. J.C.M. Meijers, Academic Medical Hospital, Amsterdam), which recognizes the apple 4 domain, coupled to CNBr-activated Sepharose. Bound proteins were eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris, pH 9. For sh-apoER2' production HEK293-EBNA cells were transfected by the DNA-PEI method according to Durocher et al [29]. Sh-apoER2' production was done in a 1 liter suspension culture (in medium containing 90% freestyle, 10% calcium free DMEM, 0.5 % fetal calf serum, Invitrogen) for 4 days. Sh-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 β_2GPI

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

Preparation of phospholipid vesicles

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

Binding of domain deletion mutants to phospholipid vesicles

Binding of DM of apple 4 – β_2GPI to PS/PC vesicles was tested in a solid phase binding assay. High binding 96 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), a generous gift of Dr. J. Arnout, Leuven, Belgium. Apple 4 – β_2GPI was used as a positive control and plasma β_2GPI as a negative control. 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, apple 4 – β_2GPI, plasma β_2GPI (final concentration 100 μg/ml) or buffer were incubated for 30 min at 4°C, followed by an incubation of 90 s at 37 °C. Clotting was initiated by the addition of 50 μl of Innovin (Dade Behring, Marburg, Germany).

Blood collection for perfusion experiments

Freshly drawn venous blood was collected from healthy donors (with informed consent) into 1/10 of volume of 3.2% 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 β_2GPI, apple 4, apple 2 – β_2GPI, apple 4 – β_2GPI, DM, or buffer were added to whole blood 5 min before the start of the perfusion at a concentration of 100 µg/ml and incubated at 37°C. The prewarmed blood was drawn through the perfusion chamber by an infusion pump (pump 22, model 2400-004; Harvard, Natrick, MA). Afterward, the coverslips were taken from the perfusion chamber and rinsed with Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4), fixed in 0.5% glutaraldehyde in PBS, dehydrated with methanol, and stained with May-Grünwald/Giemsa as described previously. Platelet deposition was evaluated with a light microscope equipped with a JAI-CCD camera (Copenhagen, Denmark) coupled to a Matrox frame grabber (Matrox Electronic Systems Ltd., Quebec, Canada) using Optimas 6.2 software (Optimas Inc., Seattle, WA) for image analysis. Evaluation for platelet adhesion was performed on 20 fields, perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Results are expressed as mean relative coverage (mean ± SD, n = 3). Statistical analysis was performed with a Student T-test.

Binding of apple 4 – β_2GPI to immobilized soluble human apoER2'

Binding of apple 4 – β_2GPI 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 β_2GPI, apple 4, apple 2 – β_2GPI, apple 4 – β_2GPI, apple 4 – β_2GPI – Trp316Ser, delta V (3 µg/mL, 50µL/well) or plasma β_2GPI (3 µg/mL) in the presence of a mouse monoclonal α-β_2GPI antibody (19H9; 1 µg/mL) in PBS/1%BSA, bound protein was detected using rabbit polyclonal anti-β_2GPI (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 apple 4 – β_2GPI. Results are expressed as mean ± SD (n = 3).

Association of apple 4 – ß$_2$GPI with sh-apoER2' in the presence of inhibiting peptides

Binding of apple 4 – β_2GPI to sh-apoER2' was further investigated using peptides with the following sequences; VSRGGMRK (representing a cationic patch at aa position 37-44 in domain I of β_2GPI), CKNKEKKC (representing a cationic patch at aa position 282-287 in domain V of β_2GPI) and EKCKNKCK (scrambled). Hydrophobic 96 well ELISA plates were coated with 5 µg/mL of sh-apoER2' in PBS (50µL/well). Wells were blocked with PBS/4% BSA (150µL/well). After incubation with apple 4 – β_2GPI (3 µg/mL, 50µL/well) with or without increasing concentrations of peptides (0 – 500 µg/mL), wells were subsequently incubated with a rabbit polyclonal anti-β_2GPI antibody (1:500; 50µL/well) and SWARPO (1:500, 50µL/well). This was followed by a staining procedure using OPD. Binding of apple 4 – β_2GPI 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 β_2GPI, apple 4 – β_2GPI, apple 4 – β_2GPI – Trp316Ser or DM of apple 4 – β_2GPI (final concentration 100 µg/mL). Incubations were performed in the presence of 3 mM $CaCl_2$. For competition experiments, proteins (final concentration 100 µg/mL) were incubated with the inhibiting peptides (final concentration 500 µg/mL) for 5 min at 37°C. As control, platelets were incubated with buffer and the inhibiting peptides. Afterwards, platelets were lysed on ice with 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid), containing 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-β_2GPI 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

Expression and Purification of domain deletion mutants

To study the effect of domain involvement of apple 4 – β_2GPI on PL-binding, clotting time and platelet adhesion, dimeric constructs of β_2GPI fused to the C terminus of the dimerization domain (apple 4) of factor XI were made. BHK cells were transfected with expression vectors containing DM of apple 4 – β_2GPI. Protein expression was confirmed by western blotting using an anti-β_2GPI monoclonal antibody. Cell lines with the highest expression were selected using a β_2GPI enzyme linked immunosorbent assay (ELISA). The proteins were purified using a monoclonal α-β_2GPI antibody (Moab 2B2) 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 apple 4 – β_2GPI (*D*), apple 4 – β_2GPI – Trp316Ser (*W*), apple 4 – β_2GPI – Δ1β_2GPI (Δ*I*), apple 4 – Δ2β_2GPI (Δ*II*) and apple 4 – Δ5β_2GPI *(*Δ*V*) migrated as monomers with an apparent molecular mass of 50 kDa. Plasma β_2GPI (*M*) migrated with a molecular mass of 45 kDa under non-reducing conditions. Under reducing conditions full-length apple 4 – β_2GPI and apple 4 – β_2GPI – Trp316Ser migrated with a molecular mass of approximately 62 kDa. Apple 4 – Δ1β_2GPI, apple 4 – Δ2β_2GPI and apple 4 – Δ5β_2GPI migrated slightly slower with a molecular mass of approximately 56 kDa. Plasma β_2GPI migrated with a molecular mass of approximately 52 kDa.

Effect of apple 4 – β_2GPI domain deletion mutants on clotting time

To study the role of individual domains of apple 4 – β_2GPI for competing with coagulation factors, we studied the effect of the DM on clotting time. For this purpose we performed the prothrombin time (PT). Concentrations of 200 µg/mL plasma derived β_2GPI, apple 4 – β_2GPI or DM, diluted in TBS, were mixed 1:1 with normal pooled plasma (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 apple 4 – β_2GPI; apple 4 – β_2GPI showed a relative prolongation of the clotting time 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 β_2GPI, delta V or apple 4 – β_2GPI – Trp316Ser to NPP did not influence the clotting time. Furthermore, the control proteins apple 4 and apple 2 – β_2GPI did not influence the clotting time.

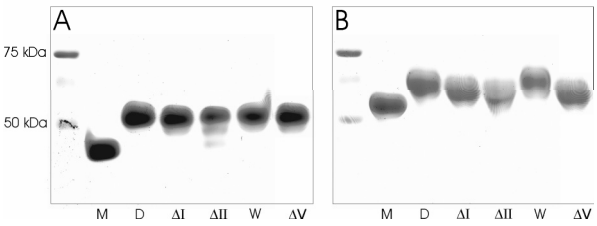

Figure 1. SDS-PAGE analysis of apple 4 – β_2GPI constructs.
Purified plasma β_2GPI (M), apple 4 – β_2GPI (D), delta I (ΔI), delta II (Δ2), delta V (ΔV) and apple 4 – β_2GPI – Trp316Ser (W) were analysed on a 10% SDS-PAGE under non-reducing- (A) and reducing (B) conditions. Gels were stained with Coommassie Brilliant Blue. The molecular masses of prestained markers are expressed in kilodalton (kDa).

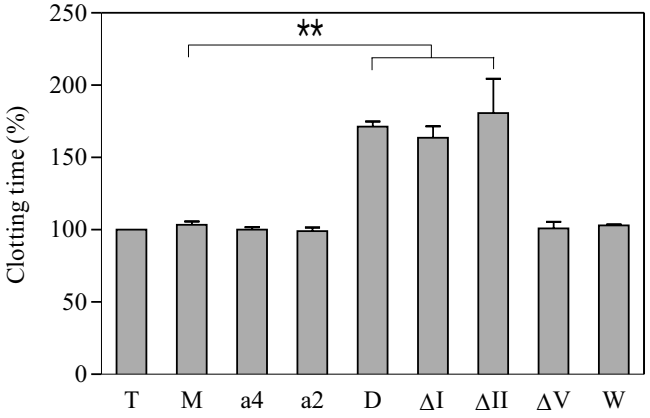

Figure 2. Effect of apple – β_2GPI constructs on the prothrombin time (PT).
Plasma-derived β_2GPI (M), apple 4 (a4), apple 2 – β_2GPI (a2), apple 4 – β_2GPI (D), delta I (ΔI), delta II (ΔII), delta V (ΔV) or apple 4 – β_2GPI – Trp316Ser (W) were 1:1 diluted with normal pooled plasma (final protein concentration 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 apple 4 – β_2GPI, delta I and delta II are not significant.

Binding of domain deletion mutants to immobilized phospholipids

The phospholipid-binding features of apple 4 – β_2GPI fusion proteins were tested in a solid phase binding assay. Phospholipid vesicles (25 µM, 20% PS/PC 80%) were immobilized on 96-well ELISA plates, and binding of plasma-derived β_2GPI and DM of apple 4 – β_2GPI was measured. As shown in *Figure 3*, half-maximal binding of apple 4 – β_2GPI 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 apple 4 – β_2GPI – Trp316Ser, half-maximal binding was observed at a concentration of 26.0 µg/mL. The presence of an aa substitution in the phospholipid-insertion loop explains why the Trp316Ser mutant hardly binds to anionic phospholipids. Plasma-derived β_2GPI showed little binding at a concentration of 16 µg/mL.

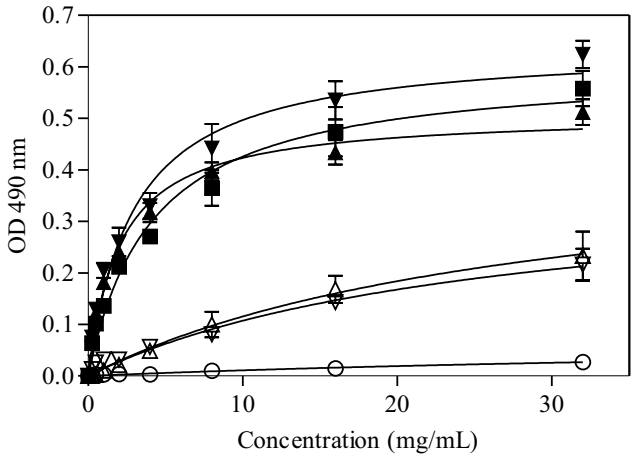

Figure 3. Binding of apple 4 – β_2GPI domain deletion mutants to immobilized PL
Phospholipid vesicles (20% PS/PC 80%, 25µM) were immobilized on high binding 96-well ELISA plates and incubated with increasing concentrations (ranging from 0.25 – 32 µg/mL) of plasma β_2GPI (•), apple 4 – β_2GPI (▫), delta I (▼), delta II (▫), delta V (△) or apple 4 – β_2GPI – 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 β_2GPI	Apple 4-β_2GPI	Apple 4-$\Delta1\beta_2$GPI	Apple 4-$\Delta2\beta_2$GPI	Apple 4-$\Delta5\beta_2$GPI	Apple 4-β_2GPI-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 β_2GPI and apple 4 – β_2GPI fusion proteins were fitted according to a one-site binding model in GraphPad. Half-maximal binding is given as apparent K_d ($K_{d(app)}$) both in µg/mL as in nmol/L (nM). Results are expressed as mean ± SD (n = 3).

Effect of domain deletion mutants on platelet deposition to collagen type III under conditions of flow

To determine which domain of apple 4 – β_2GPI is involved in platelet sensitization, we performed perfusion experiments with citrated whole blood pre-incubated with plasma β_2GPI, apple 4 – β_2GPI or DM (final concentration 100 µg/mL). To determine basal platelet adhesion to collagen type III, whole blood was 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 β_2GPI, apple 4, apple 2 – β_2GPI 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], apple 4 – β_2GPI – Trp316Ser did not induce increased platelet adhesion to collagen. In contrast, platelet adhesion increased significantly when full length apple 4 –, 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 apple 4 – β_2GPI (*Figure 4B, Panel a*), delta I (*not shown*) and delta II (*not shown*). Plasma β_2GPI, delta V (*Panel b and d, respectively*) and apple 4 – β_2GPI – Trp316Ser (*not shown*) displayed comparable effects on morphology and number of platelet aggregates as incubation with buffer (*Panel a*).

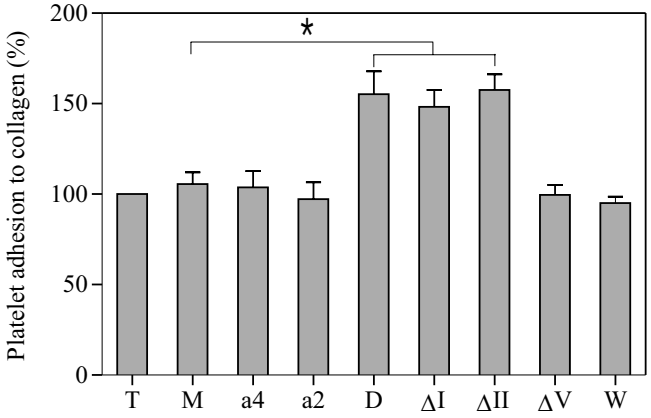

Figure 4A. Platelet deposition on collagen type III in the presence of domain deletion mutants of apple 4 – β_2GPI

Whole blood was pre-incubated at 37°C for 5 minutes with buffer (T), plasma-derived β_2GPI (M), apple 4 (a4), apple 2 – β_2GPI (a2), apple 4 – β_2GPI (D), apple 4 – β_2GPI – Trp316Ser (W) or domain deletion mutants (ΔI, ΔII and ΔV, respectively) of apple 4 – β_2GPI (10% v/v) with a final concentration of 100 µg/mL. Whole blood was perfused over collagen type III for 90 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 apple 4 – β_2GPI, delta I and delta II were not significant.

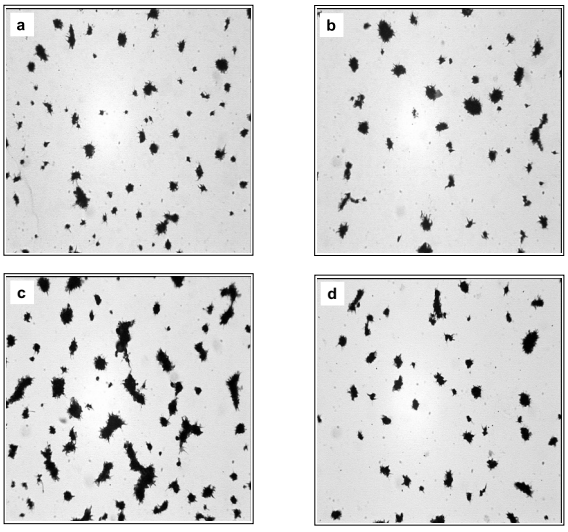

Figure 4B. Platelet morphology and aggregate formation in the presence of buffer (Panel A), plasma β_2GPI (Panel B), apple 4 – β_2GPI (Panel C) or delta V (Panel D).

There was no difference between apple 4 – β_2GPI, delta I and delta II. Platelet morphology and aggregate formation in the presence of apple 4 – β_2GPI – Trp316Ser was similar to buffer control.

Binding of apple 4 – β_2GPI and the Trp316Ser mutant to immobilized soluble human apoER2'

Binding of apple 4 – β_2GPI 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 apple 4 – β_2GPI and apple 4 – β_2GPI – 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 β_2GPI in the presence of a monoclonal α-β_2GPI antibody (19H9) displayed binding to sh-apoER2'. Half-maximal binding occurred at a concentration of 2.4 ± 0.7 µg/mL (corresponds to 21 nM). No binding was found with plasma β_2GPI, apple 4 or delta V. Apple 2 – β_2GPI displayed only slight interaction with sh-apoER2'.

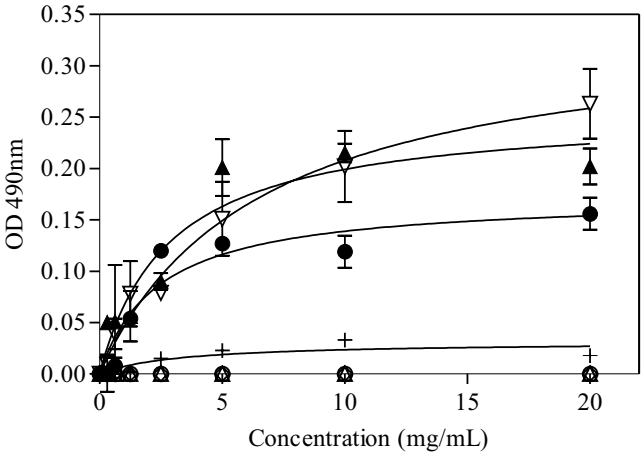

Figure 5. Binding of apple 4 – β_2GPI and the Trp316Ser mutant to immobilized apoER2'

Interaction between apple 4 – β_2GPI and sh-apoER2' was measured in a solid phase binding assay. Soluble human apoER2' was coated on a hydrophobic 96 well ELISA plate in a concentration dependent manner (0-10 µg/mL). After blocking, plasma β_2GPI (•), apple 4 (◊), apple 2 – β_2GPI (+), apple 4 – β_2GPI (▫), delta V (△), apple 4 – β_2GPI – Trp316Ser (▽) mutant and plasma β_2GPI + 19H9 (•) were incubated (3 µg/mL). Afterwards bound protein was detected with rabbit polyclonal anti-β_2GPI. Bound antibody was detected using SWARPO. Results are expressed as mean ± SD (n = 3).

Association of domain deletion mutants and the Trp316Ser mutant of apple 4 – β_2GPI with apoER2' on platelets

The only member of the LDL receptor family known to be present on platelets is apoER2'. To demonstrate that a cationic patch in domain V of β_2GPI and not the hydrophobic PL-insertion loop in domain V (Ser311-Lys317) is responsible for inter-

action with apoER2', immunoprecipitations were performed. Platelets were incubated with buffer, plasma-derived β_2GPI, full length apple 4 – β_2GPI, DM or apple 4 – β_2GPI – Trp316Ser, lysed and subjected to immunoprecipitation with an anti-apoER2' antibody. Afterwards, Western blots were incubated with a monoclonal anti-β_2GPI antibody to detect interaction between β_2GPI and apoER2'. Association with apoER2' was observed with apple 4 – β_2GPI (*Figure 6*). Hardly any association was observed when platelets were incubated with plasma β_2GPI 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 apple 4 – β_2GPI – Trp316Ser. The interaction of the Trp316Ser mutant with apoER2' on the surface of platelets was similar to that of full length apple 4 – β_2GPI.

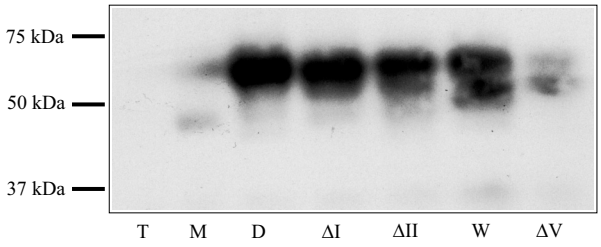

Figure 6. Immunoprecipitations with domain deletion mutants of apple 4 – β_2GPI and apoER2' on platelets
Washed platelets (300.000/μL), resuspended in Hepes/Tyrode buffer containing 3 mM $CaCl_2$ were incubated with buffer, apple 4 – β_2GPI (D), delta I (ΔI), delta II (ΔII), delta V (ΔV) or apple 4 – β_2GPI – 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-β_2GPI antibody followed by visualization using chemiluminescence. Blot represents three different experiments.

Association of apple 4 – β_2GPI with apoER2' in the presence of CKNKEKKC, EKCKNKCK (scrambled) or VSRGGMRK peptides

Association of ligands with members of the LDL receptor family is supported by electrostatic interactions. To investigate the possibility that association of apple 4 – β_2GPI with apoER2' is supported by a cationic patch in domain V of apple 4 – β_2GPI competition studies were performed with positively charged peptides. Incubation of washed platelets with apple 4 – β_2GPI and CKNKEKKC (represents a cationic patch at aa position 282-287 in domain V of β_2GPI) peptide resulted in decreased binding of apple 4 – β_2GPI to apoER2' (*Figure 7A*). This effect was not seen with the VSRGGMRK peptide (represents a cationic patch at aa position 37-

44 in domain I of β_2GPI). The decreased binding of apple 4 – β_2GPI to apoER2' in the presence of CKNKEKKC was also seen for the Trp316Ser mutant. Incubation of washed platelets with apple 4 – β_2GPI and EKCKNKCK (scrambled peptide) resulted in a minor reduction of the association between apple 4 – β_2GPI and apoER2' (*Figure 7B*).

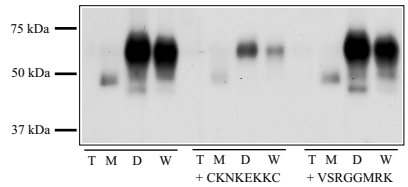

Figure 7A

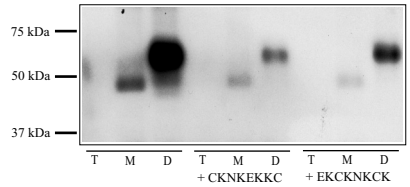

Figure 7B

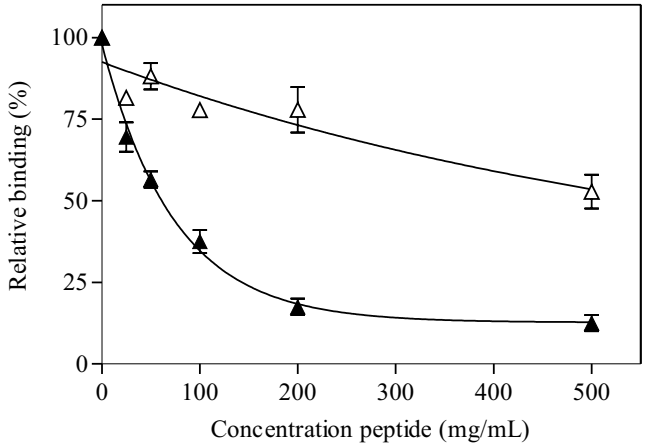

Figure 7C

Figure 7. Immunoprecipitations with apple 4 – β_2GPI and apoER2' on platelets in the presence of CKNKEKKC, EKCKNKCK or VSRGGMRK peptides

Competition experiments were performed to investigate electrostatic involvement between apple 4 – β_2GPI and apoER2'. For this purpose washed platelets were incubated with buffer (T), plasma β_2GPI (M), apple 4 – β_2GPI (D), apple 4 – β_2GPI – 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 apple 4 – β_2GPI and the peptides CKNKEKKC (□) and EKCKNKCK (Δ), binding of apple 4 – β_2GPI in the presence of the peptides was investigated in the solid phase binding assay (Panel C).

Binding of apple 4 – β_2GPI to sh-apoER2' in the solid phase binding assay in the presence of CKNKEKKC, EKCKNKCK (scrambled) or VSRGGMRK peptides

To show direct competition between apple 4 – β_2GPI and the different peptides, apple 4 – β_2GPI was able to interact with sh-apoER2' in the presence of increasing concentrations peptides VSRGGMRK, CKNKEKKC or EKCKNKCK (scrambled). As shown in *Figure 7C*, inhibition was observed for both the CKNKEKKC and the EKCKNKCK peptide. Binding of apple 4 – β_2GPI to sh-apoER2' in the presence of the CKNKEKKC peptide was reduced to 12.5 ± 3.6 %. In the presence of the scrambled peptide EKCKNKCK binding was reduced to 52.8 ± 7.4 %. In the presence of VSRGGMRK peptide no inhibition was observed (*data not shown*).

Effect of the peptides CKNKEKKC and EKCKNKCK on the prothrombin time

The inhibiting effect of the peptides on association between apple 4 – β_2GPI and apoER2' might be due to interference with binding of the apple 4 – β_2GPI constructs to phospholipids. Therefore, we investigated the influence of the peptides in the clotting time (PT), which is a phospholipid-dependent clotting assay. The clotting time in the presence of buffer was set at 100 %. Apple 4 – β_2GPI displayed a relative prolongation of the PT to 194.0 ± 2.8 % (*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 μg/mL) did not influence the clotting time in the absence of dimeric β_2GPI up to concentrations of 1 mg/mL (*data not shown*).

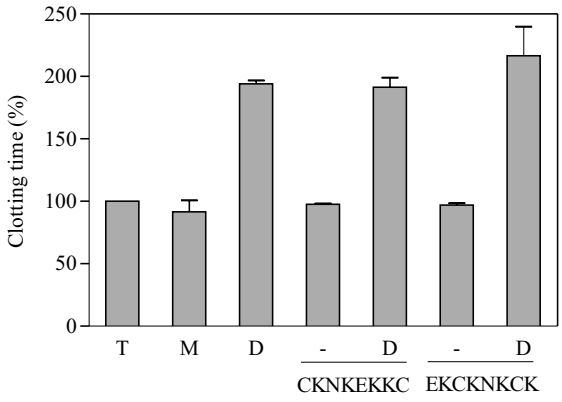

Figure 8. The influence of the inhibiting peptides CKNKEKKC and EKCKNKCK on the prothrombin time

Plasma-derived β_2GPI (M), apple 4 – β_2GPI (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 (β_2GPI) to anionic phospholipids (PL) on the cell surface can activate these cells. Therefore, a search for a cellular receptor for β_2GPI on platelets was initiated. Lutters *et al*. have demonstrated that dimerization of β_2GPI (either artificially by fusing β_2GPI with the apple 4 domain of factor XI, or physiologically by binding aPL to β_2GPI) results in increased affinity of β_2GPI for platelets (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 β_2GPI was involved. This receptor has later been identified as apoER2' (25). In the present study, the domain of β_2GPI responsible for interaction with apoER2' has been determined by using constructs of dimeric β_2GPI lacking 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 β_2GPI to anionic phospholipids [14]. In this study, we showed that a cationic patch in domain V of dimeric β_2GPI is involved in interaction with apoER2' on platelets and that, by using the 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 β_2GPI. Both domain deletion mutants (delta I and delta II) prolong the clotting time in the prothrombin-time (PT) comparable to full length dimeric β_2GPI (*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 β_2GPI (*Figure 4A*). Deletion of domain V or the Trp316Ser substitution abolished the prolongation of the clotting time induced by dimeric β_2GPI, which was to be expected, as domain V harbors the phospholipid-binding site. Indeed, both mutants show an approximate 15-fold decreased affinity for anionic phospholipids. Furthermore, in the perfusion model both mutants were not able to increase platelet adhesion to collagen, stressing the necessity of domain V in the activation of platelets.

Based on the observations in this paper, we propose the following model for the activation of platelets by β_2GPI (*Figure 9*). β_2GPI binds to platelets with a low affinity. However, when bound to the platelet membrane a conformational change is induced in domain I of β_2GPI [36 37 38 39], resulting in the exposure of a cryptic epitope in this domain. Binding of anti-β_2GPI antibodies to this newly exposed epitope in domain I takes place. When one antibody interacts with two molecules of β_2GPI,

the protein dimerizes resulting in an increased affinity for phospholipids on the outer surface of the platelet membrane. This increased affinity of the β_2GPI /anti-β_2GPI complexes is mimicked by our recombinant dimeric β_2GPI construct. The binding to the platelet membrane also results in concentration of β_2GPI on the cellular surface, which allows interaction with apoER2' due to mass action effects. Stabilization of the binding of dimeric β_2GPI 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 β_2GPI is not able to associate with the receptor (*Figure 6*). Conformational changes in domain V of β_2GPI after binding to phospholipids have been described before [40]. The interaction between β_2GPI and apoER2' takes place via a cationic region in domain V, as peptides covering cationic amino acids present in domain V are able to inhibit the binding of dimeric β_2GPI to apoER2' (*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 β_2GPI, dimerization of the receptor may occur [41]. The interaction of dimeric β_2GPI 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 44 45].

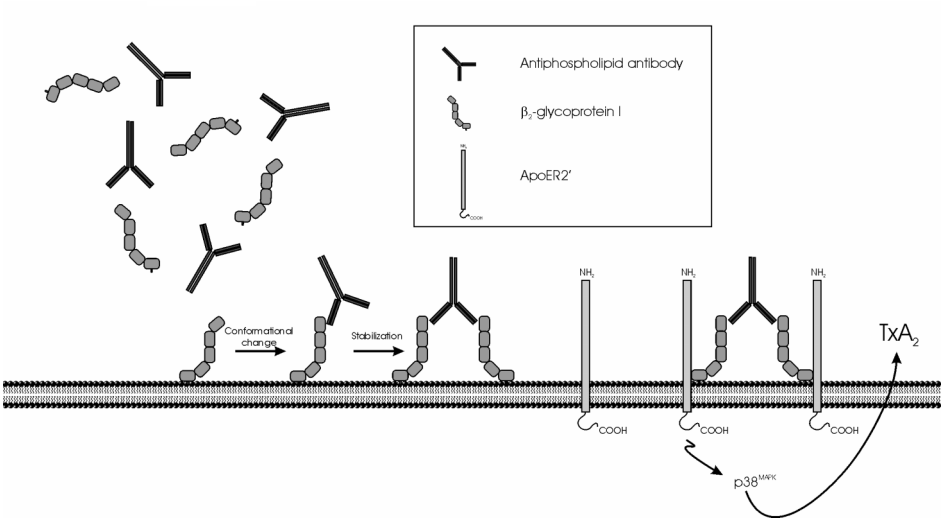

Figure 9. Proposed mechanism of platelet activation via dimerization of β_2GPI by aPL
Beta2-glycoprotein I circulates free in plasma with low affinity for phospholipids. A conformational change in domain I of β_2GPI is induced after low-affinity binding to platelets. Dimerization of β_2GPI via binding of aPL occurs followed by stabilization of the complex on the platelet surfaced which subsequently results in firm adhesion of the β_2GPI/aPL complex to PL and association with apoER2' on the surface of platelets. We cannot exclude the possibility that dimeric β_2GPI dissociates from the cellular surface after interaction with apoER2'. The interaction results in down-stream signaling, TxA2 synthesis and furtherplatelet activation.

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INTERACTION OF BETA2-GLYCOPROTEIN I WITH MEMBERS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR FAMILY

4

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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 (β_2GPI) bound to phospholipids. We have previously demonstrated that dimerization of β_2GPI 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 β_2GPI, but not monomeric β_2GPI 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 β_2GPI 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 β_2GPI 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 μM). Binding to all receptors tested was unaffected by a tryptophane to serine (W316S) substitution in domain V of β_2GPI, which is known to disrupt the phospholipid binding site of β_2GPI. 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 β_2GPI can interact with different LDL-R family members. This interaction is dependent on a binding site within domain V of β_2GPI, which does not overlap with the phospholipid-binding site within domain V.

Keywords: antiphospholipid syndrome, beta2-glycoprotein I, antiphospholipid antibodies, low-density lipoprotein receptor family, protein-receptor interaction.

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 (β_2GPI) display the highest correlation with thrombosis [3]. The cause of haemostatic dysbalance induced by β_2GPI in the presence of β_2GPI antibodies in vivo still remains unraveled. Several possibilities have been proposed, of which cell activation by β_2GPI/anti-β_2GPI antibodies has received most attention. The involvement of cellular receptors that can bind these immune complexes is essential in all these hypotheses [4-7].

β_2-Glycoprotein I is an abundant plasma protein (approximately 200 μg/mL). At present, the physiological function of β_2GPI 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 β_2GPI/antibody complexes can activate platelets. This effect could be mimicked by a recombinant form of dimeric β_2GPI. 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 β_2GPI to human platelets [13]. ApoER2 (also known as LDL receptor-related protein (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 β_2GPI

with different affinities. Furthermore, apoER2'-mediated platelet activation by β_2GPI only occurred in the presence of anti-β_2GPI antibodies. Since these antibodies possess the property to dimerize antigens, we investigated whether anti-β_2GPI antibody induced dimerization of β_2GPI 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 β_2GPI), not the primary sequences, in different ligands constitute receptor-binding domains. Therefore, we used domain deletion mutants of dimeric β_2GPI to identify which domain of β_2GPI 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 β_2GPI mutant to LRP, megalin, apoER2' and the VLDL-R.

Experimental procedures

Purification of plasma β_2GPI

Plasma β_2GPI 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, β_2GPI was dialyzed against TBS. Purity of the protein was checked on a 4-15% SDS-PAGE. Plasma β_2GPI 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 β_2GPI

Apple 4 — C321S — β_2GPI (further referred to as dimeric β_2GPI) and the non-dimeric apple 2 — β_2GPI were constructed as described previously [18]. Domain deletion mutants of dimeric β_2GPI were constructed using full-length apple 4 — C321S — β_2GPI 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 (apple 4) and β_2GPI, the

PCR product was cloned into the vector apple 4-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 apple 4 [19]. Sequence analysis was performed to confirm correct amplification of the β_2GPI 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 β_2GPI-ELISA [20]. Full-length and domain deletion mutants of dimeric β_2GPI 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). Apple 4 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 β_2GPI and apoER2' and the VLDL-R

Binding of β_2GPI 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 β_2GPI was detected using subsequently a rabbit polyclonal anti-β_2GPI 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 β_2GPI 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/mm2 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 μM $CaCl_2$ with a flow rate of 20 μl/min at 25ºC. 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 β_2GPI/anti-β_2GPI antibody complex to LDL-receptor family members

Binding of plasma β_2GPI in the presence of a monoclonal anti-β_2GPI antibody (3B7) that recognizes domain I of β_2GPI 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 β_2GPI (100 nM) was incubated with 3B7 (nM) for 30 minutes at 37ºC. 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 μM CaCl2 with a flow rate of 20 μl/min at 25ºC. Because of the complexity of the interaction between antibody/β_2GPI complexes and immobilized receptor, the kinetic parameters of this interaction cannot be calculated using the available software programs. Interaction between β_2GPI/anti-β_2GPI antibody complex and coated apoER2' and the VLDL-R (5 μg/mL) was measured using the solid-phase immunosorbent assay. In this assay, plasma β_2GPI (3 μg/mL) was incubated with 3B7 (1 μg/mL) in PBS/1%BSA. Bound β_2GPI 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 β_2GPI was pre-incubated with increasing concentrations of heparin (0 — 2 mg/mL) in TBS/0.005% Tween 20 containing 1 μM CaCl2 for 30 minutes at 37oC 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 β_2GPI. For inhibition studies with apoER2' and the VLDL-R, dimeric β_2GPI (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: $$Y = \frac{B_{\max 1} \times X}{K_{d1} + X} + \frac{B_{\max 2} \times X}{K_{d2} + X}$$

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

Binding of a β_2GPI/anti-β_2GPI antibody complex to LDL-receptor family members

To investigate the effect of dimerization of β_2GPI by an antibody on its interaction with members of the LDL-R homologues, we assessed the binding of plasma β_2GPI in the absence or presence of an anti-β_2GPI antibody (3B7) to LRP, megalin, the LDL-R, apoER2' and the VLDL-R. Interaction of β_2GPI 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 β_2GPI in the presence of 3B7 resulted in binding of the immune complex to all receptors tested, except for the LDL-R. Plasma β_2GPI or 3B7 alone had less than 20% binding to the receptors. Thus, β_2GPI binds to LRP, megalin, apoER2' and the VLDL-R, however dimerization of β_2GPI is necessary for optimal binding.

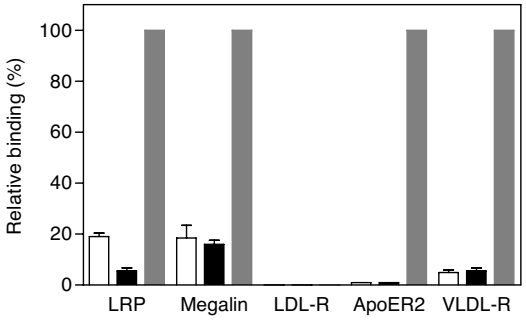

Figure 1. Binding of a β_2GPI/anti-β_2GPI antibody complex to members of the LDL-receptor family
Plasma β_2GPI was incubated with an anti-β_2GPI Moab (3B7) at 37ºC for 30 minutes. The formed immune complex could interact with LRP, megalin, apoER2' and the VLDL-R. Binding of dimeric β_2GPI (grey bars) in the presence of 3B7 to the receptors was set at 100%. Plasma β_2GPI (white bar) and 3B7 (black bar) alone hardly bound to the receptors. Results represent mean ± SD (n = 3).

Different members of the LDL-receptor family contain a binding site for recombinant dimeric β_2GPI

Interaction between β_2GPI/antibody complexes and the receptors is complicated to examine, since the analysis can be influenced by the affinity of the antibody for β_2GPI. Therefore, we studied binding of a stable, recombinant form of dimeric β_2GPI 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 β_2GPI, apple 4 did and the non-dimeric protein apple 2 — β_2GPI did not bind to LRP. In contrast, dimeric β_2GPI did bind to LRP. Upon replacement of dimeric β_2GPI with buffer (arrow), the response signal declined gradually, indicating that dimeric β_2GPI dissociates from LRP and that binding is reversible.

The interaction of dimeric β_2GPI 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 β_2GPI 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 β_2GPI 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 β_2GPI and the LDL-R (*Panel D*), apoER2' (*Panel E*) and the VLDL-R (*Panel F*) showed one class of binding sites (R2: 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 β_2GPI (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*.

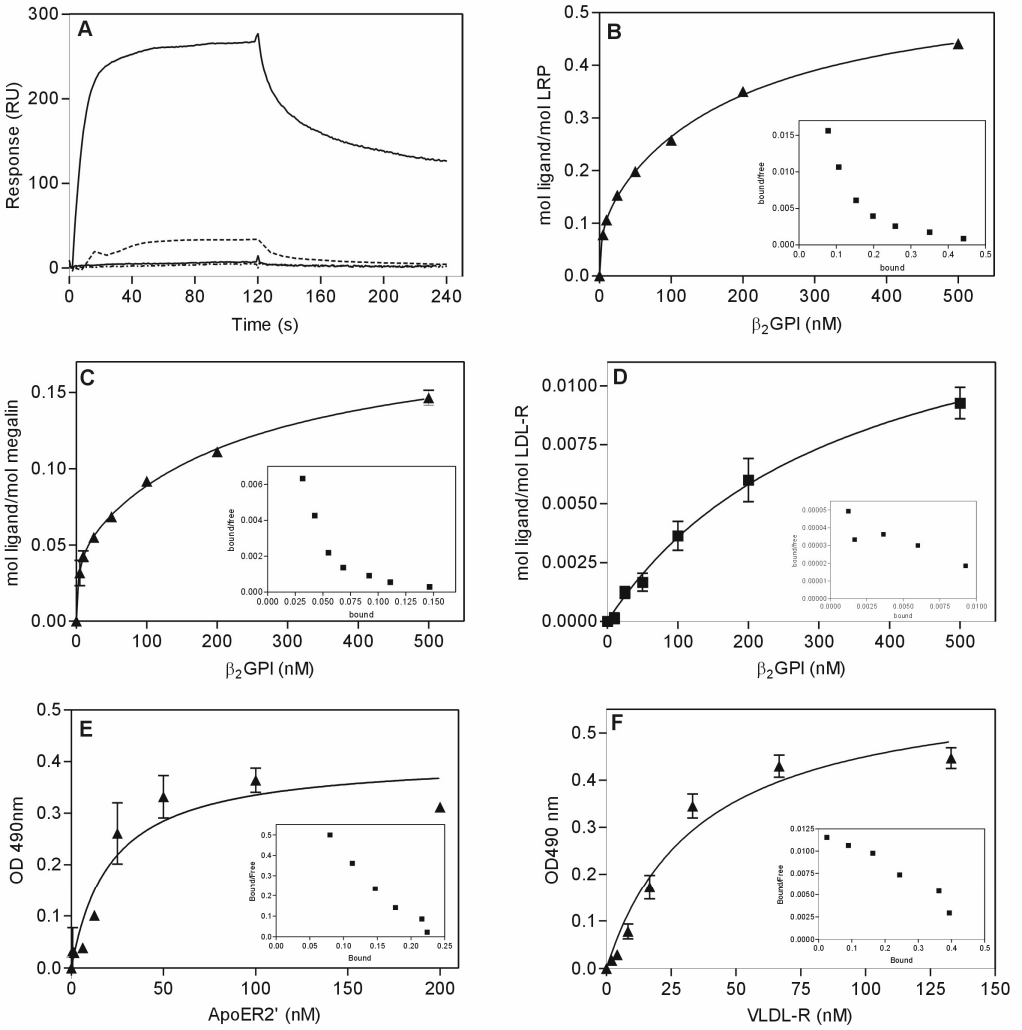

Figure 2. Interaction of plasma and dimeric β_2GPI with LDL-R homologues A, a reproducible sensorgram showing interaction of dimeric β_2GPI with LRP (solid line). *Also the control proteins apple 2 — β_2GPI (broken line), apple 4, and plasma β_2GPI are displayed. B, LRP, C, megalin or D, LDL-R was immobilized on a CM5 or C1 sensor chip at a density of 10 fmol/mm², 26 fmol/mm², or 29.4 fmol/mm² respectively. Seven different concentrations (5 — 500 nM) of dimeric β_2GPI were passed over the immobilized receptors. E, apoER2' or F, VLDL-R was coated with increasing concentrations (0 — 10 µg/mL) on a 96 wells plate and dimeric β_2GPI (3 µg/mL) was able to interact with the receptors. Lines represent the data curves and their fitted curves obtained with a one-site binding model or a two-site binding model. Scatchard plots (inserts) verify the use of the binding models. Data for LRP, megalin and the LDL-R is expressed as mol ligand bound to mol receptor. Steady state constants derived from the binding isotherms are listed in Table 1. Results represent mean ± SD (n = 3).*

Table 1: Steady state constants obtained from the binding of dimeric β_2GPI mutants
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.

receptor	dimeric β_2GPI	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

Effect of RAP on the dimeric β_2GPI — LDL-receptor family interaction

The specificity of the interactions was examined by analyzing the interaction between dimeric β_2GPI and individual receptors in the presence of RAP, a universal ligand-inhibitor for this receptor family. For SPR analysis, pre-injections with five different concentrations of RAP (0 — 200 nM) were performed prior to injection of β_2GPI. For immunosorbent analysis, dimeric β_2GPI was incubated with RAP and binding to apoER2' or the VLDL-R was analyzed. As shown in *Figure 3*, binding of dimeric β_2GPI to LRP or megalin was inhibited up to 80% by RAP. In the presence of RAP, binding of dimeric β_2GPI to apoER2' and the VLDL-R was inhibited up to 75%, and 100%, respectively.

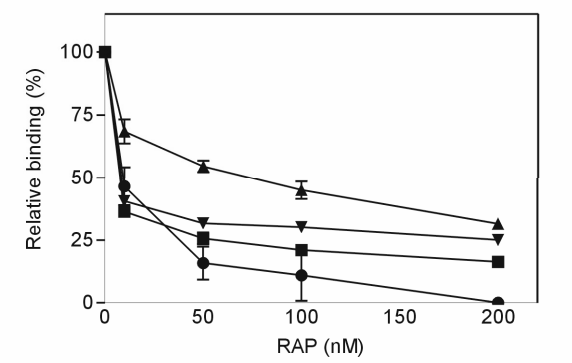

Figure 3. Inhibition of dimeric β_2GPI - LDL-receptor family interaction by RAP For LRP and megalin, SPR analysis was applied to study the effect of RAP. *Pre-injections with five different concentrations of RAP were performed for 2 minutes, directly followed by injections of 100 nM dimeric β_2GPI on immobilized LRP (■) and immobilized megalin (▲) for 2 minutes. Interaction of dimeric β_2GPI with coated apoER2' (▼) or VLDL-R (•) in the presence of RAP was measured using a solid phase immunosorbent assay. Binding of dimeric β_2GPI in the absence of RAP was set at 100 %. Results represent mean ± SD (n = 3).*

Effect of heparin on the dimeric β_2GPI — LDL-receptor family interaction

β_2-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^2). 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 β_2GPI and LRP, megalin, apoER2' and the VLDL-R. As shown in *Figure 4*, heparin inhibited the interaction of dimeric β_2GPI 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 β_2GPI 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.

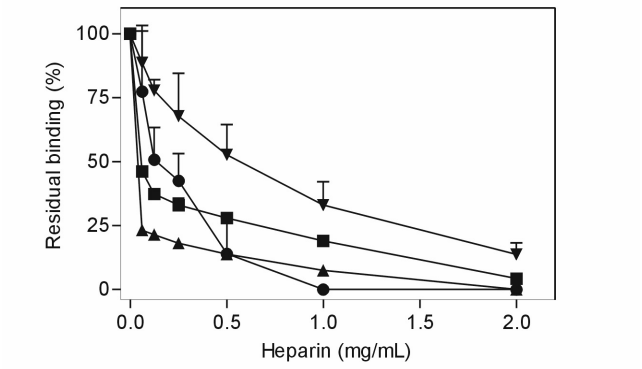

Figure 4. Effect of heparin on the interaction between dimeric β_2GPI and receptors
Dimeric β_2GPI (100 nM) was pre-incubated with increasing concentrations of heparin (0 — 2 mg/mL) at 37ºC for 30 minutes. Afterwards the effect of heparin on binding of dimeric β_2GPI to immobilized LRP (■) or megalin (▲) was investigated using SPR analysis. The effect of heparin on the interaction between dimeric β_2GPI 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).

Function of the different domains of β_2GPI in LDL-receptor interaction

To further characterize the interaction between dimeric β_2GPI 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 β_2GPI. Deletion of domain V of β_2GPI appeared to be crucial for binding to LRP and megalin. A small increase in the response signal was observed with increasing concentrations 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 β_2GPI). 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 β_2GPI. The results are summarized in *Table 1*. 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 β_2GPI (*Table 2*). 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 β_2GPI.

Interaction of dimeric β_2GPI with recombinant LRP clusters

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 β_2GPI and LRP was best characterized with a two-site binding model may suggest heterogeneity of LRP. To identify LRP regions involved in binding dimeric β_2GPI, purified recombinant cluster II and IV were used. When plasma β_2GPI was passed over both clusters II and cluster IV, no significant increase in the resonance signal was observed (*data not shown*). Binding of dimeric β_2GPI 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 3*, 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 ± 14.6 nM (K_{D2}). To further characterize the interaction, binding characteristics of the domain 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 apple 4 — β_2GPI.

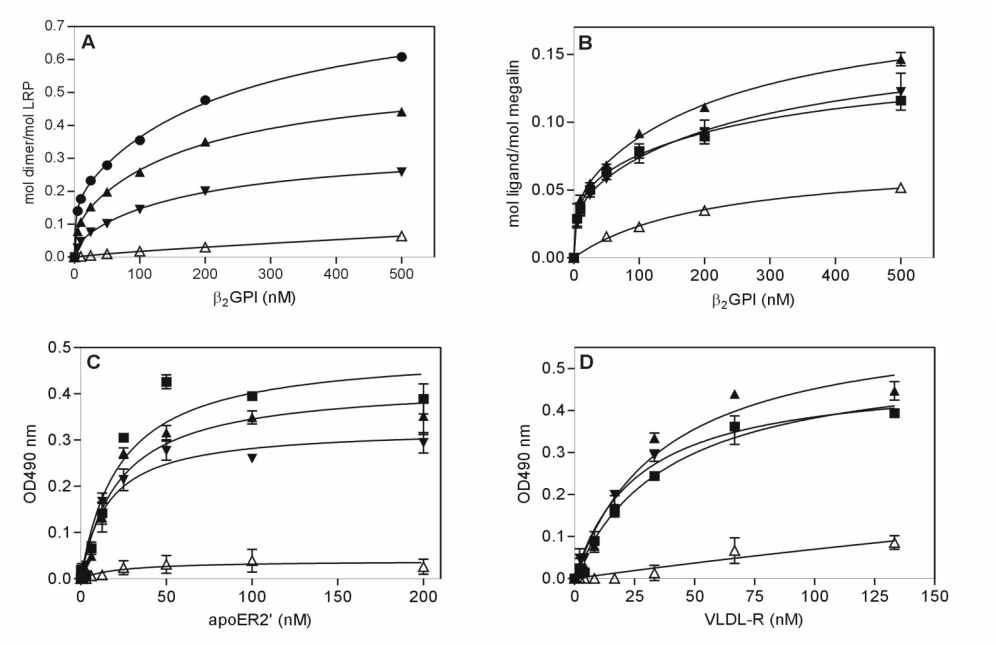

Figure 5. Binding isotherms of the domain deletion mutants for members of the LDL receptor family

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 β_2GPI (▲), 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 β_2GPI (▲), 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).

Table 2: Maximum binding ($R_{eq,max}$) for domain deletion mutants of β_2GPI
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 (K_{D1} and K_{D2}) are calculated; for a one-site binding model only one K_D is calculated. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3).

receptor	dimeric β_2GPI	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 3: Steady state constants obtained from the binding of dimeric β_2GPI mutants to 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 (K_{D1} and K_{D2}) are calculated; for a one-site binding model only one K_D is calculated. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3).

receptor fragment	dimeric β_2GPI	delta I	delta II	delta V
Cluster II of LRP	1.6 ± 0.6 159 ± 30	2.4 ± 0.8 205 ± 38	2.0 ± 0.3 157 ± 19	- 365 ± 32
Cluster IV of LRP	1.7 ± 0.4 172 ± 15	1.4 ± 1.2 249 ± 53	4.2 ± 0.7 186 ± 22	- 143 ± 7

Effect of a mutation in the phospholipid-binding site in domain V of dimeric β_2GPI

To address the possibility that binding of dimeric β_2GPI to members of the LDL-receptor family has similar characteristics as binding to anionic phospholipids, we performed binding experiments with the non-phospholipid-binding mutant of dimeric β_2GPI. 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 β_2GPI, the maximal response of

the mutant was lower for LRP and megalin. As shown in *Table 4*, 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.

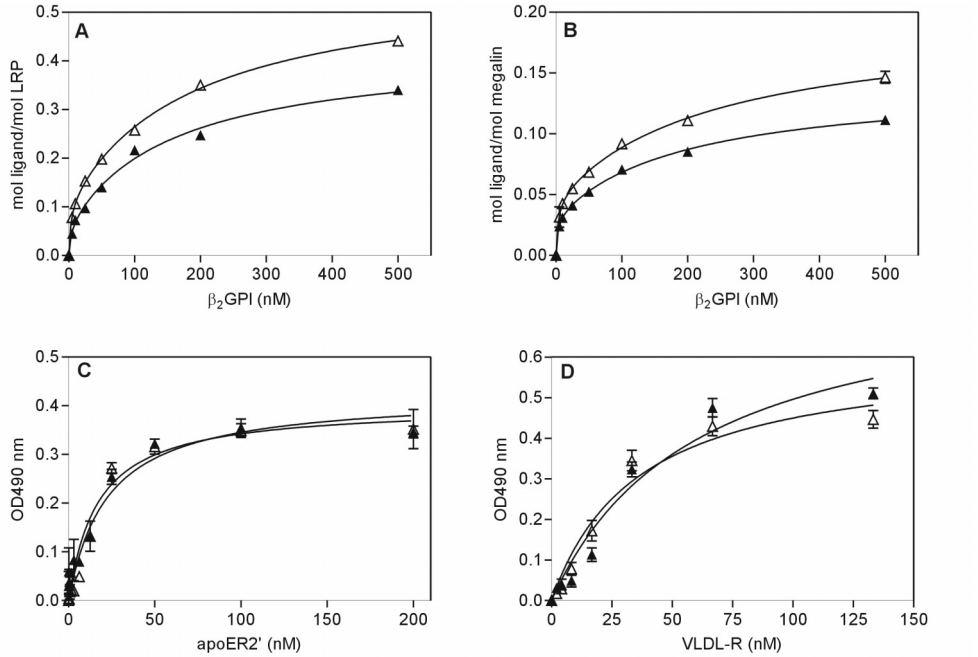

Figure 6. Effect of a mutation in the phospholipid-binding region in domain V of β_2GPI
For SPR analysis, increasing concentrations (0 - 500 nM) of dimeric β_2GPI (Δ) and Trp316Ser mutant (▲) were past over A, immobilized LRP or B, megalin. Association and dissociation was allowed for two minutes with a flow of 20 μL/min at 25ºC. 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 β_2GPI (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.

Table 4: Steady state constants obtained from the binding of the Trp316Ser mutant of β_2GPI
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).

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

Discussion

In the present study we have investigated interaction between beta2-glycopprotein I (β_2GPI) 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 b2GPI and an anti-b2GPI antibody, involving the receptor apoER2' [13]. The interaction between b2GPI 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 β_2GPI/anti-β_2GPI antibody complex. Indeed, we found that LRP, megalin and the VLDL-R recognize only a complex of β_2GPI/anti-β_2GPI but not β_2GPI alone (*Figure 1*) and

this interaction can be mimicked by our recombinant form of dimeric β_2GPI (*Figure 2*). Two binding models were identified for the interactions; interaction between dimeric β_2GPI, 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 β_2GPI. 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 β_2GPI-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 β_2GPI 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 β_2GPI. We demonstrated that domain V of β_2GPI 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 β_2GPI (*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 β_2GPI and apoER2' on platelets can be inhibited by a peptide spanning amino acids 282-287 in domain V of β_2GPI [19]. Furthermore, it has been reported that Lys (284), Lys (286) and Lys (287) in domain V are essential for the interaction of β_2GPI with heparin [27]. This implicates that both electrostatic interactions and amino acid sequences are important for the interaction between dimeric β_2GPI and LDL-R homologues. Interaction of dimeric β_2GPI 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 β_2GPI. For this purpose, we analyzed the interaction between β_2GPI 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 β_2GPI. Two domains that expose cationic charges were of interest; domain I and domain V of β_2GPI. However, delta I interacted similar with the receptors or both clusters of LRP compared to full-length dimeric β_2GPI. 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 β_2GPI/LDL-R complex.

Besides members of the LDL-R family, a number of other cellular receptors have been described for β_2GPI. The contribution of LDL-R family members in β_2GPI-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-β_2GPI 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 β_2GPI/anti-β_2GPI antibody complexes [43]. Our next challenge is to understand how cell signaling induced by anti-β_2GPI antibodies is coordinated by all these different receptors.

Besides β_2GPI, other proteins have been implicated as possible target for aPL, in particular prothrombin. However, the majority of the performed studies indicate that anti-β_2GPI 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 β_2GPI/antiβ_2GPI-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 β_2GPI/anti-b2GPI 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 β_2GPI/anti-β_2GPI antibody complexes and LDL-R family members on different cell types.

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MOLECULAR CHARACTERIZATION OF THE APOLIPOPROTEIN E RECEPTOR 2'-BINDING SITE AND PHOSPHOLIPID-BINDING SITE WITHIN BETA2-GLYCOPROTEIN I

5

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In preparation

Abstract

Previously, we have shown that recombinant dimeric β_2-glycoprotein I (β_2GPI) binds to human platelets via a cellular receptor, apolipoprotein E Receptor 2' (apoER2'). ApoER2' is a receptor belonging to the low-density lipoprotein receptor (LDL-R) family and is involved in signal transduction. The interaction between β_2GPI and apoER2' is dependent on a binding site in domain V of β_2GPI. In the present study, we have characterized the binding site in domain V of β_2GPI for apoER2' using Lys to Leu substitutions in the region between amino acid 262 and 317 in domain V of β_2GPI. We show that the lysines at position 282-287 in domain V of β_2GPI are important in the recognition of apoER2'. Apart form this region, the lysine residues at position 305-317 is also important for recognition by apoER2'. The lysine residues at position 262-276 do not participate in the recognition. In a phospholipid-dependent coagulation assay, none of the mutants was able to prolong the clotting time, which was observed for full-length dimeric β_2GPI. To study the molecular interaction of β_2GPI with phospholipids, the binding of the mutants to different anionic phospholipids was investigated. We show that the primary phospholipid-binding site in domain V of β_2GPI is located at the lysine residue at position 305-317, whereas the primary binding site for cardiolipin is located around lysine residues 282-287. From this study, we conclude that the recognition site within domain V of β_2GPI for apoER2' does not overlap the phospholipid binding site in domain V of for apoER2'.

Introduction

Beta2-glycoprotein I (β_2GPI) is a plasma protein with a calculated molecular mass of 36.3 kDa. The protein contains four potential glycosylation sites, which account for 20% (w/w) of its total molecular mass of 45 kDa determined by sodium dodecyl sulfate/polyacrilamide gel electrophoresis (SDS-PAGE). The crystal structure of β_2GPI isolated from human plasma reveals a five domain, membrane-binding protein consisting of 326 amino acids [1]. These domains are referred to as short consensus repeats (SCR) or complement control protein (CCP). The protein has an elongated J-shaped arrangement with a vertical dimension of 130 Å and a horizontal dimension of 85 Å. An overall protein dimension of 13.2 nm × 7.2 nm × 2.0 nm was found by X-ray crystallography [1]. Short interdomain linkers connect the five domains and span only three (CCP IV and CCP V) to four (other domains) residues. These linkers are often part of β-sheets, suggesting reduced interdomain flexibility [1;2]. In contrast to the crystal structure, the solution structure of β_2GPI shows an S-shaped appearance of the molecule with great interdomain flexibility between CCP III and II, suggesting reorientation of the flexible domains [3]. The five domains of β_2GPI are characterized by repeats of approximately 60 amino acids with 16 conserved residues each and two conserved disulfide bonds. These repeats have been termed as short-consensus repeats (SCR). The first four domains have common SCR folds, whereas domain V is aberrant; it is composed of 82 amino acids and has 3 potential disulfide bonds. In addition, domain V has an insertion of six residues in the region of the hyper-variable (flexible) loop. Together with a 19-residue C-terminal extension this region forms a large cationic area of 2000 Å. This patch contains among others 12 lysine residues and is located at the external curve of the J-shape. It includes four lysines within the loop Cys281-Cys288 and lysines 308 and 324, which have been implicated in cardiolipin binding [4-6]. Other residues of this cationic patch include Lys^{246}, Lys^{250}, Lys^{251}, Arg^{260}, Lys^{262}, Lys^{266}, Lys^{268} and His^{310}. The flexible loop located at Ser^{311}-Lys^{317}, containing Trp^{316}, that is important for phospholipid binding, is located within the large cationic patch. Structural data indicate a simple membrane-binding model, in which the cationic residues in domain V are crucial. In this model, domain I is positioned away from the cellular surface therefore suggested to be involved in physiological processes [7].

In previous studies, we have demonstrated that members of the low-density lipoprotein receptor (LDL-R) family recognize a cationic patch in domain V of β_2GPI [J Thromb Haem, accepted for publication]. An important step in the comprehension of receptor-mediated cellular activation is a description at the molecular level of the specific interaction that takes place between apoER2' and β_2GPI. ApoER2' is

a member of the low-density lipoprotein receptor (LDL-R) family and is identified on human platelets [8]. ApoER2 is predominantly expressed in the central nervous system, but is also expressed in the testis and vascular cells. Multiple alternative splicing variants of apoER2 exist of which one, apoER2', is expressed on platelets and megakaryocytes. ApoER2' on human platelets functions as a signalling receptor, which has been shown for oxidized LDL [9] and artificially dimerized β_2GPI [10].
In the present study, the interaction between β_2GPI and apolipoprotein E receptor 2', as expressed on human platelets, has been studied on a molecular level by substituting lysine residues into leucine residues in domain V of β_2GPI.

Experimental Procedures

A three-dimensional model illustrating the effect on the electrostatic potential after amino acid substitutions in a large cationic patch in domain V of β2GPI

A detailed molecular study of β_2GPI — LDL-R interaction was combined by a prediction of the electrostatic potential after substitution of lysine residues into leucine in the cationic patch between amino acids 262-317 in domain V of β_2GPI. The template structure was that of the fifth domain of β_2GPI as determined by X-ray crystallography [1]. The electrostatic potential on the surface β_2GPI and the mutants were calculated with Swiss-PdbViewer version 3.7b2 (Expasy), an application used to generate protein models and obtaining amino acid substitutions. This programme uses the finite difference method to solve the linearized Poisson-Boltzmann equation. Each lysine was assigned a neutral charge (leucine) because β_2GPI is active in plasma at pH of approximately 7.2. The molecular surface of domain V of β_2GPI was determined with Discovery Studio Viewer Pro version 6.0 (Accelrys Inc.).

Cloning recombinant dimeric β2GPI

The recombinant proteins apple 4 — C321S — β_2GPI (further referred to as dimeric β_2GPI), apple 4 — C321S — $\Delta 5\beta_2$GPI (delta V), the non-phospholipid binding dimer apple 4 — C321S — β_2GPI — W316S and the control proteins apple 4 (dimerization domain of factor XI) and apple 2 — β_2GPI were cloned as described previously [10].

Site-directed mutagenesis

Previously described cloned β_2GPI cDNA in eukaryotic expression vector (apple 4 — tissue — type plasminogen activator (tPA)-S478A) [10] was used to create the desired mutations using the QuikChange site-directed mutagenesis kit (Stratagene) for

which only a forward primer for each mutation is required. The sequence of dimeric β_2GPI was amplified from the vector apple 4 – C321S – β_2GPI tissue – tPA – S478A with the primers β_2GPI – *Xho* I forward (CC CT CGA GGA CGG ACC TGT CCC AAG CCA) and β_2GPI – *Xba* I reverse (CC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG). The PCR product was cloned into the pBluescript SK +/- cloning vector (Fermentas, Canada). This plasmid was used as a template to construct the pointmutations. Mutagenic primers were designed containing the desired mutation(s) in the middle of the primer. The following single lysine residues in domain V of dimeric β_2GPI were substituted into a leucine (with the primer used for mutagenesis):
Lys282Leu (5' GAT AAA GTT TCT TTC TTC TGC CTG AAT AAG GAA AAG AAG TGT AGC 3')
Lys284Leu (GTT TCT TTC TTC TGC AAA AAT CTG GAA AAG AAG TGT AGC TAT ACA)
Lys286Leu (5' TTC TTC TGC AAA AAT AAG GAA CTG AAG TGT AGC TAT ACA GAG GAT 3')
Lys287Leu (5' TTC TGC AAA AAT AAG GAA AAG CTG TGT AGC TAT ACA GAG GAT GCT 3')
In addition, in three different cationic patches in domain V of β_2GPI, three to four lysines were substituted into a leucine in the following regions: Lys262Leu, Lys264Leu, Lys268Leu and Lys276Leu (group 1; further referred to as $Lys^{262-276}$), Lys282Leu, Lys284Leu, Lys286Leu and Lys287Leu (group 2; further referred to as $Lys^{282-287}$) and Lys305Leu, Lys308Leu and Lys317Leu (group 3; further referred to as $Lys^{305-317}$). The primers for mutagenesis for these three groups were (mutated amino acids are underlined):
Group 1 ($Lys^{262-276}$): (5' CTG ATT CAG GAA CTG TTT CTG AAT GGA ATG CTA 3' for Lys262Leu, Lys264Leu, Lys268Leu and 5' AAT GGA ATG CTA CAT GGT GAT CTG GTT TCT TTC TTC TGC AAA AAT 3' for $Lys^{262-276}$)
Group 2 ($Lys^{282-287}$): (5' TTC TGC CTG AAT CTG GAA CTG CTG TGT AGC TAT ACA GAG GAT GCT 3')
Group 3 ($Lys^{305-317}$): (5' GTC CCC CTG TGC TTC CTG GAA CAC AGT TCT CTG 3' for Lys305Leu, Lys308Leu and 5' TCT CTG GCT TTT TGG CTG ACT GAT GCA TCC GAT 3' for $Lys^{305-317}$)
The mutagenic primers were used to PCR amplify the entire plasmid using QuikChange Multi enzyme (Stratagene). The mutations were identified by sequence analysis with a β_2GPI forward primer (TAT CCT GCA AAA CCA ACA C). To construct fusion proteins of apple 4 and the β_2GPI mutants, the PCR products were cloned with *Xho* I and *Xba* I into the original vector (apple 4 – C321S – β_2GPI – tPA – S478A). In this way dimeric β_2GPI with the pointmutations were constructed.

Transfection, Expression, Cell Culture and Purification of dimeric β_2GPI mutants

Recombinant full-length and mutants of dimeric β_2GPI were purified from the culture supernatant of transfected baby hamster kidney cells by affinity chromatography using monoclonal anti-β_2GPI antibody [11]. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.7. The eluted fractions were immediately neutralized with 1 M Tris, pH 9. The pooled fractions were further purified on a mono S column using fast performance liquid chromatography (FPLC) (Amersham Pharmacia Biotech). Apple 4 was purified using a monoclonal antibody (anti-XI-1). Purity of the protein fractions was determined on a 4-15% polyacryl-amide gel electrophoresis (SDS-PAGE). Concentrated eluants were dialyzed against 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.

Gel Filtration Studies

The dimeric β_2GPI constructs proteins were applied to Superdex 200 gel filtration column using FPLC equipment. The column was equilibrated with TBS, pH 7.4, at a flow rate of 1.0 mL/min. The absorbance of the eluent was monitored at 280 nm. Molecular masses were determined by comparison to a standard curve of ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

Purification of plasma β_2GPI

Plasma β_2GPI was purified as described previously [10]. In short, b2GPI was isolated from freshly, frozen citrated human plasma. Dialyzed plasma was subsequently applied to the following columns: DEAE-Sephadex A50 column, protein G-Sepharose, S-Sepharose and finally heparin-Sepharose chromatography (all Sepharoses were obtained from Amersham Pharmacia Biotech). Bound proteins were eluted with a linear salt gradient. Afterwards, b2GPI was dialyzed against buffer containing 50 mM Tris, 150 mM NaCl, pH 7.4. Purity of the protein was checked with SDS-PAGE analysis.

Preparation of phospholipid vesicles

Phospholipid vesicles containing 20% phosphatidylserine (PS), 40% phosphatidylcholine (PC) and 40% phosphatidylethanolamine (PE) were prepared according to Brunner et al. [12] with some modifications as described by Van Wijnen et al [13]. The phospholipid concentration was determined by phosphate analysis [14].

Binding of dimeric β_2GPI mutants to phospholipid vesicles

Binding of dimeric β_2GPI mutants to phosphatidylserine (PS) / phostidylcholine (PC) (20% PS/PC 80%, 5% PS/PC 95%) vesicles and cardiolipin was tested in a solid phase binding assay. High binding 96 wells ELISA plates (Costar, Corning Incorporated, 9102) were coated with PS/PC (25 μM in TBS; 50 μL/well) overnight at 4oC. Cardiolipin (50 μg/mL in ethanol; 50 μL/well) was coated under a stream of nitrogen at room temperature. Wells were blocked with TBS/0.5% gelatine (150 μL/well) for two hours at 37oC. Subsequently, wells were incubated with different concentrations of the dimeric mutants (0.25-32 μg/mL) for 1.5 hours at 37oC, followed by incubation with Moab 2B2 (3 μg/mL; 50 μL/well). Apple 4 – β_2GPI was used as a positive control and plasma β_2GPI 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 37oC), 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 the β_2GPI mutants on the clotting time

All coagulation assays were measured in a KC-10 coagulometer (Amelung, Lemgo, Germany). The prothrombin time (PT), a phospholipid-depending coagulation assay, was performed to detect effects of the lysine substitutions in domain V of β_2GPI on clotting time. In short, 25 μL of normal pooled plasma and 25 μL of buffer, plasma β_2GPI or recombinant full-length dimeric β_2GPI or mutants were incubated for two minutes at 4ºC. After an incubation of 1.5 minute at 37ºC, clotting time was initiated by the addition of 50 μL of Innovin (Dade Behring, Marburg, Germany).

Surface plasmon resonance analysis for the interaction between dimeric β_2GPI mutants and apolipoprotein E receptor 2'

Surface plasmon resonance (SPR) binding assays were performed employing a Biacore 2000 system (Biacore AB, Uppsala, Sweden). ApoER2' was immobilized on a CM1 sensor chips using the amine-coupling kit (Biacore AB, Uppsala, Sweden). Approximately 7.4 fmol/mm^2 of apoER2' was immobilized. One channel was immobilized with an irrelevant, non-binding protein and afterwards its signal was used to correct the signal from the apoER2' coated channel for non-specific binding. SPR analysis was performed in buffer containing 25 mM HEPES, 125 mM NaCl, pH 7.4 with 3 mM CaCl2 Measurements were performed with a flow rate of 20 μl/min at 25ºC. 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 ethylenediaminetetraacetic acid (EDTA) and 1 M of NaCl, pH 5.0.

Results

Three-dimensional model of β_2GPI and its mutants

A three dimensional model of the fifth domain of β_2GPI has been generated with the X-ray structure as a template [1]. The fifth domain comprises residues 244 – 326 (*Figure 1, panel A*). The structure contains a large cationic area of 2000Å, which is illustrated in *Figure 2*, panel A as a large blue area. The positively charged patch on the surface of domain V decreases when lysine residues at position Lys^{262}, Lys^{264}, Lys^{268} and Lys^{276} (group 1; $Lys^{262-276}$) are substituted by leucine residues (*Figure 2, panel B*), which is indicated by the smaller blue area in the lower part of domain V of β_2GPI. Substituting residues at position Lys^{282}, Lys^{284}, Lys^{286} and Lys^{287} (group 2; $Lys^{282-287}$) and Lys^{305}, Lys^{308} and Lys^{317} (group 3; $Lys^{305-317}$) obtain similar results (*Figure 2, panel C and panel D,* respectively).

Most of the positively charged side chains (14 of 16) are located on the surface of two regions in domain V of β_2GPI. The first of the cationic regions is defined by amino acids $Lys^{282-287}$ at the top of the molecule (*Figure 1, panel C*). In addition, mutation of these lysines is known to disrupt binding to phospholipids. The second region is defined by amino acids $Lys^{305-317}$ and the third region by amino acids $Lys^{262-276}$. All three positively charged residues are predicted to interact with apoER2'.

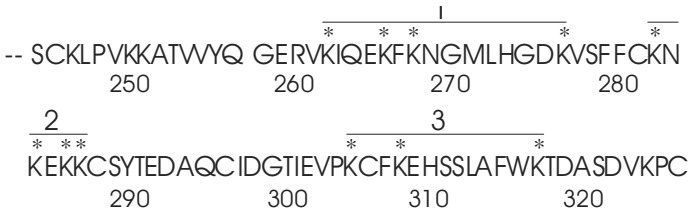

Figure 1A

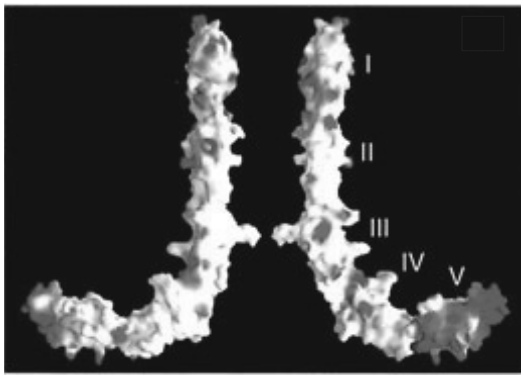

Figure 1B

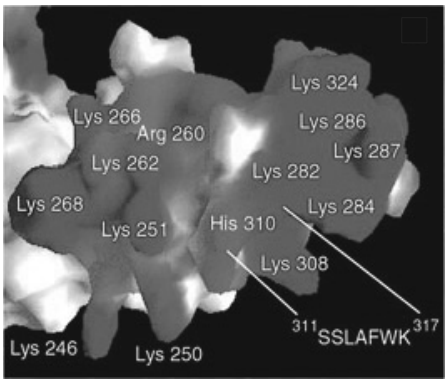

Figure 1C

Figure 1. Molecular structure of domain V of β_2GPI.
A, amino acid sequence of domain V of β_2GPI. The numbers in the bottom line refer to the residues in domain β_2GPI. The stars indicate the Lys residues that were mutated into Leu residues. The numbers on top of the lines correspond to the three groups in which three to four lysine residues are substituted. B, two views, related by 180° of the molecular model of β_2GPI highlighting the cationic patches in domain V of β_2GPI. C, view of the molecular model of the fifth domain of β_2GPI. The residues contributing to the cationic patch including the position of the phospholipid insertion (^{311}SSLAFWK^{317}) loop are indicated. Figure 1B and C are adapted from Bouma et al., 1999, EMBO J.

Expression and Purification of dimeric β_2GPI mutants

To study the molecular interaction of dimeric β_2GPI with the human platelet cellular receptor apoER2', we constructed dimeric β_2GPI mutants containing amino acid substitutions in a large cationic patch (aa 262-317) in domain V of β_2GPI. BHK cells were transfected with vectors containing the dimeric β_2GPI mutants. Protein expression was verified by western blotting using a monoclonal anti-β_2GPI antibody. Clones with the highest expression were selected using a β_2GPI enzyme linked immunosorbent assay (ELISA). Proteins were affinity purified with a monoclonal anti-β_2GPI column subsequently followed by FPLC with a mono S column. After

purification and protein quantification, the mutants were applied on a 7.5% SDS-PAGE under reducing and non-reducing conditions and stained by Coommassie Brilliant Blue. As shown in *Figure 3, panel A*, under non-reducing conditions all the mutants migrated with a molecular weight of approximately 50 kilo Dalton (kDa) comparable to that observed for full-length dimeric β_2GPI. After reduction (*Figure 3, Panel B*), the mutants migrated as monomers with a molecular mass of approximately 57 kDa.

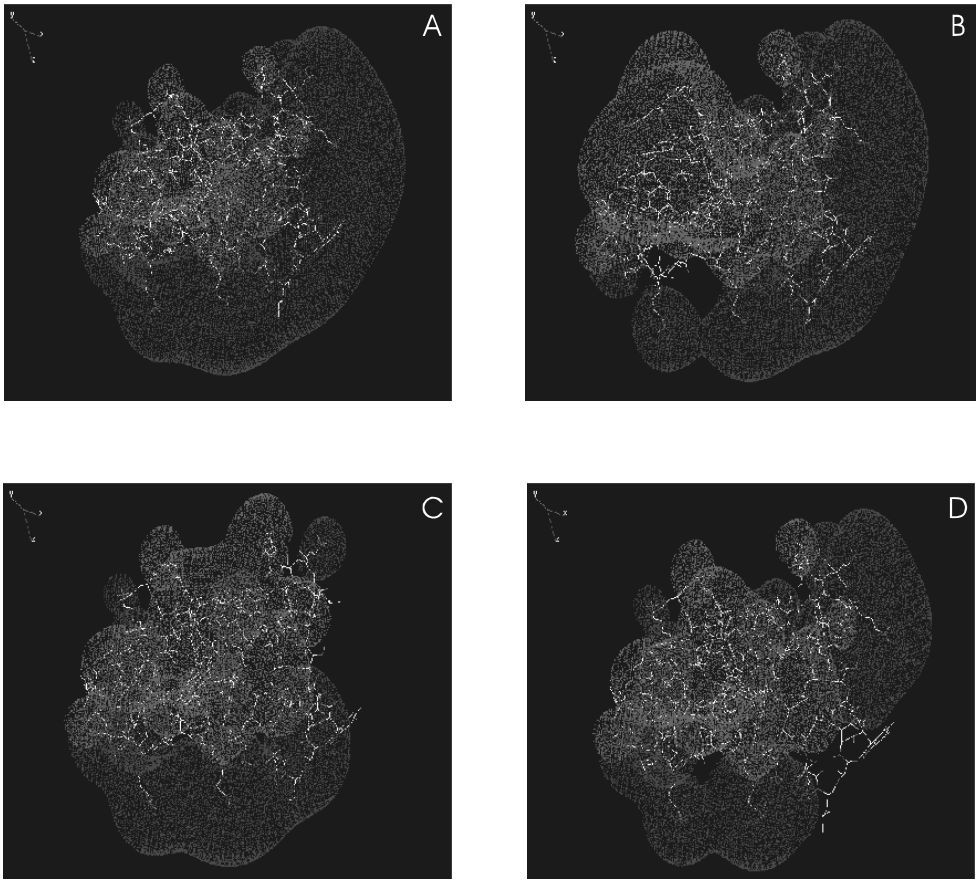

Figure 2. Electrostatic potential of the fifth domain of β_2GPI.
Electrostatic potential at the phospholipid-binding region of native domain V and mutants. The figures were prepared by program Swiss-PdbViewer. A, native domain V at neutral pH. B, $Lys^{262-276}$ mutant at neutral pH. The molecular surface of the models are coloured by electrostatic potential (positive = bleu; negative = red). C and D, $Lys^{282-287}$ and $Lys^{305-317}$ at neutral pH.

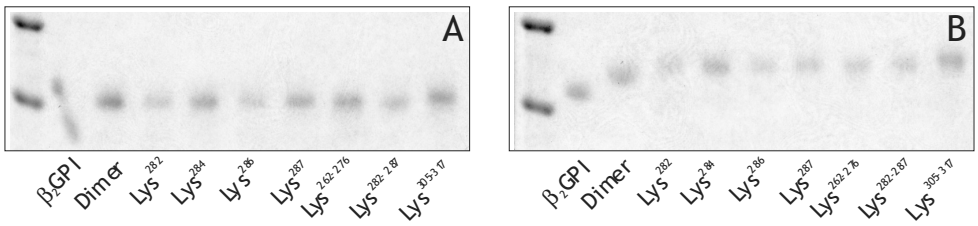

Figure 3. SDS-PAGE analysis of dimeric β_2GPI mutants.
Purified plasma β_2GPI, full-length dimeric β_2GPI and the dimeric mutants were analysed on a 7.5% SDS-PAGE under non-reducing- (A) and reducing (B) conditions. Gels were stained with Coommassie Brilliant Blue. The molecular masses of prestained markers are expressed in kilodalton (kDa).

Gel Filtration Studies

To determine whether the constructed mutants have the same conformation as full-length dimeric β_2GPI, we performed gel filtration studies under native conditions. Dimeric β_2GPI eluted with a molecular mass of 115 kilo Dalton (kDa), which was consistent with previous findings [10]. Several mutants tested eluted with a molecular mass of 115 kDa, demonstrating that both mutants also were a dimer under nondenaturing conditions (*data not shown*). These results suggest that the conformation of both mutants is similar compared to full-length dimeric β_2GPI. Plasma β_2GPI migrated with a molecular mass of 50 kDa.

Binding of mutants to immobilized phospholipids

The phospholipid binding features of the mutants were assessed in a solid-phase immunosorbent assay (ELISA). Phospholipid vesicles with different PS and PC content (20% PS/PC 80% and 5% PS/PC 95%) and cardiolipin were immobilized on 96-wells ELISA plates and binding of plasma β_2GPI and the dimeric β_2GPI mutants was measured. As shown in *Figure 4*, plasma β_2GPI did not bind to either 20% PS/PC 80% (*Panel A*) or 5% PS/PC 95% (*Panel C*), but plasma β_2GPI did bind to cardiolipin (*Panel E*). Half-maximal binding of full-length dimeric β_2GPI to phospholipids occurred at a concentration of 4.4 ± 0.1 μM for 5% PS/PC 95%, 22 ± 0.02 μM for 20% PS/PC 80% and $0.5 \cdot 10^{-3}$ ± 0.1 μM for cardiolipin (*Table 1*). Compared to the $Lys^{282-287}$ mutant, the $Lys^{305-317}$ mutant hardly bound to the PS/PC phospholipids (regardless of the PS/PC ratio), whereas the $Lys^{305-317}$ mutant showed less impaired binding to cardiolipin than the $Lys^{282-287}$ mutant. The mutant $Lys^{262-276}$ displayed only minor differences in PS- or cardiolipin-binding compared to full-length dimeric β_2GPI (*Table 1*). As

can be seen from *Figure 4, panel B and D*, the three positively charged groups in domain V of β_2GPI ($Lys^{262-276}$, $Lys^{282-287}$ and $Lys^{305-317}$) contributed to PS-binding in the order of position 305-317 > 282-287 > 262-276 for 5% PS:PC 95% and 20% PS: PC 80%. For cardiolipin, the observations were in contrast with the findings for PS containing surfaces. The contribution of cationic residues in domain V was in the order of position 282-287 > 305-317 > 262-276 (*Figure 4, panel F*). This suggests that binding of β_2GPI to cardiolipin requires other regions in domain V of β_2GPI than binding of β_2GPI to PS/PC. These results demonstrate a physiological significance of residues $Lys^{305-317}$ in domain V of β_2GPI for binding to anionic phospholipids.

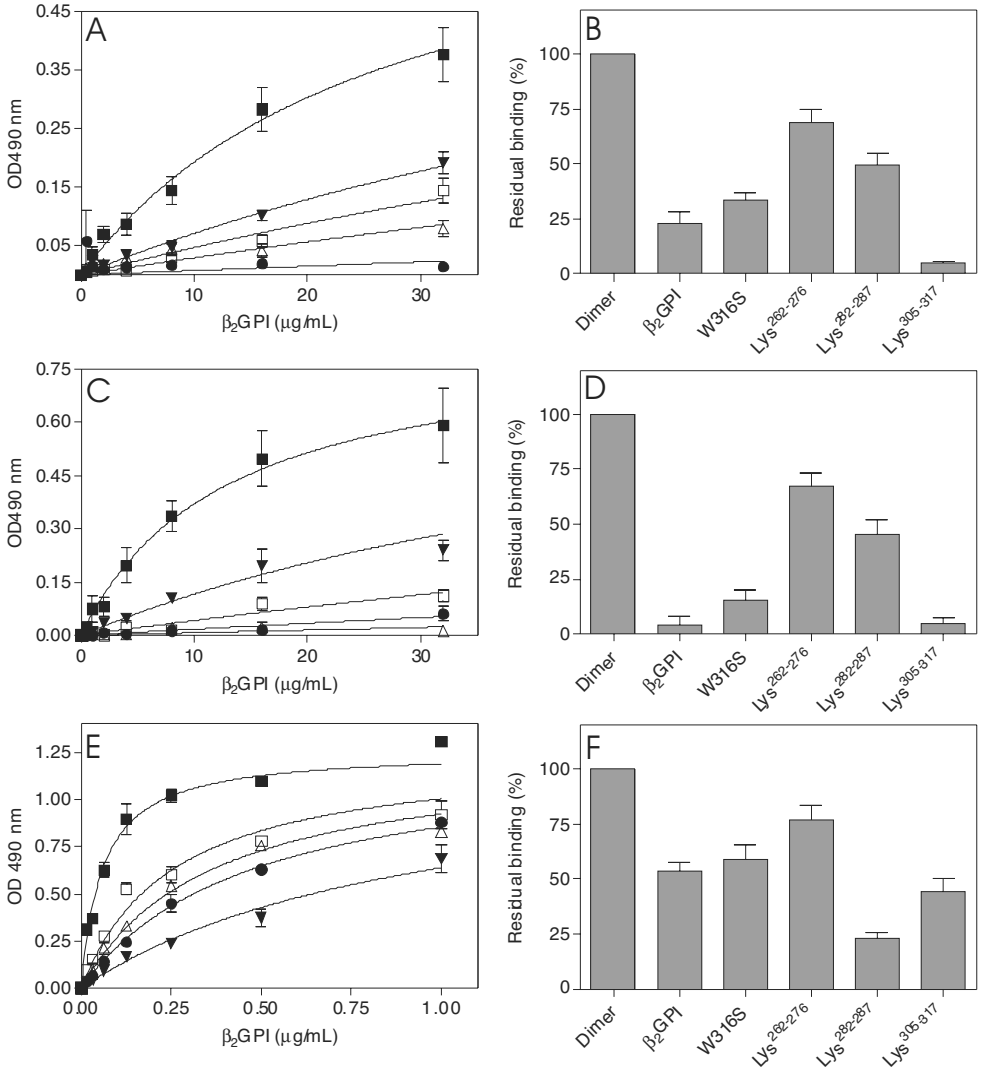

Figure 4. Binding of dimeric β_2GPI mutants to immobilized phospholipids
A and C, phospholipid vesicles (20% PS/PC 80% and 5% PS/PC 95%, 25μM) were immobilized on high binding 96-well ELISA plates. E, cardiolipin (50 μg/mL) was coated on 96-well ELISA plates under a stream of nitrogen. This was followed by incubation with increasing concentrations (ranging from 0 – 32 μg/mL for PS/PC and from 0 – 1 μg/mL) of plasma β_2GPI (Δ), full-length dimeric β_2GPI (■) and the dimeric mutants at 37ºC for 2 hours. The following constructs are shown: Lys$^{262-276}$ (▲), Lys$^{282-287}$ (▼), Lys305-317 (•) and the non-phospholipid binding dimer W316S (▫). Afterwards, bound protein was detected with Moab 2B2. Bound 2B2 was detected using OPD staining procedure. Results are expressed as mean ± SD (n = 3). B, D and E, percentage of binding to phospholipids is expressed relative to binding to phospholipids in the presence of full-length dimeric β_2GPI (set at 100%).

Table 1. Apparent dissociation constants of the dimeric β_2GPI mutants for phospholipids.
Curves of plasma β_2GPI, full-length dimeric β_2GPI and the dimeric mutants were fitted according to a one-site binding model in GraphPad. Half-maximal binding is given as apparent K_d ($K_{d(app)}$) in μmol/L (μM). Results are expressed as mean ± SD (n = 3).

Protein	Kd(app) (μM)		
	5:95	20:80	CL
β_2GPI	4.4 ± 0.1	22 ± 0.02	$7.4 \cdot 10^{-3}$ ± 0.2
fl-dimer	0.2 ± 0.03	0.1 ± 0.01	$0.5 \cdot 10^{-3}$ ± 0.1
Lys^{282}	1.8 ± 0.1	1.3 ± 0.04	$3.0 \cdot 10^{-3}$ ± 0.1
Lys^{284}	2.7 ± 0.1	1.9 ± 0.08	$1.6 \cdot 10^{-3}$ ± 0.1
Lys^{286}	2.7 ± 0.2	1.3 ± 0.05	$2.6 \cdot 10^{-3}$ ± 0.1
Lys^{287}	1.0 ± 0.1	0.7 ± 0.03	$2.6 \cdot 10^{-3}$ ± 0.1
$Lys^{262-276}$	0.5 ± 0.02	0.5 ± 0.02	$1.0 \cdot 10^{-3}$ ± 0.3
$Lys^{282-287}$	0.8 ± 0.04	0.6 ± 0.03	$8.8 \cdot 10^{-3}$ ± 0.2
$Lys^{305-317}$	7.8 ± 0.2	3.3 ± 0.2	$4.2 \cdot 10^{-3}$ ± 0.1
W316S	1.2 ± 0.05	1.6 ± 0.06	$2.1 \cdot 10^{-3}$ ± 0.1

Effect of the mutants on *in vitro* coagulation test

To study the effect of replacement of cationic charges into neutral charges in domain V of β_2GPI on coagulation, the prothrombin time (PT) with the mutants was measured. In this assay, prolongation of the clotting time was observed for full-length dimeric β_2GPI at 100 μg/mL [10]. Plasma β_2GPI, apple 4 and apple2 — β_2GPI did not prolong the clotting time (*Figure 5*). At a concentration of 100 μg/mL, full-length dimeric β_2GPI showed an increase in clotting time of 78.1 ± 2.4 %. At a concentration of 100 μg/mL, the constructs with mutations located in the KNKEKK site in domain V of β_2GPI showed a prolongation of 5.6 ± 2.8 %, 13.6 ± 2.4 %, 30.4 ± 2.4 % and 8.9 ± 3.3 %, respectively. The $Lys^{262-276}$, $Lys^{282-287}$ and the $Lys^{305-317}$ mutants showed a prolongation of 9.0 ± 1.0, 2.4 ± 0.5 % and 6.0 ± 4.0 %, respectively. As expected, delta V and the non-phospholipid binding dimer $Trp^{316}Ser$ were not able to prolong the clotting time.

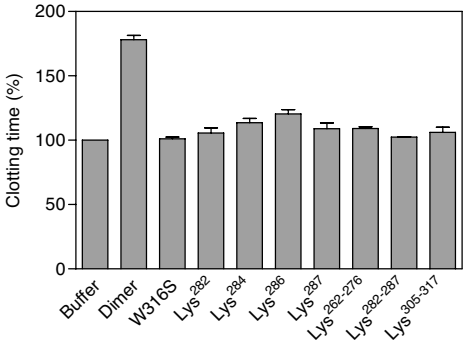

Figure 5. Effect of dimeric β_2GPI mutants on the prothrombin time (PT).
Buffer, full-length dimeric β_2GPI and the dimeric mutants were 1:1 diluted with normal pooled plasma (final protein concentration: 100 μg/mL) followed by measurement of the PT. Clotting time with buffer was set at 100%. Results represent mean clotting time ± SD in percentage (n = 3). Statistical analysis was performed using the Student T-test ($p \leq 0.001$). Differences between the pointmutations are not significant.

Interaction between dimeric β_2GPI mutants and apoER2'

Measurement of binding of the mutants $Lys^{262-276}$, $Lys^{282-287}$ and $Lys^{305-317}$ to apoER2' was performed in an ELISA. As shown in *Figure 6, Panel A,* both the $Lys^{282-287}$ and the $Lys^{305-317}$ mutant showed impaired binding to apoER2' with residual binding of 15.5 ± 1.5 % and 53.5 ± 4.5 %, respectively. The $Lys^{262-276}$ mutant showed less impaired binding to apoER2' (residual binding 82 ± 10 %). The significance of lysine residues within the KNKEKK site was further investigated using mutants with single Lys to Leu substitutions at position 282-287 in domain V of β_2GPI. In *Figure 6, panel B*, a representative sensorgram is shown, obtained from SPR analysis. Full-length dimeric β_2GPI (100 nM) associated with immobilized apoER2'. Upon replacement of full-length dimeric β_2GPI with buffer, the response started to decline gradually indicating that the protein dissociated from apoER2' and binding is reversible. Delta V (100 nM) hardly bound to apoER2'. As representative for the mutants, the sensorgram for $Lys^{282-287}$ (100 nM) is illustrated.

The interaction of the KNKEKK mutants with apoER2' was studied in more detail by calculating the steady state constants. The binding isotherms of the responses obtained at equilibrium are shown in *Figure 6, Panel C*. Full-length dimeric β_2GPI assembled efficiently with immobilized apoER2', whereas delta V displayed only minor association with the receptor. The dimeric β_2GPI mutants tested showed a clearly impaired association with apoER2'. However, there is no significant difference between the Lys^{282}, Lys^{284}, Lys^{286}, Lys^{287} and the $Lys^{282-287}$ mutants (residual binding 37.8 ± 3.1 %, 47.7 ± 5.2 % 47.7 ± 1.6 %, 48.4 ± 7.9 % and 38.7 ± 8.6 binding, respectively) (*Panel D*).

After Scatchard plot analysis and careful interpretation of the correctness of fit from the binding isotherms derived from SPS analysis, the interaction between the dimeric mutants and apoER2' showed one class of binding sites. Consequently, one affinity constant could be derived from the data for each mutant. The derived affinity constants are summarized in *Table 2*.

The three positively charged groups (Lys$^{262-276}$, Lys$^{282-287}$ and Lys$^{305-317}$) in domain V of β_2GPI contributed to apoER2'- binding in the order of position 282-287 > 305-317 > 262-276. All together, these data clearly indicate that the interaction between β_2GPI and apoER2' is due to a combined recognition site in the region Lys$^{282-287}$ and Lys$^{305-317}$, but with the most important cationic patch at position Lys$^{282-287}$ within domain V of β_2GPI.

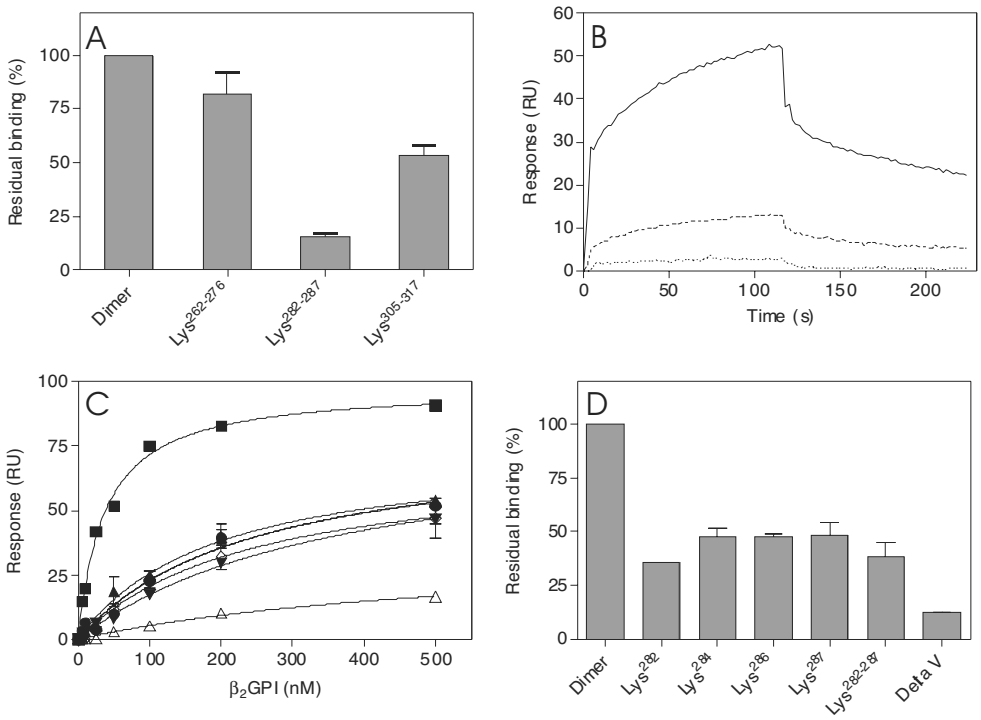

Figure 6. Binding of dimeric β_2GPI mutants to immobilized apoER2'.

SPR analysis and ELISA measured binding of full-length dimeric β_2GPI and the dimeric mutants. A, binding of the dimeric mutants Lys$^{262-276}$, Lys$^{282-287}$ and Lys$^{305-317}$ (3 μg/mL) to apoER2' (5 μg/mL) measured by ELISA relative to binding of full-length dimeric β_2GPI to apoER2' (set at 100%). B, a reproducible sensorgram showing the association phase and dissociation phase of full-length dimeric β_2GPI (—), Lys$^{282-287}$ (---) and delta V (·····). C, saturation curves derived from SPR analysis showing the dose-dependent interaction between full-length dimeric β_2GPI (■), Lys^{282} (▼), Lys^{284} (▲), Lys^{286} (•), Lys^{287} (+) and Lys$^{282-287}$ (◊). D, apoER2'-binding of the mutants with pointmutations in the KNKEKK region, as measured by SPR analysis. Expression is relative to binding of full-length dimeric β_2GPI to apoER2' (set at 100%).

Protein	K_d (nM)
fl-dimer	50 ± 14
Lys^{282}	450 ± 40
Lys^{284}	280 ± 20
Lys^{286}	320 ± 25
Lys^{287}	320 ± 25
$Lys^{262-276}$	NM
$Lys^{282-287}$	800 ± 180
$Lys^{305-317}$	240 ± 50
Delta V	> $2 \cdot 10^{-3}$

Table 2. Steady state constants of the dimeric β_2GPI mutants for apoER2'.
Binding isotherms were fitted accordingly to a one-site binding model. Steady state constants are expressed in nmol/L (nM). Data represent mean ± SD (n = 3). NM = not measured.

Discussion

Previously, we have shown that apoER2' recognizes a cationic region in domain V of β_2GPI [15] and that the same domain is crucial for recognition by anionic phospholipids (*J Throm Haem, 2006, accepted for publication*). In the present study, we have addressed the functional role of basic residues in domain V of β_2GPI for recognition by apoER2' and anionic phospholipids. To investigate the specific lysine residues within domain V of β_2GPI critical for binding to apoER2' and to anionic phospholipids, site-directed mutagenesis was performed with the three-dimensional model of the fifth domain of β_2GPI as lead. Three potential cationic patches were predicted to be involved in the interaction with apoER2' and subsequently the following dimeric mutants of β_2GPI were generated in which a single or several lysines in a particular area in domain V were substituted into a leucine: Lys^{282}, Lys^{284}, Lys^{286}, Lys^{287}, $Lys^{262-276}$ (a quatriple mutant), $Lys^{282-287}$ (a quatriple mutant) and $Lys^{305-317}$ (a triple mutant). The results of our studies are summarized in *Table 3*. Of cationic regions, $Lys^{282-287}$ proved most important for the interaction with apoER2' (K_d approximately 600 nM). A second cluster of lysine residues that is important for recognition by apoER2' is located at $Lys^{305-317}$, whereas $Lys^{262-276}$ is hardly involved in the interaction with apoER2'.

Phosphatidylcholine (PS) is one of the most important anionic phospholipids (comprises 8-15 mol% of the total phospholipid content of cellular membranes) and is thought to be involved in binding β_2GPI to cellular surfaces. In quiescent cells, PS is located at the cytoplasmic side of the plasma membrane. After activation, PS is exposed at the outer surface of cells. Several studies have investigated binding of β_2GPI to cardiolipin. Studies performed by other groups identified residues Lys^{284}, Lys^{286}, and Lys^{287} in the fifth domain of β_2GPI important for binding to cardiolipin (CL) [6;16;17]. So far, studies investigating the binding site within β_2GPI for PS surfaces, which are physiologically more important than CL, have not been conducted.

Based on the hypothesis that domain V carries the lipid-binding region within the sequence motif $C^{281}KNKEKKC^{288}$, we investigated the interaction between β_2GPI and anionic phospholipids (PS and CL) more closely using the dimeric mutants. The PS-binding site within domain V of β_2GPI is predominantly located at position $Lys^{305-317}$, whereas for cardiolipin the residue $Lys^{282-287}$ is more important. This observation was independent of the PS content tested in the binding assays. Our data are an addition on what is already known for binding of β_2GPI to anionic phospholipids. Regarding the binding of β_2GPI to cardiolipin, another group identified a crucial role for a hydrophobic region at position 313-316 in domain V of β_2GPI. This study also used recombinant β_2GPI constructed by site-directed mutagenesis [18]. However, the latter study only used recombinant β_2GPI with mutations at position 306 and 316 and did not test other regions in domain V of β_2GPI. The observed differences between PS-binding and CL-binding can be explained by the physical properties of both lipids. PS/PC forms unilamellar vesicles, whereas CL forms bilayers. The differences in lipid properties might explain the observation that various anionic phospholipids recognize different regions within domain V of β_2GPI.

A cluster of lysine residues at position $C^{281}KNKEKKC^{288}$ supports binding of β_2GPI to endothelial cells [19]. Our group has identified several members of the LDL-R family on endothelial cells (*unpublished data*). We suggest that the cationic patch at position $Lys^{282-287}$ might be involved in the interaction between β_2GPI and LDL-R homologous on endothelial cells. However, the role of the cationic patch $Lys^{282-287}$ for other members of the LDL-R family still needs to be elucidated.

Different hypotheses for the interaction of β_2GPI with membranes are discussed in the literature. One very interesting aspect outlines that the targets of β_2GPI are cell surface receptor proteins rather than lipids [20]. The underlying mechanisms for such a β_2GPI-protein interaction might be quite different from the association with anionic phospholipids and certainly require further structural characterization. Some studies stress that binding of β_2GPI to anionic phospholipids alters the conformation of the protein, thus exposing neo-epitopes [21;22]. Based on several lines of evidence, β_2GPI seems to be anchored in the lipid membrane via domain V. After a structural rearrangement of the protein, other regions in domain V might be exposed and can exert other biological functions, such as interacting with cellular receptors. An important feature of (anionic) phospholipid bilayers, present in cellular membranes, is that they are cooperative structures. They are held together by many reinforcing, covalent interactions. The β_2GPI-binding properties of apoER2' are dependent on interactions between different sites within domain V of β_2GPI, with emphasis on $Lys^{282-287}$, whereas $Lys^{303-317}$ is crucial for β_2GPI-binding to

PS. These observations suggest that β_2GPI-binding to PS cooperates β_2GPI-binding to apoER2'.

In conclusion, the interaction between *i*. β_2GPI and ii. anionic phospholipids and apoER2' involves different non-overlapping binding sites within domain V of β_2GPI. Interestingly, all residues responsible for binding to apoER2' and binding to anionic phospholipids are located at the aberrant non-SCR half of domain V of β_2GPI.

Table 3. Binding characteristics of the dimeric β_2GPI mutants
Binding characteristics of the dimeric mutants were compared with that of full-length dimeric β_2GPI and assigned a plus mark according to the strength of the interaction.

Protein	PT	PS	CL	apoER2'
fl-dimer	+	+ + +	+ + + +	+ + +
Lys^{282}	-	+	+ +	+
Lys^{284}	-	+	+ + +	+
Lys^{286}	-	+	+ +	+
Lys^{287}	-	+	+ +	+
$Lys^{262-276}$	-	+ +	+ + +	+ + +
$Lys^{282-287}$	-	+ +	+	+
$Lys^{305-317}$	-	-	+ +	+ +
Delta V	-	-	+ +	-

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PATHOGENIC ANTI-BETA2-GLYCOPROTEIN I ANTIBODIES RECOGNIZE DOMAIN I OF BETA2- GLYCOPROTEIN I ONLY AFTER A CONFORMATIONAL CHANGE

6

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Abstract

Recently, we published the existence of two populations of anti-β_2-glycoprotein I (β_2GPI) IgG antibodies. Type A antibodies recognize epitope G40-R43 in domain I of β_2GPI and are strongly associated with thrombosis. Type B antibodies recognize other parts of β_2GPI and are not associated with thrombosis. In this study, we demonstrate that type A antibodies only recognize plasma-purified β_2GPI when coated onto a negatively-charged surface, and not when coated onto a neutrally-charged surface. The affinity of type B antibodies towards plasma-purified β_2GPI was independent of the charge of the surface to which β_2GPI was coated. Type A antibodies did not recognize plasma-purified β_2GPI in solution, whereas they did recognize recombinant β_2GPI both in solution and coated onto a neutrally-charged plate. When the carbohydrate chains were removed from plasma-purified β_2GPI, we found that type A antibodies did recognize the protein in solution. This supports the hypothesis that the difference in recognition of plasma-purified and recombinant β_2GPI is caused by the difference in glycosylation and that epitope G40-R43 of plasma-purified β_2GPI is covered by a carbohydrate chain. Type A anti-β_2GPI antibodies can only recognize this epitope when this carbohydrate chain is displaced due to a conformational change. This finding has major implications both for the detection of pathogenic anti-β_2GPI antibodies and the comprehension of the pathophysiology of the antiphospholipid syndrome.

Introduction

The antiphospholipid syndrome is a systemic autoimmune disease that is characterized serologically by the presence of antiphospholipid antibodies in plasma of patients, and clinically by vascular thrombosis and/or recurrent pregnancy morbidity [1,2,3]. In 1990 it was shown that these so-called antiphospholipid antibodies do not recognize phospholipids directly, but a phospholipid-binding protein: β_2-glycoprotein I (β_2GPI) [4]. Ever since, antiphospholipid antibodies with affinity for a large number of other phospholipid-binding proteins have been described. Only antiphospholipid antibodies with affinity for β_2GPI are thought to be clinically relevant [5]. Several assays are available to detect anti-β_2GPI antibodies. The most common one is the anticardiolipin enzyme-linked immunosorbent assay (ELISA). In this assay, the antibodies recognize β_2GPI bound to immobilized cardiolipin [6]. A second assay is the lupus anticoagulant assay, which detects anti-β_2GPI antibodies with capacity to prolong phospholipid-dependent coagulation assays (LAC) [7]. A third method is an ELISA-based method in which antibodies recognize β_2GPI coated directly onto ELISA plates [3].

β_2GPI (formerly known as apolipoprotein H) is a protein of 44 kDa with a plasma concentration of approximately 150 μg/ml. β_2GPI is highly glycosylated as it contains 4 N-linked carbohydrate side-chains. Together these carbohydrate side-chains account for approximately 15% (w/w) of the total molecular mass of β_2GPI. The protein β_2GPI consists of 326 amino acids organized in 5 complement control protein domains [8,9]. Each domain consists of 60 amino acids, except for domain V. Domain V consists of 82 amino acids, caused by a C-terminal extension of 19 amino acids and an insertion of 6 amino acids forming a hydrophobic loop. This specific structure of domain V is responsible for the binding properties of β_2GPI to anionic phospholipids [8,9].

Although the three-dimensional structure of β_2GPI is known for over 5 years, the mechanism by which anti-β_2GPI antibodies recognize β_2GPI is unclear [9,10]. Two main theories have been proposed to explain the binding of antiphospholipid antibodies to β_2GPI. The first and most accepted hypothesis is known as the "dimerization theory"; one antibody must bind two β_2GPI molecules to obtain considerable avidity [11-14]. To achieve this, a high density of β_2GPI on an ELISA plate is essential. It has been reported that the coated amount of β_2GPI must exceed a certain threshold before antiphospholipid antibodies are able to bind [15]. The studies in favor of the "dimerization theory" seem rather convincing but several observations remain unexplained, and are in favor of a second hypothesis. This hypothesis is based on the recognition of a cryptic epitope by antiphospholipid antibodies. This epitope is

only exposed after binding of β_2GPI to a negatively charged surface [16-18]. Supporting this latter hypothesis is the fact that the structure of β_2GPI in solution differs from that of crystallized β_2GPI [9,10,19]. Moreover, no β_2GPI-antibody complexes can be detected in the circulation of patients with the antiphospholipid syndrome (*unpublished observations*). Furthermore, it was shown by circular dichroism measurements that cardiolipin can induce a conformational change in β_2GPI [16].
We have recently published that the population of anti-β_2GPI antibodies recognizing epitope G40-R43 (type A antibodies) cause LAC and strongly correlate with thrombosis (odds ratio 18.9, 95% confidence interval: 53.2 - 6.8). The other group of anti-β_2GPI antibodies (type B antibodies) recognized other parts of β_2GPI and did not correlate with thrombosis (1.1, 95% confidence interval: 2.8 – 0.4). In this study we have further investigated the epitope(s) on β_2GPI recognized by type A and type B anti-β_2GPI antibodies.

Experimental Procedures

Patients

52 patient plasmas positive for anti-β_2GPI IgG antibodies were included in this study; 36 patients with systemic lupus erythematosus (SLE), 11 patients with lupus like disease (LLD) and 5 patients with primary antiphospholipid syndrome. Patients with SLE meet at least 4 ACR (American College of Rheumatology) criteria for the classification of SLE and patients with LLD 1 to 3 of these criteria [20]. Patients with primary antiphospholipid syndrome have antiphospholipid antibodies (LAC and/or anticardiolipin antibodies) and a history of thrombosis in the absence of any sign of a systemic autoimmune disease. By chart review, the number of objectively verified thromboembolic events was recorded for each patient [5]. For the diagnosis of thrombosis of intracerebral vessels, computed tomographic scanning or magnetic resonance imaging was used. Myocardial infarction was diagnosed by typical electrocardiographic features and an elevated fraction creatine kinase myoglobin (CK-MB). Peripheral arterial thrombosis and thrombosis of the distal aorta was diagnosed by arteriography. Retinal thrombosis was documented by funduscopy and fluorescence angiography. Deep vein thrombosis (DVT) was diagnosed by ultrasonography or venography, pulmonary embolism by radionuclide lung scanning. Portal vein thrombosis was diagnosed by angiography. A patient was diagnosed with superficial thrombophlebitis if the manifestation was diagnosed clinically. Blood was drawn from these patients at an arbitrary visit at the lupus clinic of the University Medical Center Utrecht, Utrecht, the Netherlands. The

Institutional Review Board of the University Medical Center Utrecht approved this study, and informed consent was obtained from all patients.

Serological assays

Anti-β_2GPI IgG antibody ELISA

Antibodies against β_2GPI were measured in an ELISA as described before (inter-assay variability 11.5%) [21]. In short, plasma-purified β_2GPI (10 μg/ml diluted in a Tris based solution (TBS): 50 mM Tris in 100 mM NaCl) was incubated onto hydrophilic ELISA plates (Costar cat. nr. 9102, New York, USA). After one hour, the plates were incubated with 4% bovine serum albumin (BSA) in TBS for one hour. This was followed by incubation with patient plasma (1:50 diluted in blocking solution) for 1 hour. Then the plates were incubated with a goat-anti-human IgG alkaline-phosphatase-labeled antibody (diluted 1:1000, Biosource, Camarillo USA) followed by staining with para-nitrophenyl phosphatase (PnPP, 0.6 mg/ml diluted in diethynolamine (DEA) buffer, Sigma, St. Lious, MO, USA). Plasma samples were regarded positive when the absorbed value exceeded the cut-off value (mean +3SD of 40 healthy volunteers) and corrected for the absorption of standard positive plasma.

LAC

To determine LAC activity, an APTT and a Dilute Russell Viper Venom Time (DRVVT) were performed [5]. For the APTT (PTT-LA, inter-assay variability 2.0%, Diagnostica Stago, Gennevilliers, France), 50 μl patient plasma of patients was diluted 1:1 with normal pool plasma of 40 healthy volunteers and incubated with 50 μl APTT reagent. Coagulation was initiated by the addition of 50 μl $CaCl_2$ (25 mM). As a control, samples were tested in an actin-FS based APTT (APTT-FS, Dade Behring, Marburg, Germany) which is a LAC insensitive assay. Patients were considered positive when the ratio PTT-LA/APTT-FS was greater than 1.20 (10). The DRVVT was performed according to the instructions of the manufacturers (inter-assay variability 3.2%, Gradipore Ltd, North Ryde, Australia) and considered positive when LAC screen/LAC confirm > 1.20. A patient was considered LAC positive if one of the two LAC assays was positive.

Anticardiolipin antibody ELISA

Anticardiolipin antibodies were measured in an ELISA as described before (inter-assay variability 6.9%) [22]. Nine IgG/IgM calibrators were used to report

anticardiolipin antibody levels as GPL or MPL units. Levels above 10 GPL or GPM units were considered positive.

Anti-domain I IgG ELISA

We recently described a method in which we were able to discriminate between anti-β_2GPI IgG antibodies with reactivity towards epitope G40-R43 in domain I (type A antibodies) and anti-β_2GPI IgG antibodies with other reactivity (type B antibodies) [23]. In short, hydrophilic plates (Costar cat. nr. 9102, New York, USA) and hydrophobic plates (Costar cat. nr. 2595, New York, USA) were coated with domain I of β_2GPI (5 μg/ml diluted in TBS) for 1 hour at 37ºC. After every incubation step, the plates were washed 3 times with 0.1% Tween/TBS. The plates were blocked with 4% BSA/0.1% Tween/TBS and subsequently incubated with patient plasma (diluted 1:100 in blocking solution) containing anti-β_2GPI IgG antibodies. The bound IgG antibodies were detected by a goat-anti-human IgG alkaline-phosphatase-labeled antibody (diluted 1:1000, Biosource). This was followed by staining with PnPP (0.6 mg/ml in DEA solution). Anti-β_2GPI IgG antibodies were regarded as type A antibodies when these showed decreased affinity for domain I coated onto hydrophilic plates compared to that with domain I coated onto hydrophobic plates. Anti-β_2GPI IgG antibodies were regarded as type B antibodies when they showed equal affinity for domain I on both plates. A ratio of greater than 2 between the OD measured with the hydrophobic plate and the OD with the hydrophilic plate discriminates between type A and type B anti-β_2GPI IgG antibodies. This ratio is an indication for the relative amount of anti-β_2GPI IgG antibodies that recognize the positive charge (epitope G40-R43) on domain I (inter-assay variability 3.8%).

Purification of β_2GPI from plasma

Human plasma was dialyzed against a solution containing 0.04 M Tris, 0.01 M succinate, 0.005% polybrene, 1 mM EDTA, 1mM benzamidin, 43 mM NaCl and 0.02% NaN_3. Then, the plasma was added to DEAE-Sephadex column, and the flow-through was collected, pooled and added to a protein-G-Sepharose column. Then the effluent pool was added to a mono-S Sepharose column. The bound β_2GPI was eluted by a linear salt-gradient (138 mM NaCl – 550 mM NaCl), and checked for purity by SDS-PAGE [24]. The purified β_2GPI was analyzed with gel filtration and showed a single peak at the expected molecular weight.

Recombinant β_2GPI, and domain deleted mutants of β_2GPI

Recombinant full-length β_2GPI (DI-V) and 8 domain deletion mutants of β_2GPI

(comprising domain I (DI), domains I and II (DI-II), domains I, II and III (DI-III), domains I, II, III, and IV (DI-IV), domains II, III, IV and V (DII-V), domains III, IV and V (III-V), domains IV and V (DIV-V) and domain V (DV)) were obtained by a generous gift from Dr. Iverson of La Jolla Pharmaceutical Company [25]. The recombinant full-length β_2GPI was analyzed with gel filtration and showed a single peak at the expected molecular weight. On SDS-PAGE electrophoresis, the molecular weight of recombinant β_2GPI was slightly lower than the molecular weight of plasma-purified β_2GPI.

Purification of immunoglobulin G (IgG) antibodies from plasma samples

Blood samples were collected by venipuncture using plastic tubes containing 3.8% trisodium citrate (0.129 M) as the anticoagulant (9:1, v/v). To obtain platelet poor plasma the samples were centrifuged twice at 2000 g for 10 minutes, and subsequently stored at -50ºC until further use. The IgG fraction was purified by the use of a protein G-Sepharose column. By adding the IgG fraction to a column coated with β_2GPI, anti-β_2GPI antibodies were purified. The purity of the (affinity-) purified IgG fractions was checked by using SDS-PAGE.

Binding studies with β_2GPI coated onto hydrophobic plates or hydrophilic plates

Hydrophilic plates (Costar cat. nr. 9102, New York, USA) and hydrophobic plates (Costar cat. nr. 2595, New York, USA) were incubated with 50 µl plasma-purified β_2GPI or recombinant β_2GPI (10 µg/ml in TBS) for one hour at 37ºC. Subsequently, the plates were washed three times with washing solution (TBS/0.1% Tween) and blocked with 150 µl blocking solution (4% BSA)/TBS/0.1% Tween) for one hour at 37ºC. The plates were washed 3 times, and incubated with either patient plasma (diluted 1:50), patient IgG antibodies (type A and type B), monoclonal antibody 4F3, monoclonal antibody 2B2 or monoclonal antibody 27G7 in various concentrations (diluted in blocking buffer), for one hour at 37ºC [26,27]. Monoclonal antibodies 2B2 and 27G7 were kindly provided by Dr. Tincani and Dr. J. Arnout, respectively. To detect the bound monoclonal antibodies, the plates were washed and incubated with 50 µl rabbit-anti-mouse peroxidase-labeled antibody (diluted 1:1000, Dako, Glostrup, Denmark), followed by staining with an ortho-phenylene diamine solution (OPD solution; 4 mg/ml OPD diluted in 0.1 M $NaH_2PO_4.2H_2O$/0.1 M $Na_2HPO_4.H_2O$). The coloring reaction was stopped with the addition of 1 M H_2SO_4, and absorbance was measured at 490 nm. To detect the bound patient IgG antibodies, the plates were incubated with an alkalinephosphatase-labeled goat-anti-human IgG antibody

(diluted 1:1000, Biosource, Camarillo USA). PnPP was used as coloring substance. The reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm.

Binding of patient-derived IgG antibodies to recombinant and plasma-purified β_2GPI in solution

Hydrophilic ELISA plates (Costar cat. nr. 9102) were coated with 10 μg/ml plasma-purified β_2GPI, for 1 hour at 37ºC. The plates were washed 3 times with washing solution (TBS/0.1% Tween) and blocked with 150 μl blocking solution (4% BSA/ TBS/0.1% Tween) for one hour at 37ºC. Then, the patient-derived IgG type A antibodies (total IgG fraction) were incubated to the plate in the presence of various concentrations of plasma-purified β_2GPI and recombinant β_2GPI. The patient-derived IgG antibodies bound to the plate were detected by using an alkaline-phosphatase-labeled goat-anti-human IgG antibody (diluted 1:1000, Biosource, Camarillo USA). PnPP (0.6 mg/ml diluted in DEA buffer) was used as coloring substance. The reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm.

Recognition of plasma-purified β_2GPI in solution in the presence of cardiolipin vesicles and phosphatidylserine/phosphatidylcholine (PS/PC) vesicles

Affinity-purified patient IgG type A antibodies (20 μg/ml) of patient 1 and patient 2, were diluted in 150 mM $Na_2CO_3.10H_2O$/350 mM $NaHCO_3$ (pH 9.6) to reach a final concentration of 20 μg/ml, and were coated onto hydrophobic ELISA plates (Costar cat. nr. 2595) for 12 hours at 4ºC. The plates were washed three times with TBS/0.1% Tween and subsequently blocked with 4% BSA/TBS/0.1% at 37ºC. After 1 hour the plates were washed 3 times, and incubated with plasma-purified β_2GPI (10 μg/ml), plasma-purified β_2GPI (10 μg/ml) in the presence of cardiolipin vesicles (20 μM), or plasma β_2GPI (10 μg/ml) in the presence of PS/PC vesicles (20%/80%, 20 μM), all diluted in blocking solution. The cardiolipin and PS/PC vesicles were prepared as described before [7,28]. Then the plates were washed and incubated with a polyclonal goat-anti-human anti-β_2GPI antibody (10 μg/ml). After washing, the plates were incubated with a rabbit-anti-goat peroxidase-labeled antibody (diluted 1:1000, Biosource). Coloring was performed by using OPD. The coloring reaction was stopped by the addition of 1 M H_2SO_4, and absorbance was measured at 490 nm.

The influence of aggregating plasma-purified β_2GPI on the recognition by type A antibodies

To aggregate plasma-purified β_2GPI, the protein was incubated with 1% glutaraldehyde for 15 minutes at room temperature. Then, the solution was dialyzed against TBS and analyzed by using SDS-PAGE. The aggregated β_2GPI, plasma-purified β_2GPI and recombinant β_2GPI were coated onto a hydrophobic ELISA plate (Costar cat. nr. 2595), at a concentration of 10 ug/ml for one hour at 37ºC. Then, the plates were blocked with 4% BSA/TBS/0.1% Tween. After washing the plates, they were incubated with 2 type A plasma samples (1:100 diluted in blocking solution) for one hour at 37ºC. The bound IgG antibodies were detected with an alkaline-phosphatase-labeled goat-anti-human IgG antibody (diluted 1:1000, Biosource) and coloring was performed by using PnPP. The coloring reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm. For the inhibition ELISA we coated 10 ug/ml plasma-purified β_2GPI to a hydrophilic ELISA plate (Costar cat. nr. 2595). The plates were washed three times with TBS/0.1% Tween and subsequently blocked with 4% BSA/TBS/0.1% at 37ºC. After 1 hour the plates were washed 3 times, and incubated with 10 μg/ml anti-β_2GPI antibodies of patient 1 (type A) in the presence of either 100 μg/ml plasma-purified β_2GPI, 100 μg/ml recombinant β_2GPI or 100 μg/ml glutaraldehyde-treated β_2GPI. The antibodies bound the coated β_2GPI were detected with an alkaline-phosphatase-labeled goat-anti-human IgG antibody (diluted 1:1000, Biosource) and coloring was performed by using PnPP. The coloring reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm.

Removal of the carbohydrate side-chains of plasma-purified β_2GPI

β_2GPI was deglycosylated by incubating 50 μl sample (β_2GPI, 1.1 mg/ml diluted in TBS) with 5 units N-Glycosidase F (Roche, Mannheim, Germany) for 24 hours at 37ºC. The extent of deglycosylated β_2GPI was analyzed by using SDS-PAGE. The deglycosylated β_2GPI was analyzed with gel filtration and showed a single peak at the expected molecular weight.

Production of F(ab) fragments of monoclonal antibody 4F3

First, monoclonal antibody 4F3 was dialyzed against 100 mM sodiumacetate (pH 6.2). Then cysteïn, EDTA, sodiumazide were added to the antibody solution to obtain a final concentration of subsequently 50 mM, 1mM and 0.05%. This was followed by the addition of papaïn (10 μg papaïn per 1 mg antibody) (Sigma, P3125). After incubating the solution for 8 hours at 37ºC, iodoacetamide was added

(final concentration: 75 mM). By using a protein G coupled sepharose column the Fc parts of the antibodies were removed from the solution and the purity of the F(ab) fragments was analyzed by using SDS-PAGE.

Binding of patient IgG to plasma-purified β_2GPI, deglycosylated β_2GPI and recombinant β_2GPI in solution

Affinity-purified patient IgG type A antibodies (20 μg/ml) of patient 1, patient 2, monoclonal antibody 4F3 (3 μg/ml), F(ab) fragments of monoclonal antibody 4F3 (15 μg/ml) and monoclonal antibody 2B2 (3 μg/ml) were diluted in 150 mM $Na_2CO_3.10H_2O$/350 mM $NaHCO_3$ (pH 9.6) and coated onto hydrophobic ELISA plates (Costar cat. nr. 2595), for 12 hours at 4ºC. The plates were washed 3 times with TBS/0.1% Tween and subsequently blocked with 4% BSA/TBS/0.1% at 37ºC. After 1 hour the plates were washed 3 times, and incubated with different concentrations of either plasma-purified β_2GPI, deglycosylated plasma-purified β_2GPI or recombinant β_2GPI, all diluted in blocking solution. Then the plates were washed and incubated with a polyclonal goat-anti-human anti-β_2GPI antibody. After washing, the plates were incubated with a rabbit-anti-goat peroxidase-labeled antibody (diluted 1:1000). Coloring was performed by using OPD. The coloring reaction was stopped by the addition of 1 M H_2SO_4, and absorbance was measured at 490 nm.

Domain specificity of patient IgG antibodies and monoclonal antibodies 4F3 and 2B2

The reactivity of antibodies against β_2GPI domain deletion mutants was tested by coating 96-well hydrophobic ELISA plates (Costar cat. nr. 2595) with deletion mutants in a concentration of 10 μg/ml, for 1 hour at 37ºC. The plates were washed 4 times with TBS/0.1% Tween and subsequently blocked with a 4% BSA/TBS solution. Patient IgG fractions (10 μg/ml) and monoclonal antibodies 4F3 and 2B2 (both 3 μg/ml) were diluted in blocking solution, and added to the wells (50μl/well, 1 hour at 37ºC). The plates were washed 4 times (0.1% Tween/TBS), and incubated with alkalic-phosphatase-labeled goat-anti-human IgG antibodies (diluted 1/1000, Biosource) to detect the bound patient IgG antibodies (1 hour at 37ºC). Staining was performed with PnPP (Sigma, St. Louis, MO, USA) at a concentration of 0.6 mg/ml diluted in DEA buffer. The coloring reaction was stopped with 2.4 M NaOH, and absorbance was measured at 405 nm. The bound monoclonal antibodies were detected by peroxidase-labeled rabbit-anti-mouse antibodies, and coloring was performed with an OPD solution. The coloring reaction was stopped by 1 M H_2SO_4, and absorbance was measured at 490 nm.

Influence of increasing NaCl concentration on the recognition of β_2GPI by anti-β_2GPI IgG antibodies

Hydrophilic and hydrophobic plates (Costar cat. nr. 9102; Costar cat. nr. 2595) were incubated with 10 μg/ml plasma-purified β_2GPI or recombinant β_2GPI for 1 hour at 37ºC. After washing, the plates were blocked with 4% BSA/TBS/0.1%Tween at 37ºC for one hour. Subsequently the plates were washed and incubated with patient plasma diluted (1:100) in blocking solution containing either 150 mM NaCl or 5 M NaCl. After washing, the plates were incubated with a alkaline-phosphatase-labeled goat-anti-human IgG antibody (diluted 1:1000). Coloring was performed by using PnPP. The coloring reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm.

Results

Serological and clinical characterization of the patient population

Thirty (58%) out of 52 plasma samples with anti-β_2GPI IgG antibodies tested positive in the anti-domain I IgG ELISA (type A antibodies). Twenty-two (42%) plasma samples tested negative in the anti-domain I IgG ELISA (type B antibodies). All 52 (100%) plasma samples tested positive for anticardiolipin antibodies. Thirty-four (65%) plasma samples showed LAC activity. Of these, 28 (82%) were positive for type A and 6 (18%) for type B antibodies.

Twenty-five (83%) out of 30 patients with type A antibodies had a history of thrombosis. Of these, 16 (53%) patients had a history of venous thrombosis and 12 (40%) of arterial thrombosis. Seven (32%) patients with type B antibodies had a history of thrombosis. Of these, 5 (23%) had a history of venous thrombosis and 4 (18%) a history of arterial thrombosis.

The recognition of β_2GPI when coated onto different types of plates

To investigate whether different coating conditions influence the recognition of β_2GPI by type A and type B IgG antibodies, plasma-purified β_2GPI was coated onto hydrophilic and hydrophobic ELISA plates. Type A patient plasmas (n=30) recognized plasma-purified β_2GPI coated onto a hydrophilic plate much better than when coated onto a hydrophobic plate (median OD 0.262 vs. median OD 0.010, p=0.001). This is a reduction of 96% (OD 0.252). For type B plasmas (n=22) we did not observe a difference in recognition when plasma-purified β_2GPI coated onto either a hydrophilic plate or a hydrophobic plate (median OD 0.091 vs. median OD 0.076, no significant difference). This is a reduction of 16% (OD 0.015). For both

type A and type B plasma, a typical example is shown in figure 1A.
For total IgG, we performed this type of experiment with 3 total IgG samples of each type of plasma. All type A patients we tested had suffered from venous thrombosis in the past, and 1 of the 3 type B patients had suffered from venous thrombosis. We observed the same pattern as with the plasma samples; Type A IgG antibodies showed a high affinity for plasma-purified β_2GPI coated onto hydrophilic plates, but showed negligible affinity for plasma-purified β_2GPI coated onto hydrophobic plates. Type B IgG antibodies showed equal affinity for plasma-purified β_2GPI coated onto hydrophilic or hydrophobic plates. Both type A and type B IgG antibodies had similar affinity for recombinant β_2GPI coated onto hydrophilic or hydrophobic plates. For both type A and type B IgG a typical example is shown in figure 1B.
Monoclonal antibody 4F3 showed the same recognition pattern as type A IgG antibodies (Figure 1C). For two other monoclonal antibodies, 2B2 and 27G7, the recognition of β_2GPI was independent of the type of plate (Figure 1C). This indicates that both types of ELISA plates bound comparable amounts of plasma-purified β_2GPI and recombinant β_2GPI.

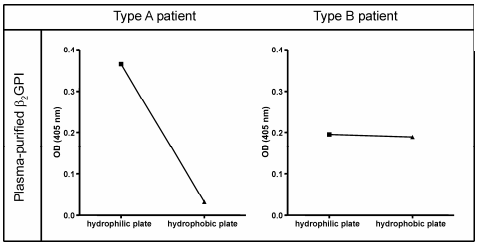

Figure 1A

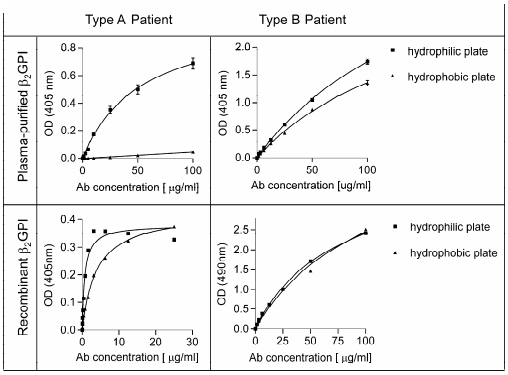

Figure 1B

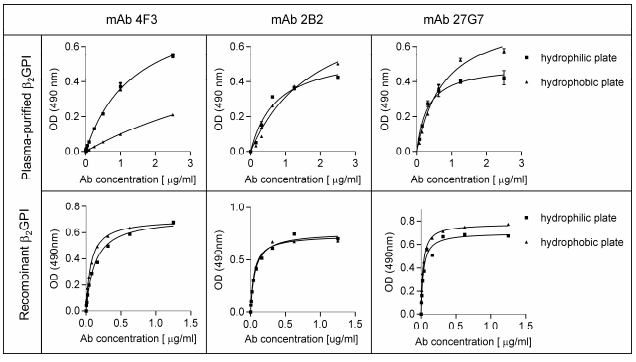

Figure 1C

Figure 1*. Patient-derived antibodies (and monoclonal antibody 4F3) do not recognize plasma-purified* β_2*GPI coated onto a hydrophobic plate. Plasma-purified* β_2*GPI and recombinant* β_2*GPI were coated onto hydrophilic and hydrophobic plates. Then, the plates were incubated with either patient plasma (A), patient IgG antibodies (B), monoclonal antibody 4F3, monoclonal antibody 2B2 or monoclonal antibody 27G7 (C). The bound mAbs were detected by a rabbit-anti-mouse peroxidase-labeled antibody, and coloring was performed by OPD. To detect the bound patient IgG antibodies, the plates were incubated with an alkaline-phosphatase-labeled goat-anti-human IgG antibody. PnPP was used as coloring substance. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers ((A) hydrophilic plate, OD: 0.087; hydrophobic plate, OD: 0.130). Error bars represent mean ± S.E.M. of duplicate points. Ab: antibody, OD: optical density.*

The recognition of β_2GPI in solution

To further investigate the differences in recognition of plasma-purified β_2GPI and recombinant β_2GPI by type A IgG antibodies, we performed several experiments with β_2GPI in solution. We coated plasma-purified β_2GPI to a hydrophilic ELISA plate, and studied whether plasma-purified β_2GPI and recombinant β_2GPI were able to inhibit the binding of patient type A IgG antibodies to the coated plasma-purified β_2GPI on the plate. Both type A patients tested in this experiment had suffered from venous thrombosis in the past. Figure 2 shows that the binding to the coated β_2GPI could be completely inhibited when patient type A IgG antibodies were pre-incubated with recombinant β_2GPI. In contrast, the binding of patient type A IgG antibodies to coated β_2GPI could only be inhibited for approximately 25% by plasma-purified β_2GPI.

To expand this observation, we coated affinity-purified patient type A IgG antibodies to a hydrophobic ELISA plate. Subsequently, the plate was incubated with plasma-purified β_2GPI, plasma-purified β_2GPI in the presence of cardiolipin vesicles, and with plasma-purified β_2GPI in the presence of PS/PC vesicles. We found that the patient type A IgG antibodies recognized plasma-purified β_2GPI only in the presence of cardiolipin vesicles (Figure 3).

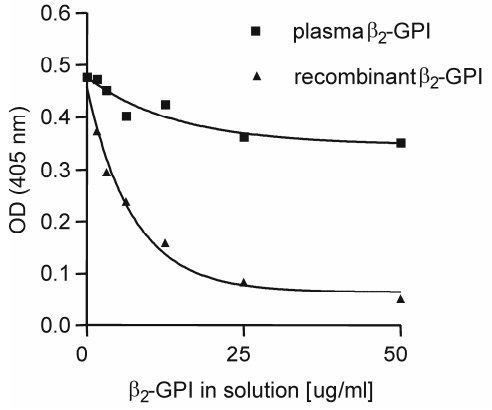

Figure 2. Binding of type A IgG antibodies to β_2GPI in solution.
Hydrophilic ELISA plates were coated with 10 μg/ml plasma-purified β_2GPI, and patient type A IgG antibodies were incubated to the plate in the presence of various concentrations of plasma-purified β_2GPI and recombinant β_2GPI. The patient type A IgG antibodies bound to the plate were detected by using an alkaline-phosphatase-labeled goat-anti-human IgG antibody. PnPP was used as coloring substance. The reaction was stopped by the addition of 2.4 M NaOH, and absorbance was measured at 405 nm. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers (OD:0.168).

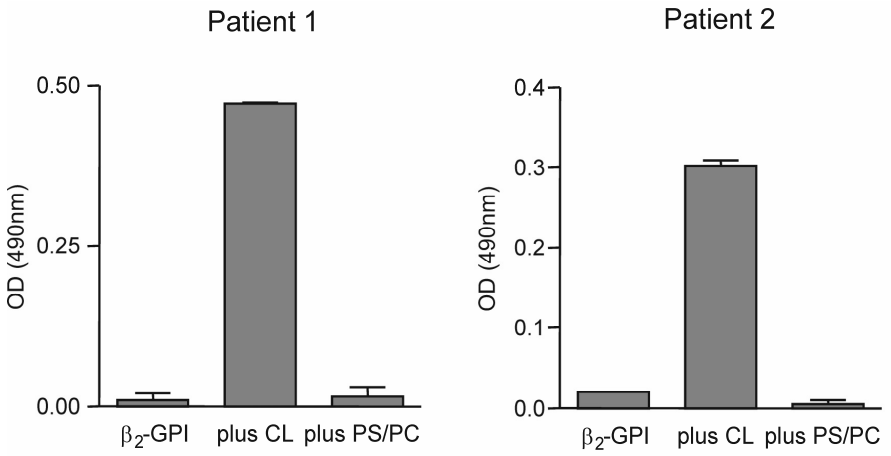

Figure 3. Type A IgG antibodies recognize plasma-purified β_2GPI in solution only in the presence of cardiolipin.
Affinity-purified type A IgG antibodies (20 μg/ml) of patient 1 and patient 2 were coated onto hydrophobic ELISA plates, and incubated with plasma-purified β_2GPI, plasma-purified β_2GPI in the presence of cardiolipin vesicles, or plasma β_2GPI in the presence of PS/PC vesicles (20%/80%). Then, the plates were incubated with a polyclonal goat-anti-human anti-β_2GPI antibody. Subsequently, the plates were incubated with a rabbit-anti-goat peroxidase-labeled antibody. Coloring was performed by using OPD. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers (incubation with plasma β_2GPI, OD:0.086; incubation with PS/PC, OD:0.087; incubation with CL, OD:0.088). CL: cardiolipin vesicles. Error bars represent mean ± S.E.M. of triplicate points.

The influence of aggregating of β_2GPI on the recognition of β_2GPI by type A antibodies

Galazka et al. described that modifying β_2GPI with glutaraldehyde induces the exposure of a cryptic epitope that is recognized by anti-β_2GPI antibodies 29. We investigated whether this modification influences the recognition of plasma-purified β_2GPI by type A antibodies. We coated plasma-purified β_2GPI, recombinant β_2GPI and plasmaβ_2GPI modified by glutaraldehyde onto a hydrophobic ELISA plate. Two randomly chosen plasma samples positive for type A antibodies (with a history of venous thrombosis) were incubated to the plate, and the bound IgG antibodies were detected. As with purified IgG antibodies in figure 1B, we found that both plasma samples recognized recombinant β_2GPI (Figure 4). Plasma-purified β_2GPI was hardly recognized by both samples, but treatment with glutaraldehyde induced binding of both samples to plasma-purified β_2GPI, to an extent that was comparable to recombinant β_2GPI.

We performed an inhibition experiment with the anti-β_2GPI antibodies of patient 1. Plasma-purified β_2GPI was coated to a hydrophilic plate and antibodies of patient 1 were incubated to the plate in the presence of either plasma-purified β_2GPI, recombinant β_2GPI or glutaraldehyde-treated plasma-purified β_2GPI. We observed a maximal decrease in signal of 15.2 % when the antibodies were incubated with plasma-purified β_2GPI. For glutaraldehyde-treated plasma-purified β_2GPI we observed a decrease of 66.7 % which was comparable to recombinant β_2GPI (63.0 %).

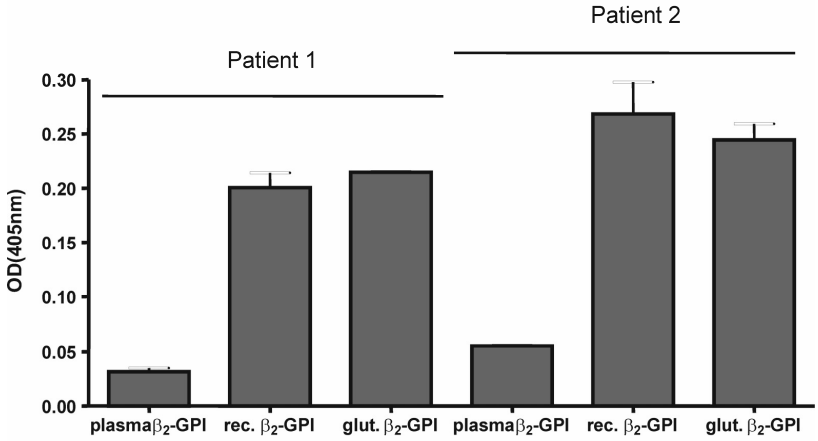

Figure 4. Type A IgG antibodies recognize plasma-purified β_2GPI after treatment with glutardialdehyde.
A hydrophobic ELISA plate was coated with plasma-purified β_2GPI (10 μg/ml), recombinant β_2GPI (10 μg/ml), and plasma-purified β_2GPI treated with glutaraldehyde (10 μg/ml). After blocking the plates, plasma of 2 type A patients was added to the wells (1:100 dilution in blocking solution). The bound patient IgG antibodies were detected with an alkaline-phosphatase-labeled goat-anti-human IgG antibody. PnPP was used as coloring substance. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers (OD: 0.126).

The importance of carbohydrate side-chains for the recognition of β_2GPI in solution

There is no difference in amino acid composition between plasma-purified β_2GPI and recombinant β_2GPI (data not shown), therefore the difference in recognition by monoclonal antibody 4F3 and patient type A IgG antibodies might be due to a difference in glycosylation. By using N-Glycosidase F we removed the carbohydrate side-chains from plasma-purified β_2GPI (Figure 5A). Then, affinity-purified patient type A IgG antibodies (both with a history of venous thrombosis) were coated onto a hydrophobic ELISA plate, and incubated with recombinant β_2GPI, plasma-

purified β_2GPI, or deglycosylated plasma-purified β_2GPI. Figure 5B shows that the coated patient type A IgG antibodies did recognize recombinant β_2GPI and plasma-purified β_2GPI without the carbohydrate chains, in contrast to plasma β_2GPI (with carbohydrate chains). We obtained comparable results with monoclonal antibody 4F3 as with the patient type A IgG antibodies (Figure 5C). We found no difference in binding pattern between monoclonal antibody 4F3 and F(ab) fragments of this antibody (figure 5D). Monoclonal antibody 2B2 recognized all forms of β_2GPI in solution (Figure 5E).

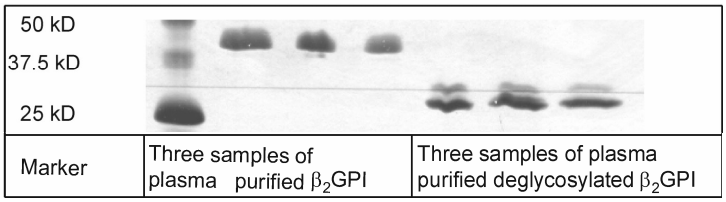

Figure 5A

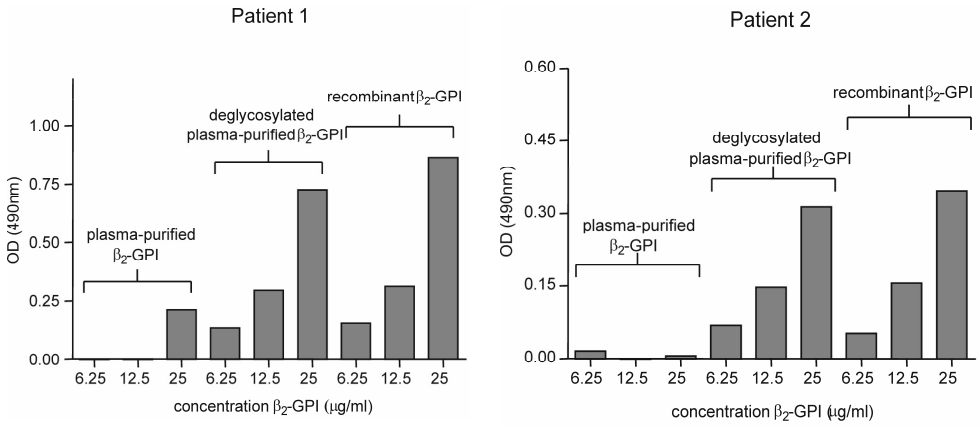

Figure 5B

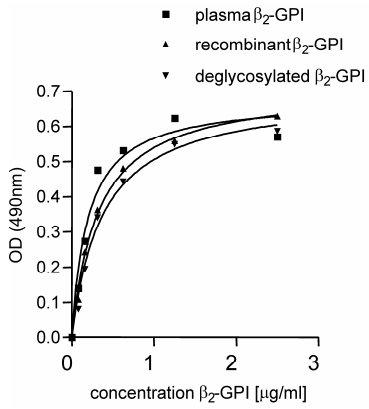

Figure 5C

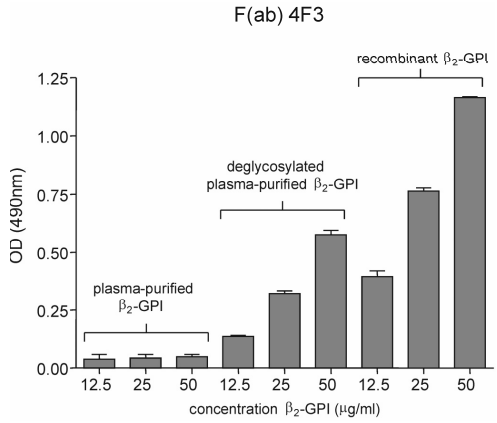

Figure 5D

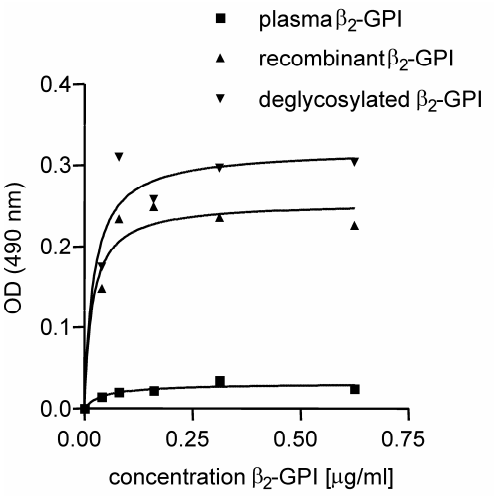

Figure 5E

Figure 5. Recognition of recombinant β_2GPI and (deglycosylated) plasma-purified β_2GPI in solution.

Type A IgG antibodies of patient 1 and patient 2 (B), monoclonal antibodies 4F3 (3 μg/ml) (C), F(ab) fragments of monoclonal antibody 4F3 (15 μg/ml) (D), and monoclonal antibody 2B2 (3 μg/ml) (D) were coated onto hydrophobic ELISA plates. Then, the plates were incubated with different concentrations of either plasma-purified β_2GPI, (A) deglycosylated plasma-purified β_2GPI or recombinant β_2GPI. Subsequently, the plates were washed and the bound β_2GPI was detected by a polyclonal goat-anti-human anti-β_2GPI antibody. After washing, the plates were incubated with a rabbit-anti-goat peroxidase-labeled antibody. Coloring was performed with OPD. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers ((B) OD:0.179; (D) OD:0.117).

Domain-specificity of the antibodies

To investigate the specificity of type A IgG antibodies, type B IgG antibodies, monoclonal antibody 4F3 and monoclonal antibody 2B2, we coated domain deletion mutants of β_2GPI to a hydrophobic ELISA plate. All patient type A IgG antibodies (n=30) showed a high avidity for all domain deletion mutants that contained domain I (Figure 6A). Patient type B antibodies (n=22) recognized full-length recombinant β_2GPI, domains I and V, and also domains I and V extended with additional domains (Figure 6B). Monoclonal antibody 4F3 showed a moderate affinity for domain deletion mutant I-II, but showed no reactivity when domain I was not present. This indicates that monoclonal antibody 4F3 recognizes the intersection between domain I and domain II. Monoclonal antibody 2B2 predominantly recognized the domain deletion mutants that contained domain III.

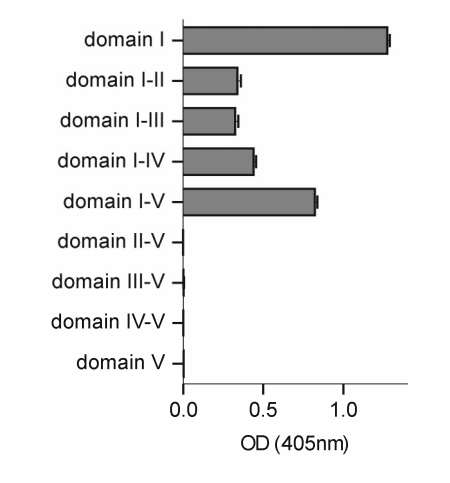

Figure 6A

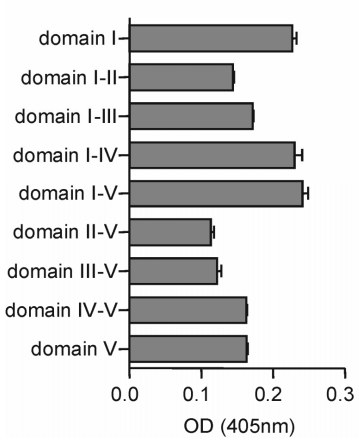

Figure 6B

Figure 6. Reactivity of patient-derived IgG antibodies, and monoclonal antibodies 4F3 and 2B2 towards the different domain deletion mutants of β_2GPI.

Domain deletion mutants of β_2GPI were coated onto an ELISA plate to test the domain specificity of: (A) patient-derived type A IgG fractions and (B) patient-derived type B IgG fractions. The patient-derived IgG fractions and monoclonal antibody 4F3 and monoclonal antibody 2B2 were incubated to the plates. Subsequently, the plates were washed and incubated with alkaline-phosphatase labeled goat-anti-human IgG Abs to detect the bound patient IgG antibodies. Staining was performed by using PnPP. The coloring reaction was stopped by 2.4 M NaOH, and absorbance was measured at 405 nm. The obtained optical density was corrected for the optical density obtained with total IgG isolated from pooled plasma from 40 healthy volunteers ((A+B) OD:0.101).

Influence of NaCl concentration on the recognition of β_2GPI by anti-β_2GPI IgG antibodies

Hammel *et al*. recently showed that a carbohydrate side-chain was positioned on domain I of β_2GPI, probably covering epitope G40-R43 19. Because epitope G40-R43 is positively charged and the carbohydrate side-chain is negatively charged, we investigated whether an increase in salt concentration might induce a displacement of this carbohydrate side chain. Figure 7A shows that type A anti-β_2GPI IgG antibodies showed a decreased affinity for plasma β_2GPI compared to recombinant β_2GPI when coated onto a hydrophobic plate. In the presence of 5 M NaCl, plasma β_2GPI and recombinant β_2GPI were equally recognized by type A antibodies. This recognition pattern was not seen when β_2GPI was coated onto hydrophilic plates (figure 7B). For type B antibodies, we observed no residual binding in the presence of 5 M NaCl for either recombinant β_2GPI or plasma β_2GPI (data not shown). For both type A and B patients, we tested 2 randomly chosen patient samples in this experiment. Both type A patient samples had a history of venous thrombosis, both type B patient samples did not have a history of thrombosis.

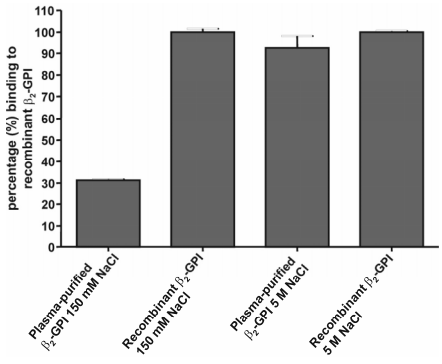

Figure 7A

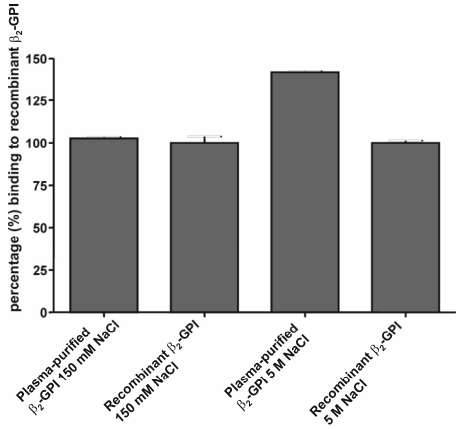

Figure 7B

Figure 7. Influence of NaCl concentration on the recognition of β_2GPI by anti-β_2GPI IgG antibodies.

Plasma-purified β_2GPI and recombinant β_2GPI were coated to hydrophobic (A) and hydrophilic (B) plates. Subsequently, the plates were blocked with 4% BSA/TBS/Tween, and incubated with (type A) patient plasma that was diluted in blocking solution with either 150 mM NaCl or 5 M NaCl. To detect the bound patient IgG antibodies, the plates were incubated with an alkaline-phosphatase-labeled goat-anti-human IgG antibody. PnPP was used as coloring substance. Error bars represent mean ± S.E.M. of quadruplicate points.

Discussion

Whether antiphospholipid antibodies recognize a cryptic epitope on β_2GPI has been the subject of a lengthy debate. Based on this study, we conclude that a conformational change in β_2GPI is necessary for type A antibodies to recognize the protein. This conformational change is induced by binding of β_2GPI to a negatively charged surface via a positive charged patch in domain V of β_2GPI.

We recently published that the population of anti-β_2GPI antibodies that binds domain I of β_2GPI at position 40-43 highly correlates with thrombosis [23]. This area of domain I is probably covered by a carbohydrate chain in solution, as could be concluded from the studies by Hammel *et al.* [19]. We hypothesized that this carbohydrate chain impedes antiphospholipid antibodies to bind epitope G40-R43 in solution. An anionic surface may induce a conformational change in β_2GPI which results in the displacement of the carbohydrate side-chain. Antiphospholipid antibodies are then able to bind epitope G40-R43. Indeed, the removal of the carbohydrate chains with N-glycosidase F resulted in an increased affinity for plasma-purified β_2GPI in solution by our monoclonal antibody 4F3 and patient type A IgG antibodies. Furthermore, by increasing the concentration of NaCl we were able to induce binding of anti-β_2GPI IgG antibodies to plasma β_2GPI on a hydrophobic plate. This indicates that the carbohydrate side chain no longer covers epitope G40-R43, and is now accessible for antibodies to bind. This hypothesis is further supported by the fact that proteins produced in insect cells have truncated carbohydrate side-chains instead of complex carbohydrate side-chains (as produced in mammalian cells). Due to these shorter carbohydrate side-chains, recombinant β_2GPI exposes epitope G40-R43 constitutively.

From our studies, it is clear that anti-β_2GPI antibodies bind a cryptic epitope that is only exposed on β_2GPI when bound to a negatively charged surface. This by no means excludes a role for dimerization of β_2GPI. Antibody-induced dimerization of β_2GPI seems important for functional activity because LAC activity is not seen when Fab(1) fragments of anti-β_2GPI antibodies are used [30]. Furthermore, we have also shown that dimerized β_2GPI and not monomeric β_2GPI induces LAC activity [11]. We postulate that the recognition of β_2GPI by anti-β_2GPI antibodies occurs as follows (Figure 8): β_2GPI has a relatively low affinity for anionic phospholipids, and a certain concentration of β_2GPI is necessary for binding. After binding of β_2GPI to anionic phospholipids via domain V, the protein undergoes a conformational change. This results in the exposure of several cryptic epitopes, including epitope G40-R43 in domain I, enabling antibodies to bind this epitope [17,23,31]. When the amount of β_2GPI bound to the anionic phospholipids reaches a certain density,

one antibody can bind two β_2GPI molecules, thereby increasing the affinity of the interaction with anionic phospholipids and stabilizing the complex.
This model has several consequences for the assays we use to detect anti-β_2GPI antibodies. It is obvious that for the LAC assay, both dimerization and exposure of the cryptic epitope are necessary. Clotting assays use PS as catalytic surface, but the affinity of β_2GPI for PS is low, thus stabilization of the interaction via dimerization is essential [3,13]. In contrast to the LAC assay, in the anticardiolipin antibody ELISA cardiolipin is used as binding surface for β_2GPI. The affinity of β_2GPI for cardiolipin is 30-40 times higher than the affinity for PS [13]. This relatively high affinity enables β_2GPI of becoming stabilized on the cardiolipin, and there is no need for dimerization via the antibodies to increase the affinity of β_2GPI for the surface. Only the exposure of a cryptic epitope is essential in this assay. In the ELISA in which β_2GPI is directly coated onto the plate, the situation is more complex [32-34]. Some brands of ELISA plates are unable to induce the conformational change (due to a difference in structure or charge), and are unsuitable for use in an anti-β_2GPI ELISA [33]. Other brands of ELISA plates do induce the conformational change, but their affinity for β_2GPI is rather low. These influences of different ELISA plates on our test results emphasize the need for standardization of the anti-β_2GPI ELISA. In this respect it is interesting to mention that we observed differences in binding of β_2GPI onto plates of different lot numbers of the same brand (unpublished data).
Another consequence of our results concerns the use of different sources of β_2GPI. We found that patient type A IgG antibodies (and monoclonal antibody 4F3) did show reactivity towards recombinant β_2GPI coated onto hydrophobic plates, in contrast to plasma-purified β_2GPI coated onto hydrophobic plates. We also found that patient type A IgG antibodies (and monoclonal antibody 4F3) did recognize recombinant β_2GPI in solution, but showed hardly any reactivity towards plasma-purified β_2GPI. This difference in binding characteristics between plasma-purified β_2GPI and recombinant β_2GPI can be explained by the difference in glycosylation. Furthermore, bovine β_2GPI has an additional glycosylation site at position 73 which can easily influence the binding of epitope G40-R43 by type A anti-β_2GPI antibodies [19]. In addition to this, it was already suggested that aggregation of β_2GPI promotes the binding of certain anti-β_2GPI antibodies [35]. Galazka et al. found that a cryptic epitope was exposed on β_2GPI when it was treated with glutaraldehyde [29]. We found that these 2 features also hold true for type A antibodies. The importance of the carbohydrate chains and conformation of β_2GPI has major consequences for the binding of antiphospholipid antibodies. One should check for aggregates of β_2GPI in a (plasma) sample, and one should be careful in using β_2GPI isolated from

other sources than human plasma when studying the interaction between β_2GPI and anti-β_2GPI antibodies.

In conclusion, we found that pathogenic antiphospholipid antibodies bind a cryptic epitope on domain I of β_2GPI. This epitope (G40-R43) is accessible for antiphospholipid antibodies only after a conformational change.

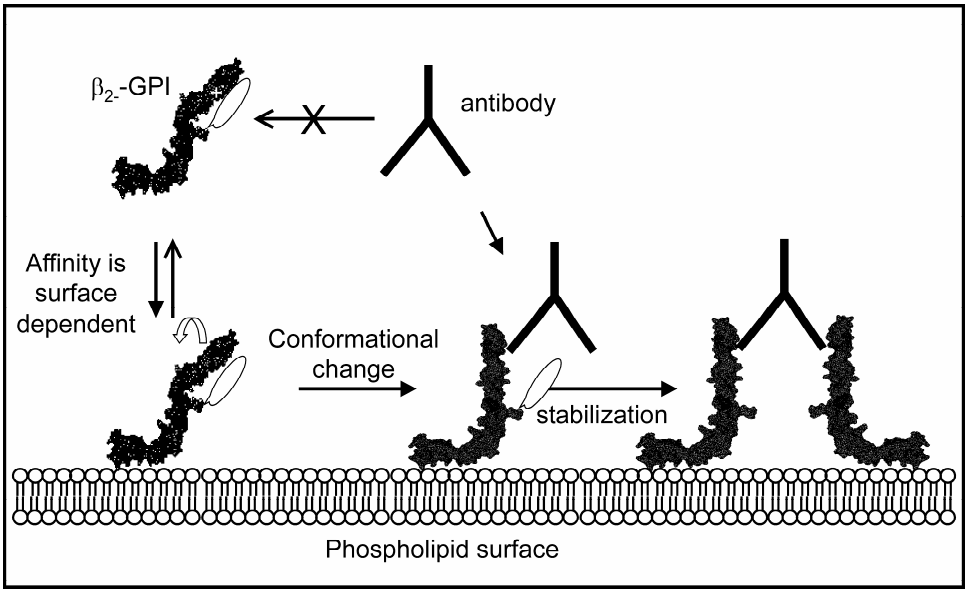

Figure 8. Mechanism describing the binding of antiphospholipid antibodies to β_2GPI. *Our proposed mechanism is based on our findings in this study together with both the crystal structure and the NMR structure of β_2GPI in solution: (i) Antiphospholipid antibodies can not bind β_2GPI in solution because epitope G40-R43 is covered by one of the carbohydrate chains. (ii) Binding to a phospholipid membrane induces a conformational change in β_2GPI. (iii) As a result, the carbohydrate chain is not able to cover epitope G40-R43 anymore and is now able to bind antiphospholipid antibodies. aPL: antiphospholipid antibodies.*

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General Discussion 7

Introduction

The pathophysiology of the antiphospholipid syndrome (APS) is still not understood. It is difficult to envision that low affinity anti-β_2GPI antibodies that affect one single biological activity, not to mention several biologic pathways, cause the syndrome. A link must exist how anti-β_2GPI antibodies induce prolongation of an in vitro clotting assay and the thrombotic manifestations observed in APS patients with circulating anti-β_2GPI antibodies. There are two theories explaining this paradox. One is the "dimerization theory". Due to binding of anti-β_2GPI antibodies to β_2GPI, the protein dimerizes resulting in increased affinity for cellular surfaces. The other one is the "cryptic epitope" theory. Due to binding of β_2GPI to anionic phospholipids, a cryptic epitope within β_2GPI that is normally shielded off, is exposed thereby facilitating the binding of anti-β_2GPI antibodies to β_2GPI [1]. The affinity of the formed immune complex is high enough to compete with clotting factors on a cellular surface. We know how the antibodies induce the observed in vitro lupus anticoagulant (LAC).

Several hypotheses have been put forward to explain the arterial and/or venous thrombosis in APS patients. We cannot exclude that arterial and venous thrombosis is the result of different actions of β_2GPI/anti-β_2GPI antibody complexes on cells. A number of interesting studies identified mechanistic links between anti-β_2GPI antibodies and thrombosis. Now, attention is mainly focussed on the interference of such immune complexes with antithrombotic pathways, such as fibrinolysis [2-4] and the Protein C pathway [2]. However, impaired Protein C pathway results in venous thrombosis [5;6], while in APS venous and arterial thrombosis are present.

It is difficult to envision that simple binding to anionic phospholipids can activate cells. A second hit must occur to activate cells. An important observation is that β_2GPI/anti-β_2GPI antibodies complexes not only have an increased affinity for anionic phospholipids but also for cellular surfaces. Binding to and activation of endothelial cells, platelets and monocytes by β_2GPI/anti-β_2GPI antibody complexes has been reported. There must be an additional mechanism how dimerization of β_2GPI can explain the observed cellular activation.

β2GPI-induced platelet activation

Research into the pathology of APS was obscured by the idea that anionic phospholipids are crucial for binding β_2GPI/antiβ_2GPI antibody complexes. We now know that cellular activation by β_2GPI/anti-β_2GPI antibody complexes is the consequence of classic receptor-ligand interactions. These interactions represent a basis for understanding the pathology of APS. Previous studies of our group revealed that platelets are sensitized by β_2GPI/anti-β_2GPI antibody complexes or

recombinant dimeric β_2GPI under conditions of arterial flow using a whole blood perfusion model [7]. The binding of dimeric β_2GPI to platelets is mediated by the platelet receptor apolipoprotein E receptor 2' (apoER2'). Platelet activation is among others dependent on the synthesis of thromboxane A_2 (TxA_2) [7-9]. Release of TxA_2 and binding to its receptor on the platelet membrane results in further platelet activation. Several investigators observed increased levels of thromboxane breakdown products in the urine of APS patients, indicating the existence of activated platelets in the circulation of APS patients. This is supported by the observation that activated platelets do circulate in APS patients [10;11]. Apart form apoER2', other receptors have been suggested for β_2GPI, annexin A2 on human platelets and endothelial cells [12] and members of the toll-like receptor family (TLR) on endothelial cells, platelets and monocytes [13-15]. Further studies should elucidate the role of different receptors.

Several studies show that the addition of aPL to human platelets results in an increase in phosphorylation of p38 mitogen-activated protein kinase ($p38^{MAPK}$) [9;16]. $P38^{MAPK}$ is a member of a family of proline-directed Ser/Thr kinase that is dual-phosphorylated on residues Thr^{180} and Tyr^{182}. Platelets contain members of the MAPKs, including ERK-1 ($p44^{MAPK}$), ERK-2 ($p42^{MAPK}$) and $p38^{MAPK}$. Activated $p38^{MAPK}$ phosphorylates and activates cyclic phospholipase A_2 ($cPLA_2$), resulting in the release of arachidonic acid (AA), a precursor in the formation of TxA_2. Increased phosphorylation of $p38^{MAPK}$ is also found when platelets are activated by dimeric β_2GPI (*unpublished data*). The $p38^{MAPK}$ phosphorylation is transient and reaches its maximum at one minute. This indicates that binding of dimeric β_2GPI to apoER2' on the platelet membrane results in phosphorylation and activation of $p38^{MAPK}$. Synthesis of TxA_2 supports further platelet activation. Because of its known low rate of endocytosis and ligand degradation, apoER2' is probably involved in signal transduction. Whether apoER2' is involved in endocytosis by platelets remains to be determined.

Molecular regions within β2GPI that are recognized by apoER2' on platelets

It is proposed for members of the LDL-R family that positive electrostatic surface potentials, not the primary sequences, in different ligands constitute receptor-binding domains. For example, electrostatic interaction between basic residues in the lectin-like domain of LOX-1, a scavenger receptor sharing similar ligand-binding properties as the LDL-R family, and negatively charged oxidized LDL (oxLDL) is critical for the binding activity of LOX-1 [17]. Specific cationic residues within the RAP molecule [18] facilitate binding of receptor-associated protein (RAP)

to low density lipoprotein receptor-related protein (LRP). A specific domain within apoB100 mediates LDL binding to the LDL-R [19]. This domain is termed the B-site and encodes a patch of cationic amino acids consisting of lysine and arginine residues. These residues are essential for binding of LDL to the LDL-R. Modification of these residues results in reduced signaling in platelets [20].

Instead of a short primary sequence, a patch rich in cationic residues thus compose a receptor-binding domain within ligands. Lysines and arginines on the outer surface of ligands comprise the cationic potential required for binding to LDL-R family members. X-ray diffraction analysis supports this proposal. The LDL-R recognizes specific basic amino acid residues within apolipoprotein E (apoE) [21]. These basic amino acids are clustered into a surface patch on one large helix. For lipoprotein C lipase, amino acids 378-448 within the C-terminal binding domain are crucial for binding to LRP [22-24]. Thus, a positive electrostatic potential exposed on the outer surface of different ligands may constitute a domain that is recognized by various members of the LDL-R family. β_2GPI is composed of five sushi or complement control protein (CCP) domains. The cationic charge in domain I and domain V is the result of multiple lysine and arginine residues. Domain V displays the largest cationic patch (2000 Å^2) and might be essential for binding to apoER2'. Indeed, we show in Chapter 3 the importance of a cationic patch within domain V (amino acid 282-287) of β_2GPI for the interaction with apoER2'. This is in accordance with other groups who found that lysine residues constitute a ligand recognition site for members of the LDL-R family (*Table 1*).

Table 1: Amino acid sequences in several ligand important for binding to LDL-R homologous

Ligand	Receptor	Amino acids within ligand	Residues important for binding	References
ApoB100	LDL-R	3359-3369	RLTRKRGLKLA	19;775
apoE	LDL-R	134-150	RVRLASHLRKLRKRLLR	217
β_2-macroglobulin	LRP	1366-1392	FIPLKPTVKMLERSNHVSRTEVSSNHV	784
PAI-1	LRP	69-87	DKGMAPALRHLYKELMGPWN	793
Lipoprotein lipase	LRP	403-425	KIRVKAGETQKKVIFCSREKVSHL	802
aprotinin	LRP	38-47	CRAKRNNFKSA	811

Members of the Low-Density Lipoprotein Receptor family and their role in the antiphospholipid syndrome

The LDL-R gene family consists of structurally closely related transmembrane glycoproteins that participate in a wide range of biological activities, such as lipoprotein metabolism, protection against atherosclerosis, neurodevelopment

and in the transport of nutrients, vitamins and drugs [25]. At present, 12 members of the LDL-R family in mammals are identified. The function of a receptor present on one cell type may not be restricted to a single function. Cellular distribution of LDL-R homologous is not restricted to specific cells or tissues. The LDL-R, for example, is distributed throughout the human body and is expressed in hepatocytes, macrophages and the central nervous system. Like the LDL-R, also the low-density lipoprotein receptor related protein (LRP) is expressed by a wide range of cell types and tissues, among others hepatocytes, macrophages and vascular smooth muscle [26]. Megalin is expressed in the apical plasma membrane of secretory epithelia facing transcellular fluid [27]. Because of the various expression patterns of members of the LDL-R family, one can envision a role for these receptors in the pathophysiology of APS. The first paper published on apoER2' in platelets showed that this receptor might be involved in platelet inhibition [28]. However, further research illustrated that human platelets are activated by LDL or dimeric β_2GPI, which is mediated by apoER2' [7;19;29]. Megalin recognizes complexes of β_2GPI and phospholipids, but also β_2GPI alone [30]. We show that several members of the LDL-R family recognize complexes of β_2GPI/anti-β_2GPI antibody complexes (*Chapter 4*). Apart from apoER2', these receptors include the VLDL-R, megalin and LRP. Besides these receptors, dimeric β_2GPI is also able to bind with low affinity to the LDL-R. The LDL-R is known to be involved in ligand uptake and endocytosis [31;32], whereas a role in signal transduction is still unclear. Therefore, we believe that the LDL-R has little role in the pathophysiology of APS.

RAP dose-dependently inhibited binding of dimeric β_2GPI to members of the LDL-R receptor family, indicating a specific interaction. In accordance with data for apoER2', domain V of β_2GPI comprises the main recognition site for members of the LDL-R family. Heparin is able to inhibit the interaction of dimeric β_2GPI with members of the LDL-R family (*Chapter 4*). Lys^{284}, Lys^{286} and Lys^{287} in domain V of β_2GPI are essential for the interaction of b2GPI with heparin [33], suggesting an involvement of this region in the interaction with LDL-receptor family members. Despite data indicating a crucial role for domain V of β_2GPI, our group previously showed that a monoclonal anti-β_2GPI antibody (4F3), that recognizes domain I of β_2GPI, could inhibit platelet adhesion to a collagen surface [7]. This suggests that domain I is involved in platelet activation. However, the data presented in *Chapter 4* shows that domain I of β_2GPI is not involved in the interaction with members of the LDL-R family. The observed inhibition with 4F3 can be due to steric interference; binding of 4F3 induces a conformational change within β_2GPI that abrogates binding of β_2GPI to apoER2'.

Members of the LDL-R family are widely distributed in cells and tissues and are associated with a number of metabolic processes and disorders such as embryonic development of the brain, Alzheimer disease (AD), viral and bacterial infections and lipid metabolism. For example, familial hypercholesterolemia (FH), which is one of the most common human inborn errors of metabolism, is caused by loss-of-function mutations in the LDL-R gene [34]. LRP mediates critical biological pathways in AD such as the clearance of denatured proteins (beta-amyloid) [35;36]. As mentioned, several cell types that are involved in the pathology of APS, among others platelets, monocytes and endothelial cells, express LDL-R family members. It is of interest to study whether dimeric β_2GPI can activate these cells. This is currently under investigation. In conclusion, data presented here suggests that the antiphospholipid syndrome can be added to the expanding list of pathological conditions mediated by members of the LDL-R family.

Identification of ligand binding sites within LRP

The identification of ligand-binding sites within a member of the LDL-R family has revealed multiple sites for protein interaction throughout the whole of the receptor ligand binding domains. Information of the different ligand-binding sites is often provided from studies of how certain inhibitors with known binding sites displace the ligand of interest or how a potential ligand influences binding of well-established ligands. Two small cysteine-rich complement type (CR) domains within a receptor are sufficient for high affinity ligand binding. Some ligands bind to identical or partially overlapping sites, which results in competitive receptor binding, while other ligands have unique binding sites that are suggestive of multiple ligand epitopes. Also, in the extremely large receptors containing multiple CR domains (among others LRP and megalin) most, if not all, of the CR domains can mediate ligand binding.

It is of interest to establish how many CR domains contribute to the direct binding of a ligand. Analysis of LRP minireceptors comprised of isolated cluster II and cluster IV (8 and 11 CR domains, respectively), as well as the VLDL-R and the LDL-R clusters (7 and 8 CR domains, respectively) demonstrate that a ligand can bind with high-affinity to several of these CR domains. For example, RAP can bind with high-affinity to cluster II and cluster IV of LRP [37-39], which displays only minor differences in ligand-binding properties [40]. In *Chapter 4* we demonstrate that *i*. RAP can inhibit dimeric β_2GPI binding to LRP and *ii*. dimeric β_2GPI can bind to both cluster II and cluster IV, which comprise the high-affinity recognition regions within LRP. The domain deletion mutants, except for the domain V deletion mutant, bind with

comparable affinities (high- and low affinity binding site) to these minireceptors. Therefore, LRP might recognizes two cationic patches in domain V of β_2GPI, containing a low- and a high affinity binding site. The high-affinity binding site may be located at position $Lys^{282-287}$ within domain V of β_2GPI, as we show in *Chapter 3* that this region is important for recognition by apoER2'.

NMR and crystallization studies of LRP revealed that flexible linkers connect the CR domains of LRP. The combination of several CR domains may comprise a suitable ligand-binding site. The observation that multiple CR domains (for example cluster II and IV) mediate ligand binding is in accordance with the findings for LRP (*Chapter 4*). The cationic patch in domain V of β_2GPI measures 2000 $Å^2$. Given the fact that the overall dimensions of a CR domain is 20 × 20 × 27 Å [17], domain V of β_2GPI can accommodate more than one CR domain. This is already true for binding of RAP and α_2-macroglobulin (α_2-M) to LRP; ligand-binding repeats from two clusters cooperate to generate a high affinity-binding site for RAP (cluster II and cluster IV) and α_2-M (cluster I and cluster II) [40;41].

Receptor dimerization is an important process before signal transduction can occur. Bivalent antibodies directed to the extracellular part of LRP, but not Fab fragments of the same antibody, are able to induce calcium influx in cultured neurons, indicating the necessity of receptor dimerization 42. Interestingly, intact LRP interacted with dimeric β_2GPI in a 1:2 stoichiometric (*Chapter 4*). These data provide biochemical evidence that dimeric β_2GPI may require simultaneous association with two LRP molecules. This observation suggests that binding of dimeric β_2GPI to LRP induces receptor multimerization on the cell surface, bringing signaling factors bound to the cytoplasmic tail of the individual receptors into close proximity. Receptor dimerization is already reported for apoER2; after binding of Reelin, apoER2 on neuronal cells is able to dimerize involving several CR domains. This is followed by subsequent signal transduction [43]. The fact that soluble receptors are not able to associate with Reelin supports the receptor dimerization hypothesis.

Mutational studies mapping the interaction between β_2GPI and apoER2'

Site-directed mutagenesis identifies a cluster of lysine residues recognized by apoER2'

Since a cationic peptide spanning amino acid residue $Lys^{282-287}$ in domain V of β_2GPI was able to inhibit binding of dimeric β_2GPI to apoER2' on human platelets (*Chapter 3*), it is of interest to explore domain V for potential recognition sites

for apoER2'. One approach to determine which residues are of importance for protein-protein interaction is site directed mutagenesis. Cationic residues within ligands are important for recognition by members of the LDL-R family, as has been established for several ligands (*Table 1*). These recognition sites mainly comprise lysine and arginine residues. Therefore, we predicted that lysine residues in domain V of β_2GPI contain the receptor-binding site. A detailed molecular study for the interaction between β_2GPI and apoER2' is preceded by a prediction of the electrostatic surface potential of domain V after substation of lysine residues into neutral leucine residues (*Chapter 5*). Analysis of a three-dimensional molecular structure of domain V revealed that regions $Lys^{262-276}$, $Lys^{282-287}$ and $Lys^{305-317}$ are located at the outer surface of the putative apoER2'-interacting surface area. This observation raises the question to what extent these regions contribute to the interaction with apoER2'. Employing site-directed mutagenesis in which lysine residues were replaced for leucine residues, we observed reduced binding of dimeric β_2GPI to apoER2' (*Chapter 5*). We clearly show that the $Lys^{282-287}$ residue is crucial for interaction with apoER2' and that a second recognition site resides at residue $Lys^{305-317}$. We propose that instead of a short patch of a primary sequence in domain V of β_2GPI, a cationic secondary or tertiary organization may comprise a receptor-binding domain. The cationic electrostatic potentials encompass stretches of lysine residues in domain V of β_2GPI. These cationic residues may bind to several CR domains of apoER2'.

The major binding site for RAP on apoER2 on neuronal cells is mapped to Asp^{35} on the first CR domain of apoER2 and a minor role for the third CR domain [43]. Only the third RAP domain (amino acid 216-323) binds to apoER2. It is of interest to study which CR domain of apoER2' on human platelets is crucial for recognition of dimeric β_2GPI. Therefore, binding studies with different complement-type repeats in CR domains of apoER2' should be performed.

The fifth domain of β_2GPI contains the phospholipid-binding site

Domain V of β_2GPI differs significantly from the other four CCR domains. It folds into a central β-spiral and an extended C-terminal loop region. This loop contains several hydrophic residues and is implicated in binding to anionic phospholipids. Apart from phospholipids, domain V might be involved in binding to hydrophobic ligands [44] and dextran sulphates [45]. Using synthetic peptides the major phospholipid-binding site is mapped to the basic patch of lysine residues ($Cys^{281-288}$) that is located on the outer surface of the fifth domain of β_2GPI. Lys^{284}, Lys^{286} and Lys^{287} are crucial for the electrostatic interaction with anionic phospholipids [46;47]. However, the optimal

integrity of the C-terminus and the nearby-located Leu$^{313-316}$ residue is required for optimal interaction with anionic phospholipids [48;49]. Based on the hypothesis that domain V comprises the phospholipid-binding site within the residue Cys$^{281-288}$, we performed phospholipid-binding assays with several dimeric mutants of β_2GPI (*Chapter 5*). Lys$^{282-287}$ is a critical residue within β_2GPI for binding to cardiolipin, as shown by site-directed mutagenesis [50]. In that study, the investigators used β_2GPI with mutations in the KNKEKK site and not in the region after the KNKEKK site. The same group confirmed their results by performing competition experiments with peptides spanning regions within domain V of β_2GPI [46]. In *Chapter 5*, this finding is supported by data with the dimeric β_2GPI mutants. However, we provide evidence that the residue Lys$^{305-317}$ is more crucial for binding to phosphatidylserine (PS), a natural occurring phospholipid in cellular membranes than the Lys$^{282-287}$ residue.

Different hypotheses exist explaining the interaction between β_2GPI and anionic phospholipids. An interesting observation outlines that the targets for β_2GPI are cell surface receptors rather than phospholipids [51]. However, evidence exist that β_2GPI interacts directly with anionic phospholipids, which alters the conformation of the protein [52]. In *Chapter 6*, we show that a conformational change within β_2GPI is necessary for anti-β_2GPI antibodies to recognize the protein. This conformational change is induced by the binding of β_2GPI to an anionic surface via a cationic patch in domain V of β_2GPI (*Chapter 6*).

The fifth domain of β_2GPI is important for binding to apoER2' on human platelets, but is also important for binding to phospholipids. Interestingly, Reelin binding to phospholipids might promote signal transduction via apoER2 by ensuring that Dab1, an intracellular adaptor protein, integrates into the signaling complex in a suitable orientation [53]. This raises the question whether anionic phospholipids are required for binding and proper orientation of β_2GPI before it can interact with apoER2' and induce signal transduction in platelets. In the kinetic studies, several members of the LDL-R family recognized both dimeric β_2GPI and a β_2GPI/antiβ_2GPI antibody complex. In the absence of phospholipids, LDL-R family members recognize both dimers in a purified system (*Chapter 4*). We used a monoclonal antibody and not a patient derived IgG, as patients IgG do not recognize β_2GPI in solution. Moreover, the non-phospholipid binding dimeric β_2GPI did interact with the receptors (*Chapter 3 and 4*). This suggests that the recombinant dimeric β_2GPI does not necessarily need phospholipids to bind to members of the LDL-R family. The following hypothesis is proposed. In patients with APS, monomeric (plasma) β_2GPI circulates free in plasma at a concentration of approximately 200 μg/mL. Low-affinity binding of β_2GPI to anionic phospholipids mediated by a cationic patch at position Lys$^{305-317}$ is

a prerequisite before anti-β_2GPI antibodies can bind to and dimerize β_2GPI. This leads to formation and stabilization of β_2GPI/anti-β_2GPI antibody complexes on the cellular surface, followed by binding of the complex to apoER2' on platelets. This latter interaction involves region $Lys^{282-287}$ in domain V of β_2GPI. The hypothesis that plasma β_2GPI needs to be dimerized by anti-β_2GPI antibodies on an anionic phospholipid surface is supported by the fact that β_2GPI/anti-β_2GPI antibody complexes are not detected in the plasma of APS patients.

Implications for the antiphospholipid syndrome

Involvement of the receptors in the antiphospholipid syndrome

The understanding of the LDL-family has broadened since the discovery of the LDL-R as a cargo receptor. It is clear now that other members of the LDL-R family have additional functions in regulating signal transduction. A direct relation between endocytosis and signal transduction has been shown for megalin, whereas the role for the VLDL-R and apoER2' seems to lie in signal transduction with a limited role for endocytosis.

The crucial role of these receptors in the control of several physiological processes makes them attractive targets. Knowledge of the molecular basis for ligand recognition and of their many functions, members of the LDL-R family are attractive for the development of specific inhibitors. For example, if binding of β_2GPI/antiβ_2GPI–antibody complexes to LDL-R family members induces signal transduction, designing a compound selectively inhibiting this interaction would be attractive. This approach might be more efficient or can cause fewer side effects.

Anticoagulant therapy and antiphospholipid antibodies

Frequent thrombotic manifestations in the antiphospholipid syndrome are deep venous thrombosis (DVT), pulmonary emboli (PE), ischaemic stroke and transient ischaemic attacks (TIA). Thrombosis is reported in almost any vessel in the body

Initial treatment of deep venous thrombosis (DVT) is the administration of low molecular weight heparin (LMWH), unfractionated heparin (UFH) and vitamin K antagonists (VKA) [54]. The starting dose for treatment of DVT is a bolus injection of 5.000 Units (U) unfractionated heparin (UFH), followed by a continuous infusion of 30.000 U for a period of 24 hours. Subsequent doses should be standardized according to weight. For administration with LMWH, a therapeutic range is 0.6 to

1.0 IU/mL. The target range is less clear in patients treated with LMWH once a day, but a level between 1.0 IU/mL and 2.0 IU/mL seems reasonable.
Initial treatment for VTE in patients with APS is the administration of low-molecular weight heparin (LMWH), overlapped with VKA or aspirin treatment [53;55;56]. Based on the results from retrospective studies [54;57;58], for long-term treatment, high-intensity VKA is recommended for preventing recurrent VTE. In general, patients with ischaemic strokes receive antiplatelet drugs, notably low-dose aspirin. Low-dose aspirin may also be effective in patients with ischaemic strokes and aPL [59]. In summary, a life-long prophylaxis to patients with thromboembolic events and prophylaxis with LMWH during risk periods is recommended.
Several possibilities exist to explain the therapeutic effect of heparin toward decreasing the thrombotic risk in APS patients: *1.* has an antithrombotic effect via antithrombin (AT) independent of aPL *2.* modulates cell-mediated events to prevent production or alter the action of aPL or *3.* facilitates the elimination of antiphospholipid antibodies [60-62]. It is reported that unfractionated heparin and LMWH reduce the *in vitro* binding of aPL [63]. As shown in *Chapter 4*, heparin can abrogate binding of dimeric β_2GPI to members of the LDL-R family. Half-maximal binding of dimeric β_2GPI to LRP or megalin is observed at 0.06 mg/mL of unfractionated heparin (corresponds to 11 IE/mL). We also observed inhibition with LMWH for LRP and megalin with half-maximal binding at approximately 0.05 mg/mL LMWH, which corresponds to 8 IE/mL (*unpublished data*). In conclusion, we show that heparin performs its therapeutic effect by binding to domain V of β_2GPI. Thereby heparin interferes with binding of β_2GPI to members of the LDL-family and possibly to other cellular receptors. Finally, β_2GPI is not able to activate cells involved in APS (platelets, monocytes, endothelial cells).

Future prospects

Soluble receptors; a marker of the antiphospholipid syndrome?

An interesting feature of the LDL-R family is the reports of soluble forms of these receptors. A soluble form of LRP has been identified in human plasma that retains LRP-ligand binding ability [64 65 66]. This soluble form is probably the result of proteolytic cleavage of the LRP form the cellular surface by a metalloproteinase. In addition, soluble megalin is present in urine that remains the ability to bind ligands [64]. However, the physiological relevance of soluble megalin finding is unclear.

For other receptors, cleavage from the cell membrane after ligand binding is reported. Several other cell surface proteins are shed from the cell membrane, including L-selectin [67], GpIb [68], interleukin receptors [69] and transferrin receptor [70]. The hypothesis exist that these soluble receptors are the result of proteolytic cleavage after binding of ligands.

There is no data, but we assume that members of the LDL-R family might be cleaved from the cell membrane (platelets, monocytes, endothelial cells) after interaction with β_2GPI/antiβ_2GPI antibody complexes. Soluble receptors in plasma of patients with APS can serve as a diagnostic marker for cellular activation and thus they might be a predictive tool for thrombosis. For several receptors, the diagnostic value has already been established. Measurement of soluble LOX-1, a scavenger receptor sharing similarities with the LDL/R family with regard to ligand binding, or soluble Fc gamma RIIIa (CD16) in vivo may provide a novel diagnostic tool for the evaluation and prediction of atherosclerosis and vascular disease [71;72]. Interestingly, among other markers, soluble P-selectin in plasma, can be used in the assessment of platelet activation status [73]. In summary, measurement of soluble members of the LDL-R family might serve as a diagnostic tool for APS.

Cellular activation in the antiphospholipid syndrome

Until know, we only have clear evidence that dimeric β_2GPI can activate platelets via apoER2'. This is followed by phosphorylation of apoER2', phosphorylation and activation of p38 MAPK and thromboxane synthesis. Recently, our group has identified on endothelial cells several members of the LDL-R that recognize dimeric β_2GPI, suggesting a role for the LDL-R family in dimeric β_2GPI induced endothelial cell activation (unpublished data). Other groups have shown the involvement of other receptors (annexin A2, toll-like receptors) in cellular activation and signal transduction in the antiphospholipid syndrome [13;15]. However, dimeric β_2GPI-induced cellular activation is still under investigation.

Animal models

How β_2GPI dimers increase platelet deposition *in vitro* [74] and thrombus formation in vivo is subject for ongoing research. Passive administration of anti-β_2GPI antibodies to pregnant BALB/c mice induced clinical findings consistent with APS (increased fetal resorptions, reduced platelet counts, and prolonged activated partial thromboplastin time) [75]. In another study, infusion of anti-β_2GPI antibodies promoted thrombus formation in a photochemically induced thrombosis model [76]. Cellular activation via the Fc portion of β_2GPI/anti-β_2GPI antibody complexes was not essential, because F(ab')(2) fragments of the same antibodies still promoted thrombus formation. However, it is still unclear which receptors are involved in the induction of thrombosis in animal models.

We have now clear evidence that members of the LDL-R family recognize dimeric β_2GPI and this interaction results in cellular activation (*Chapter 3*). This involves a high-affinity interaction that can compete with binding for other ligands of this receptor family. Whether the contribution of the LDL-R family is physiologically relevant for development of thrombosis needs to be established. To explore the role of the LDL-R family in the pathogenesis of APS, studies with LDL-R family knockout animal models are essential.

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Summary 8

Beta2-glycoprotein I (β_2GPI), also known as apolipoprotein H (apoH), is a membrane-adhesion protein that circulates in plasma (100-300 μg/mL) in a free and a bound form to lipoproteins. The molecular weight of β_2GPI is approximately 37 kDa. It consist of 326 amino acids residues with four carbohydrates attached to domain III and domain IV, contributing to ~20% (w/w) of its total molecular weight of 45 kDa. The β_2GPI gene is located on chromosome 17q23-24 and consists of 8 exons (~1.2 kb) that are separated by large sequences (~16.2 kb). β_2GPI belongs to a super family of mainly complement proteins. This family of proteins is characterized by repeating units, called short consensus repeats (SCR), Sushi domains or complement control proteins and is involved in protein-protein interactions. β_2GPI consists of five of these SCR domains of which the first four domains structurally resemble each other (~60 amino acids and two conserved disulfide bridges). The fifth domain is aberrant and is comprised of 82 amino-acids and three internal disulfide bonds. β_2GPI has been implicated in blood coagulation and is a principal target for antiphospholipid antibodies present in plasma of patients with the antiphospholipid syndrome (APS). APS is a condition associated with venous/arterial thrombosis accompanied by pregnancy complications and the presence of anti-β_2GPI antibodies in plasma of affected patients. Several studies have shown a significant correlation between thrombotic complications and anti-β_2GPI antibodies. The pathological mechanism by which β_2GPI/anti-β_2GPI antibody complexes exert their biological function remains unknown. Several reports clearly demonstrate that anti-β_2GPI antibodies are implicated in the activation of platelets via the apolipoprotein receptor E' (apoER2') and activation of endothelial cells by binding to β_2GPI complexed with annexin II or members of the Toll-like (TLR) receptor family.

We have previously shown that dimerized β_2GPI can bind to apoER2' on human platelets. ApoER2' belongs to a super-family of multi-ligand receptors, called the low-density lipoprotein receptor (LDL-R) family. This receptor family is expressed on a variety of cells and tissues, among others brain, heart, liver, lung, platelets, monocytes and smooth muscle cells (SMS). This diverse expressing pattern assumes their role in several pathological processes. In this thesis we have addressed the following questions regarding β_2GPI and the LDL-R family. 1.) Which domain of β_2GPI is recognized by the human platelet receptor apolipoprotein E receptor 2' (apoER2')? 2.) Do other members of the LDL-R family recognize β_2GPI? 3.) Is their a specific sequence within β_2GPI that is recognized by LDL-R homologous or is it merely a case of electrostatic potential? To answer these questions we have conducted molecular studies combined with cellular- and kinetic studies.

The interaction between β_2GPI and apoER2' on human platelets is investigated using domain deletion mutants of recombinant dimeric β_2GPI (*Chapter 3*). Based on the assumption that ligand-binding to LDL-R homologous is governed by electrostatic interactions we expected that domain V, having the most positively charged residues, is involved in the receptor interaction. In a first screen, the effect of domain deletion mutants of recombinant dimeric β_2GPI on binding to apoER2' was assessed by solid phase immunosorbent assays. When we tested dimeric β_2GPI lacking domain I or domain II, no effect on binding was measured. The calculated affinity constants (steady state constants) were similar to full-length dimeric β_2GPI. In contrast, dimeric β_2GPI lacking domain V displayed impaired binding to apoER2'. These results indicated the importance of this domain in the interaction with apoER2'. These results were further confirmed with isolated platelets expressing apoER2' on their surface. Competition studies employing peptides spanning cationic regions in domain I and domain V of β_2GPI, showed that a specific residue in domain V (Cys^{281}-Cys^{288}) is recognized by apoER2'. This residue does not overlap the phospholipid binding site in domain V of β_2GPI (Ser^{311}-Lys^{317}). The results in this chapter argue a role for a specific residue within domain V of β_2GPI that is crucial for the interaction with the platelet receptor apoER2'.

Considering the wide cell- and tissue expression of members of the LDL-R family, we further explored the interaction between dimeric β_2GPI and several LDL-R homologous, including the LDL-R, the very low-density lipoprotein receptor (VLDL-R), megalin and LDL-R related protein (LRP). In *Chapter 4*, kinetic studies were employed to investigate whether other LDL-R family members can recognize dimeric β_2GPI. These studies particularly addressed the measurement of steady state constants for β_2GPI-receptor interactions. Surface plasmon studies (SPR) were performed with soluble forms of the receptors tested. These analyses revealed that dimeric β_2GPI bound to all receptors tested. However, the steady state constant derived from the interaction between the LDL-R and dimeric β_2GPI was remarkably low compared to the other receptors tested. To show that not only recombinant dimeric β_2GPI bound to the receptors but also β_2GPI/antiβ_2GPI antibody complexes, we tested binding of β_2GPI in the presence of a monoclonal anti-β_2GPI antibody. This complex bound to all receptors, except for the LDL-R. These results were consistent with the results obtained with dimeric β_2GPI. From literature data we know that the LDL-R is only involved in ligand-binding and not in ligand endocytosis and cannot be involved in signal transduction. Consistent with finding for apoER2', the other receptors tested also recognized a cationic residue

within domain V of dimeric β_2GPI, as 1) heparin, a mucopolysaccharide recognizing Lys^{282}, Lys^{284} and Lys^{287} in domain V of β_2GPI, could block binding of dimeric β_2GPI to all receptors tested and 2) the domain V deletion mutant hardly bound to the receptors. Furthermore, this cationic residue did not overlap the phospholipid binding site within domain V; a non-phospholipid binding mutant of dimeric β_2GPI showed no impaired binding to the receptors tested.

In *Chapter 5*, the interaction between dimeric β_2GPI and apoER2' is further investigated on a molecular level. Using dimeric β_2GPI with several amino acid substations in domain V, we addressed the β_2GPI-binding site for apoER2'. SPR analysis showed that the region Lys^{282}-Lys^{287} within domain V is crucial for recognition by apoER2', whereas the region Lys^{305}-Lys^{317} plays a minor role in the interaction. Another mutant tested, the Lys^{262}-Lys^{276} mutant hardly showed impaired binding to apoER2'. These results were consistent with the competition experiment using the peptides spanning region Cys^{281}-Cys^{288} discussed in *Chapter 3*.
Binding of β_2GPI to a cellular surface via anionic phospholipids and subsequent dimerization by anti-β_2GPI antibodies is a prerequisite before the dimerized protein can bind to apoER2' on the platelet surface. In this chapter, we investigated the interaction between dimeric β_2GPI and apoER2' on a molecular level, but it is also important to elucidate the interaction of dimeric β_2GPI with anionic phospholipids. Several groups have investigated binding of β_2GPI to cardiolipin. They have concluded that the region Cys^{281}-Cys^{288} in domain V is crucial for this interaction. However, binding of β_2GPI to anionic phospholipids, such as phosphatidylserine (PS) present on (slightly) activated platelets and other cells, has not yet been performed. We know from X-ray data that the phospholipid-binding region is located at Ser^{311}-Lys^{317}. We conducted phospholipid-binding experiment with the pointmutations employing solid phase immunosorbent assays. We showed that the cardiolipin-binding region within domain V is not similar to the PS-binding region; replacement of the residues Lys^{282}-Lys^{287} did not affect binding to apoER2' in such an extent as the mutant Lys^{305}-Lys^{317}. Taken together, our findings indicate that binding of β_2GPI to a cellular surface occurs in a two-step process. 1.) Binding of dimeric β_2GPI to anionic phospholipids (PS) accompanied by the residues Ser^{311}-Lys^{317}. 2.) Subsequent binding of dimeric β_2GPI to apoER2' supported by the cationic residue Cys^{281}-Cys^{288} within domain V.

The interaction of β_2GPI with anionic phospholipids is believed to be correlated with the biological function of the protein. Whether binding of β_2GPI to anionic

phospholipids induces a conformational change within β_2GPI, has been a subject of many investigations. Several investigators have put forward data that support this theory. Hammel *et al*. have shown that a structural rearrangement of β_2GPI after binding to anionic phospholipids, and not neutral phospholipids, can result in the exposure of a cryptic epitope located in the several domains. This conformational change may induce oligomerization of β_2GPI on the lipid surface serving as the basis for binding of anti-β_2GPI antibodies. In contrast, Krilis *et al*. have shown that binding of anti-β_2GPI antibodies is dependent on the density of the antigen and not on cryptic (neo)-epitope formation. The study described in *Chapter 6* shows that anti-β_2GPI antibodies only bind a cryptic epitope in domain I that is exposed after β_2GPI-binding to anionic phospholipids. These domain specific antibodies do not recognize β_2GPI in solution. However, after removal of the carbohydrate side chains, attached to domain II and domain IV (deglycosylation), these antibodies do recognize β_2GPI in solution. This indicated that the carbohydrate side chains no longer cover the cryptic epitope located in domain I. The cryptic epitope has been mapped to location Gly40-Arg43 in domain I of β_2GPI. Therefore, it is supposed that the antibodies might actually bind to the new determinants of β_2GPI that were specifically exposed during the interactions between the protein and anionic phospholipids.

In conclusion, the results in this thesis show that the interaction between β_2GPI and LDL-R family members is based on the recognition of a cationic patch within domain V of β_2GPI by LDL-R homologous. Molecular studies have identified a specific region within domain V of β_2GPI (Lys^{282}, Lys^{284}, Lys^{286} and Lys^{287}) that is recognized by the platelet receptor apoER2'. Furthermore, the studies demonstrate that the apoER2' recognition site does not overlap the phospholipid-binding site (the flexible loop located at Ser^{311}-Lys^{317}) within domain V of β_2GPI. These observations may learn us how β_2GPI binds to cellular surfaces containing anionic phospholipids. First, low-affinity binding of β_2GPI to anionic phospholipids via the flexible loop located at Ser^{311}-Lys^{317} occurs, inducing a conformational change within β_2GPI. These structural changes results in binding of anti-β_2GPI antibodies to an exposed epitope in domain I of β_2GPI followed by protein dimerization. Now, high-affinity binding of β_2GPI to LDL-R family members takes place, probably via the residue Lys^{282}-Lys^{287} within domain V of β_2GPI. However, several questions still not to be answered.
So far, the studies described in this thesis show the relevance of β_2GPI dimerization in cellular activation in *in vitro* and in biochemical experiments. Animal models, in which thrombosis can be studies, should be used to investigate the

physiological role of β_2GPI dimerization. In addition, future studies must be performed to investigate the role of the LDL-R family in β_2GPI/anti-β_2GPI induced thrombosis. This can be accomplished using LDL-R knockout mice.

Nederlandse Samenvatting 9

Beta2-glycoproteïne I (β_2GPI), ook wel bekend als apolipoproteine H (apoH), is een membraanadhesie eiwit dat in plasma circuleert (100-300 μg/mL) in een vrije- en een gebonden vorm. De molecuulmassa van β_2GPI is ongeveer 37 kDa. Het eiwit is opgebouwd uit 326 aminozuren. Vier suikergroepen gebonden aan domein III en domein IV dragen voor ongeveer 20% bij aan de totale molecuulmassa van 45 kDa. Het gen van β_2GPI ligt op chromosoom 17q23-24 en bestaat uit 8 exonen (~1.2 kilobasen (kb)) die door grote sequenties (intronen; ~16.2 kb) van elkaar worden gescheiden. β_2GPI behoort tot een superfamilie van voornamelijk complement eiwitten. Deze familie van eiwitten wordt gekenmerkt door herhalende delen (units), genaamd short consensus repeats (SCR), Sushi domains of complement control proteins, welke betrokken zijn bij eiwit-eiwit interacties. β_2GPI is opgebouwd uit vijf SCR domeinen waarvan de eerste vier domeinen gelijk zijn aan elkaar (~60 aminozuren en twee geconserveerde disulfide bruggen). Het vijfde domein is afwijkend en is opgebouwd uit 82 aminozuren en drie disulfide bruggen.

β_2GPI schijnt betrokken te zijn bij bloedstolling en is een antigeen voor antifosfolipiden antistoffen die aanwezig zijn in plasma van patiënten met het antifosfolipiden syndroom (APS). APS wordt gekenmerkt door arteriële en/of veneuze trombose in combinatie met zwangerschap complicaties en de aanwezigheid van anti-β_2GPI antistoffen in plasma van APS patiënten. Verschillende studies hebben een significante correlatie tussen trombotische complicaties en anti-β_2GPI antistoffen aangetoond. Het pathologische mechanisme via welke β_2GPI/anti-β_2GPI antistof complexen hun biologische functie uitoefenen, is onbekend. Meerdere studies tonen aan, dat anti-β_2GPI antistoffen betrokken zijn bij de activatie van bloedplaatjes via de apolipoprotiëne E receptor 2' (apoER2') en activatie van endotheelcellen door binding van β_2GPI gecomplexeerd met annexine II of leden van de Toll-like receptor (TLR) familie.

Recentelijk heeft onze groep aangetoond dat gedimerizeerd β_2GPI bindt aan apoER2' op humane bloedplaatjes. ApoER2' behoort tot een superfamilie van multi-ligand receptoren, genaamd de low-density lipoprotein receptor (LDL-R) familie. Deze familie van receptoren is geëxpresseerd op diverse cellen en weefsels, waaronder de hersenen, het hart, de lever, de longen, bloedplaatjes, monocyten en gladde spiercellen. Deze uitgebreide expressie suggereert dat de LDL-R familie in verschillende pathologische processen een rol kan spelen. In dit proefschrift zijn de volgende vragen met betrekking tot β_2GPI en de LDL-R familie gesteld. 1.) Welk domein van β_2GPI wordt herkend door apoER2' op humane bloedplaatjes? 2.) Zijn andere leden van de LDL-R familie in staat β_2GPI te herkennen? 3.) Wordt een specifieke sequentie in β_2GPI herkent door leden van de LDL-R familie of is er eerder

sprake van een elektrostatische interactie? Om deze vragen te beantwoorden zijn moleculaire studies gecombineerd met cellulaire- en kinetische studies.

De interactie tussen β_2GPI en apoER2' op humane bloedplaatjes is onderzocht met behulp van domein deletie mutanten van recombinant dimeer β_2GPI (*Hoofdstuk 3*). Gebaseerd op de veronderstelling dat binding van liganden aan leden van de LDL-R familie afhankelijk is van elektrostatische interacties, was domein V van β_2GPI, welke de meeste positieve lading bevat, het meest interessant om te onderzoeken. In een eerste experimentele opzet werd binding van de geconstrueerde domein deletie mutanten aan geïmmobiliseerd apoER2' gemeten in een enzym-linked immunosorbent assay (ELISA). Binding aan apoER2' van dimeer β_2GPI zonder domein I of domein II was vergelijkbaar met binding van wild-type dimeer β_2GPI. De affiniteit constanten van de domein I en domein II deletie mutant waren niet afwijkend ten opzichte van wild-type dimeer β_2GPI. In tegenstelling tot deze twee mutanten, vertoonde de domein V deletie mutant een aanzienlijk verminderde binding aan apoER2'. Deze resultaten tonen aan dat domein V belangrijk is voor de interactie met apoER2'. Deze resultaten werden geverifieerd met geïsoleerde, humane bloedplaatjes welke apoER2' expresseren. Competitie studies, gebruikmakende van peptiden die positief geladen gebieden in domein I en domein V van β_2GPI omtreffen, toonden aan dat een specifiek residu in domein V (Cys^{281}-Cys^{288}) wordt herkend door apoER2'. Dit residu overlapt niet het fosfolipiden-bindend gebied in domein V van β_2GPI (Ser^{311}-Lys^{317}). De resultaten in dit hoofdstuk tonen aan dat een specifiek residu in domein V van β_2GPI van belang is voor de interactie met de bloedplaatjes receptor apoER2'.

Gezien de wijde expressie van leden van de LDL-R familie op cellen en weefsel, werd de interactie tussen dimeer β_2GPI en andere leden van de LDL-R familie onderzocht, waaronder de LDL-R, de very low-density lipoprotein receptor (VLDL-R), megaline en de LDL-R related protein (LRP). In *Hoofdstuk 4*, worden kinetische studies toegepast om te onderzoeken ofdat bovengenoemde leden van de LDL-R familie dimeer β_2GPI herkennen. Deze studies omvatten voornamelijk het berekenen van affiniteit constanten voor β_2GPI-receptor interacties. Surface plasmon resonance (SPR) studies werden uitgevoerd met soluble vormen (zonder transmembraan deel) van de receptoren. Deze analyses toonden aan dat dimeer β_2GPI aan alle geteste receptoren bond, maar de affiniteit constante voor de β_2GPI/LDL-R interactie was lager in vergelijking met de andere receptoren. Niet alleen dimeer β_2GPI bond aan de receptoren, maar ook β_2GPI/anti-β_2GPI complexen bonden aan alle receptoren,

behalve aan de LDL-R. Deze resultaten zijn consistent met de behaalde resultaten gebruikmakende van dimeer β_2GPI. Van data uit de literatuur is het bekend dat de LDL-R betrokken is bij binding van liganden en niet in de endocytose van gebonden liganden en niet betrokken is in signalering. Consistent met data voor apoER2', herkenden de overige geteste receptoren ook een positief geladen residu in domein V van β_2GPI, omdat 1) heparine, een mucopolysaccharide die Lys^{282}, Lys^{284} en Lys^{287} in domein V van β_2GPI, binding van dimeer β_2GPI aan de receptoren kon remmen en 2) de domein V deletie mutant bond nauwelijks aan de receptoren. Deze positief geladen residu overlapt niet de fosfolipiden-bindend gebied in domein V van β_2GPI, aangezien een dimeer mutant die niet in staat is aan fosfolipiden te binden, wel een normale interactie laat zien met de geteste receptoren.

In *Hoofdstuk 5*, is de interactie tussen β_2GPI en apoER2' op een moleculair niveau onderzocht. Door positief geladen lysine residuen in domein V van β_2GPI te substitueren voor neutrale aminozuren, is het gebied dat apoER2' herkent, verder ontrafeld. SPR analyse heeft aangetoond dat het gebied Lys^{282}-Lys^{287} in domein V van β_2GPI van cruciaal belang is voor binding aan apoER2', terwijl het gebied Lys^{305}-Lys^{317} van minder belang is voor de eiwit-receptor interactie. Een ander dimeer β_2GPI mutant heeft aangetoond, dat de lysine residuen tussen Lys^{262}-Lys^{276} geen significante rol spelen in de interactie met apoER2'. Deze resultaten zijn consistent met de competitie studies zoals beschreven in *Hoofdstuk 3*.
β_2GPI binding aan negatief geladen fosfolipiden en opeenvolgend dimerizatie door anti-β_2GPI antistoffen is van belang voor binding aan apoER2' op bloedplaatjes. In dit hoofdstuk is de moleculaire interactie tussen β_2GPI en apoER2' bestudeerd, maar het is ook van belang om de interactie tussen β_2GPI en negatief geladen fosfolipiden te onderzoeken. Uitgaande van diverse studies heeft men geconcludeerd dat het gebied Cys^{281}-Cys^{288} in domein V van β_2GPI belangrijk is voor de interactie met cardiolipine. Echter, binding van β_2GPI aan negatief geladen fosfolipiden, waaronder fosfatidylserine (PS) aanwezig op (licht) geactiveerde bloedplaatjes en diverse andere cellen, is nog niet bestudeerd. X-ray data hebben aangetoond dat het fosfolipiden-bindend gebied in domein V van β_2GPI gelokaliseerd is in het residu Ser^{311}-Lys^{317}. In dit hoofdstuk is de interactie tussen β_2GPI en negatief geladen fosfolipiden onderzocht in een ELISA opzet, gebruikmakende van de dimeer β_2GPI mutanten. Deze studies hebben aangetoond dat domein V van β_2GPI niet met hetzelfde gebied bindt aan cardiolipine en PS; het residu Lys^{282}-Lys^{287} is meer van belang voor binding van β_2GPI aan cardiolipine, terwijl Lys^{305}-Lys^{317} belangrijker is voor binding aan PS. Samenvattende, deze bevindingen tonen aan dat β_2GPI binding

aan membranen gebaseerd is op twee opeenvolgende processen. 1.) Binding van β_2GPI aan negatief geladen fosfolipiden via Ser^{311}-Lys^{317} gevolgd door dimerizatie. 2.) Binding aan apoER2' via de positief geladen residu Cys^{281}-Cys^{288} in domein V van β_2GPI.

De veronderstelling bestaat dat de interactie tussen β_2GPI en negatief geladen fosfolipiden gecorreleerd is aan de biologische functie van het eiwit. Ofdat binding van β_2GPI aan deze fosfolipiden een conformationele verandering in β_2GPI induceert, is al reeds een lange tijd een discussie. Diverse onderzoeken ondersteunen deze hypothese. Hammel *et al*. hebben aangetoond dat een structurele verandering van β_2GPI na binding aan negatief (en niet positief) geladen fosfolipiden kan resulteren in het vrijkomen van een cryptische epitoop gelegen in meerdere domeinen van β_2GPI. Deze conformationele verandering kan resulteren in oligomerizatie van β_2GPI op het lipide oppervlak waardoor anti-β_2GPI antistoffen aan β_2GPI kunnen binden. In tegenstelling, Krilis *et al*. laten zien dat binding van anti-β_2GPI antistoffen afhankelijk is van de dichtheid van het antigeen (β_2GPI) en niet afhankelijk is van het vrijkomen van cryptische epitopen in β_2GPI. De studie beschreven in *Hoofdstuk 6* toont aan dat anti-β_2GPI antistoffen alleen aan β_2GPI kunnen binden, indien een cryptische epitoop (Gly^{40}-Arg^{43}) in domein I is vrijgekomen na binding van β_2GPI aan negatief geladen fosfolipiden. Deze domein specifieke antilichamen herkennen β_2GPI niet in oplossing. Echter, na deglycosylatie, herkennen de anti-β_2GPI antistoffen wel het antigeen in oplossing, omdat de cryptische epitoop niet meer wordt afgeschermd door de suikergroepen.

In conclusie, de resultaten in dit proefschrift hebben aangetoond dat de interactie tussen β_2GPI en leden van de LDL-R familie gebaseerd is op de herkenning van een positief geladen residu in domein V van β_2GPI. Moleculaire studies hebben een specifiek gebied in domein V gekarakteriseerd (Lys^{282}, Lys^{284}, Lys^{286} and Lys^{287}) welke door de LDL-R familie wordt herkend. Dit positief geladen residu overlapt niet het fosfolipiden-bindend gebied in domein V van β_2GPI (het flexibele gebied op positie Ser^{311}-Lys^{317}). Deze bevindingen geven ons een beter inzicht hoe β_2GPI aan cellulaire oppervlakten bindt. Ten eerste, er vindt binding met een lage affiniteit plaats tussen Ser^{311}-Lys^{317} in domein V van β_2GPI en negatief geladen fosfolipiden, dat vervolgens een conformationele verandering in het molecuul teweegbrengt. Deze structurele verandering resulteert in dimerizatie van het eiwit door binding van anti-β_2GPI antistoffen aan de epitoop Gly^{40}-Arg^{43} in domein I van β_2GPI. Nu kan β_2GPI met een hoge affiniteit binden aan leden van de LDL-R familie via de positief

geladen residu Cys^{281}-Cys^{288} in domein V van β_2GPI. Meerdere vragen blijven nog steeds onbeantwoord. De studies beschreven in dit proefschrift hebben de relevantie van β_2GPI dimerizatie laten zien uitgaande van experimenten waarin is gekeken naar cellulaire activatie. Tevens is met behulp van biochemische en kinetische experimenten het belang van β_2GPI dimerizatie aangetoond. Diermodellen, waarin β_2GPI-geïnduceerde trombose kan worden onderzocht, moeten de fysiologische relevantie van dimerizatie van β_2GPI aantonen. Toekomstige studies zullen moeten uitsluiten in hoeverre de LDL-R familie *in vivo* betrokken is in de interactie met β_2GPI. Dit kan worden gerealiseerd door LDL-R knockout muizen te incorporeren in de diermodellen, waarin β_2GPI-geïnduceerde trombose wordt onderzocht.

Appendices 10

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Curriculum vitae

Menno van Lummel werd geboren op 26 januari 1978 te Maassluis. De middelbare school werd in 1996 afgerond met het behalen van het diploma hoger algemeen voortgezet onderwijs aan de openbare scholengemeenschap 's Gravenlant te Schiedam. In datzelfde jaar werd begonnen met de studie klinische chemie aan de Hogeschool Rotterdam en omstreken te Delft. In 2000 heeft hij zijn bachelor diploma klinische chemie behaald met een afstudeerstage bij het Leids Universitair Medisch Centrum onder begeleiding van Dr. R. Nieuwland en Prof. dr. A. Sturk. In datzelfde jaar is hij gestart als specieel-analist op de afdeling Metabole Ziekten van het Wilhelmina Kinderziekenhuis te Utrecht. Vervolgens vergaarde hij werkervaring in de research bij Prof. dr. A.A. ten Have-Opbroek en Dr. W.J. de Vree op de afdeling Longziekten van het Leids Universitair Medisch Centrum te Leiden. Van mei 2002 tot juni 2006 heeft hij gewerkt aan het in dit proefschrift beschreven promotie-onderzoek onder supervisie van Prof. dr. Ph. G. De Groot en Dr. R.H.W.M. Derksen. Het promotie-onderzoek werd uitgevoerd op de afdeling Haematologie van het Universitair Medisch Centrum Utrecht. Tijdens deze periode ontving hij de '*Young Investigator Award*' van het XXth Congress of the International Society on Thrombosis and Haemostasis in Sydney, Australië in 2005. Momenteel is hij werkzaam als postdoc bij de groep van Dr. W.J. van Blitterswijk op de afdeling Cellulaire Biochemie, Nederlands Kanker Instituut te Amsterdam.

Dankwoord

Het einde van het ene
Is altijd het begin van iets nieuws

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bij het interpreteren van SPR data. Hier is een mooi artikel uit gekomen. Ton Lisman, jouw niet wetenschappelijke discussies waren vaak een goede afleiding van het onderzoek. Je Fokke & Sukke plaatjes trouwens ook.

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Menno