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### Beta-2 glycoprotein1: function in health and disease

Spyridon Miyakis

*University of New South Wales*, [smiyakis@uow.edu.au](mailto:smiyakis@uow.edu.au)

Bill Giannakopoulos

*University of New South Wales*

S A. Krilis

*St. George Hospital, Sydney*

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### Abstract

Beta-2 glycoprotein I ( $\beta$ 2GPI) is the principal target of autoantibodies in the antiphospholipid syndrome (APS). It is abundant in human plasma and shares high homology between different mammalian species. Although the exact physiological function of  $\beta$ 2GPI has not been fully elucidated, several interactions have been described with other proteins and with negatively charged surfaces, such as anionic phospholipids, dextran and heparin.  $\beta$ 2GPI is involved in the coagulation pathway, exerting both procoagulant and anticoagulant activities. Plasma from  $\beta$ 2GPI-deficient mice exhibits impaired thrombin generation in vitro. Recently, it has been demonstrated that  $\beta$ 2GPI binds factor (F) XI in vitro at concentrations lower than those of the protein in human plasma, and this binding inhibits FXI activation to FXIa by thrombin and FXIIa. Proteolytic cleavage of the fifth domain of  $\beta$ 2GPI abolishes its inhibition of FXI activation and results in reduced ability of the cleaved  $\beta$ 2GPI to bind phospholipids. It retains its ability to bind FXI. In vivo activation of FXI by thrombin is thought to be an important mechanism by which coagulation is accelerated via components of the contact activation pathway. Thus  $\beta$ 2GPI may attenuate the contact activation pathway by inhibiting activation of FXI by thrombin. Moreover, because  $\beta$ 2GPI is the dominant autoantigen in patients with APS, dysregulation of this pathway by autoantibodies may be an important mechanism for thrombosis in patients with APS.

### Keywords

beta, function, 2, health, disease, glycoprotein1

### Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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# Beta 2 glycoprotein I—function in health and disease

Spiros Miyakis, Bill Giannakopoulos, Steven A. Krilis\*

*Department of Immunology, Allergy and Infectious Diseases, University of New South Wales, St. George Hospital, 2 South Street, Kogarah, NSW 2217, Australia*

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Factor XI activation;  
Plasmin;  
Fibrinolysis

**Abstract** Beta-2 glycoprotein I ( $\beta_2$ GPI) is the principal target of autoantibodies in the antiphospholipid syndrome (APS). It is abundant in human plasma and shares high homology between different mammalian species. Although the exact physiological function of  $\beta_2$ GPI has not been fully elucidated, several interactions have been described with other proteins and with negatively charged surfaces, such as anionic phospholipids, dextran and heparin.  $\beta_2$ GPI is involved in the coagulation pathway, exerting both procoagulant and anticoagulant activities. Plasma from  $\beta_2$ GPI-deficient mice exhibits impaired thrombin generation in vitro. Recently, it has been demonstrated that  $\beta_2$ GPI binds factor (F) XI in vitro at concentrations lower than those of the protein in human plasma, and this binding inhibits FXI activation to FXIa by thrombin and FXIIa. Proteolytic cleavage of the fifth domain of  $\beta_2$ GPI abolishes its inhibition of FXI activation and results in reduced ability of the cleaved  $\beta_2$ GPI to bind phospholipids. It retains its ability to bind FXI. In vivo activation of FXI by thrombin is thought to be an important mechanism by which coagulation is accelerated via components of the contact activation pathway. Thus  $\beta_2$ GPI may attenuate the contact activation pathway by inhibiting activation of FXI by thrombin. Moreover, because  $\beta_2$ GPI is the dominant autoantigen in patients with APS, dysregulation of this pathway by autoantibodies may be an important mechanism for thrombosis in patients with APS.

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## Introduction

Beta-2 glycoprotein I ( $\beta_2$ GPI) is the major antigen bound by antibodies demonstrable in the antiphos-

pholipid syndrome (APS) [1].  $\beta_2$ GPI was first described in the early 1960s as a component of the beta-globulin fraction of human serum [2]. This molecule was classified as an apolipoprotein, and it was initially termed apolipoprotein H. Thirty years following its discovery, clear evidence emerged that  $\beta_2$ GPI is the major antigenic target for antiphospholipid antibodies (aPLab) circulating in the plasma of APS patients [3]. This breakthrough

\* Corresponding author. Tel.: +612 93502955; fax: +612 93503981.

E-mail address: s.krilis@unsw.edu.au (S.A. Krilis).

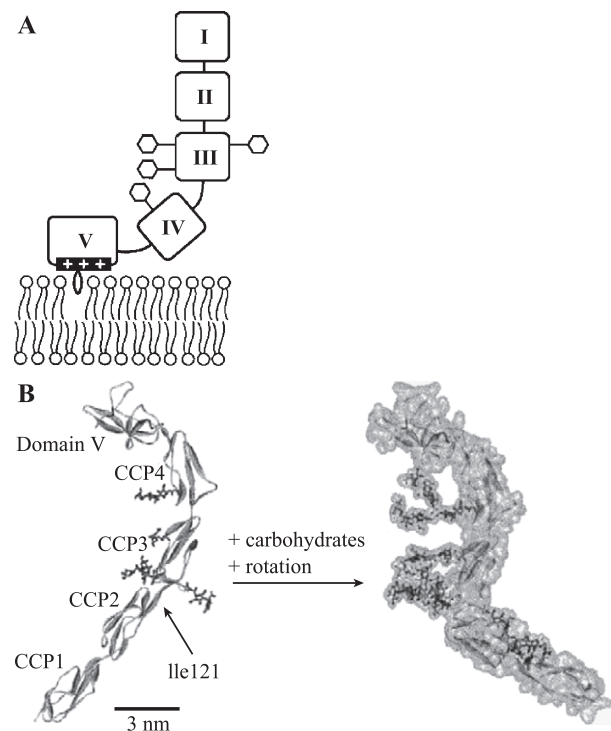
generated great interest in  $\beta_2$ GPI, and its role in the pathogenesis of autoantibodies underlying APS. The role of this protein in the coagulation system both in healthy people and patients with APS is being increasingly elucidated. For example, an anticoagulant effect of this protein has been demonstrated in *in vitro* systems [4]. Moreover,  $\beta_2$ GPI has varied and interesting interactions with several other proteins and with negatively charged surfaces, such as heparin, DNA, dextran and anionic phospholipid, independent of any involvement with autoantibodies [5,6]. The report that follows will discuss the main properties of  $\beta_2$ GPI, summarizing existing evidence and theories on the physiological role of this protein, as well as its involvement in the pathogenesis of the APS.

### $\beta_2$ GPI—general considerations

$\beta_2$ GPI is a single-chain protein with 326 amino acids, containing four N-linked glycosylation sites. Its average molecular weight measured by reduced SDS-PAGE is 54.2 kDa. The oligosaccharides in  $\beta_2$ GPI are highly heterogenous in structure. Despite the fact that enzymatic deglycosylation of  $\beta_2$ GPI seems to modify its secondary structure, alterations in glycosylation in recombinant human  $\beta_2$ GPI do not appear to affect cardiolipin binding nor the binding of  $\beta_2$ GPI dependent anticardiolipin antibodies [7]. The gene encoding  $\beta_2$ GPI maps to chromosome 17q23-24, and its promoter has been characterized recently [8].  $\beta_2$ GPI is highly conserved among mammals. Human, bovine, canine and mouse proteins all have five domains with 60–80% amino acid sequence homology, domain V being the most conserved [9,10].

### Molecular structure of $\beta_2$ GPI

$\beta_2$ GPI consists of five repeating sequences, also known as short consensus repeats (SCR; Fig. 1). Thus,  $\beta_2$ GPI belongs to the complement control protein (CCP) superfamily, which also includes molecules like complement receptor 1 and 2, C4b binding protein, complement FH, clotting FXIII (subunit-b), IL2 receptor and haptoglobin 2. The first four SCRs have 60 amino acid residues, including four cysteines each. Disulphide bridges join the first to the third and the second to the fourth cysteines to form a “looped-back” structure, known also as sushi domains [9]. Domains III and IV have three and one n-linked glycosylation sites, respectively [11].



**Figure 1** (A) A schematic representation of the  $\beta_2$ GPI structure at the domain level. The putative binding site for anionic phospholipid surface on the fifth domain is illustrated, so are the proposed glycosylation sites on domains III and IV (three and one glycosylation sites, respectively). Domain V also carries the lysine rich positively charged region, as well as the hydrophobic mobile insertion loop. Modified and reproduced with permission from Bouma et al. [11]. (B) The crystal structure of  $\beta_2$ GPI (left) is shown in comparison with the atomic model with complete carbohydrate chains and transformed to the best fit to the experimental data (right). For details see Ref. [18]. Reproduced with permission from Hammel et al. [18].

The fifth domain contains a conserved positively charged (multiple lysine) region between cysteines 281 and 288 that is critical for phospholipid binding [12]. Many of the positively charged side chains are located in a particular surface region of domain V, consisting of three loops (Fig. 1A). Site-directed mutagenesis, either reducing the net positive charge of the central loop in this region (lysine residues within positions 284–287 of the  $\beta_2$ GPI molecule) or disrupting the position of this loop (by mutating the flanking cysteines) inhibits the binding of  $\beta_2$ GPI to negatively charged phospholipids [12]. In addition, the fifth domain has an extra 20 amino acid tail, terminating in a cysteine, that can form a disulphide link with an extra cysteine found midway between the standard second and third cysteine residue positions [9]. This 20-residue tail-loop (amino acids 307–326) is conserved between human, bovine and canine species, differing only in

one residue in the rat and in two residues in the mouse [13]. Much of this tail is highly mobile and projects from the main surface of the fifth domain. Cleavage by both plasmin and (less effectively) FXa has been demonstrated to occur at this region (Lys<sup>317</sup>–Thr<sup>318</sup>; [14,15]). This cleavage affects the position of the lysine-rich region. Heparin can bind to this lysine-rich region within the fifth domain of  $\beta_2$ GPI [6]. Moreover, heparin at therapeutic concentrations is able to enhance the plasmin-mediated cleavage of the C-terminal region of  $\beta_2$ GPI [6]. The lysine residues in the region Cys<sup>281</sup>–Cys<sup>288</sup> on the fifth domain are involved in binding of  $\beta_2$ GPI to FXI (Shi et al. unpublished data). Plasmin-mediated cleavage of domain V abolishes the inhibitory action of  $\beta_2$ GPI on FXI activation, although cleaved  $\beta_2$ GPI is able to bind FXI [16]. Finally, polymorphisms of domain V have been described, which affect phospholipid binding, either by disrupting the disulphide bridge forming the terminal tail or by directly impairing binding to anionic phospholipids [5].

In addition to the phospholipid binding region in domain V, a secondary interaction between domain I and phospholipid has also been suggested, which—in contrast with domain V binding—occurs only at low ionic strengths [17]. Whether this low-ionic-strength interaction is of relevance physiologically is still unclear. Thus,  $\beta_2$ GPI 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 domains V and I.

In summary, the structure of  $\beta_2$ GPI suggests a lipid membrane insertion area on the fifth domain, two domains (III and IV) protected from proteolysis by glycosylation and two further domains (I and II) projecting away from the lipid surface into the extracellular space, thus able to interact with other proteins and/or antibodies (Fig. 1). Both hydrophobic and electrostatic interactions appear necessary for  $\beta_2$ GPI and anionic phospholipids to bind.  $\beta_2$ GPI does not bind to nonpolar phospholipids such as phosphatidylcholine, suggesting that the hydrophobic loop is insufficient alone. Cleavage of the hydrophobic loop makes only a small difference to heparin binding [6], suggesting that with heparin, the electrostatic interactions are relatively more important.

Evidence exists that the structure of  $\beta_2$ GPI in solution may be different from the crystal structure, the former being less elongated and more bulky [18]. Differences in the overall shape between crystal and solution structure are attributed to the carbohydrate units and to the flexibility of the CCP domains (Fig. 1B and C). It has been

proposed that transitions between conformational states directly correlate with the functional properties of the protein because the solution structure shows less immunological activity [19]. The interaction of  $\beta_2$ GPI with anionic phospholipids, plastic microtitre plates or cellular receptors may reorientate the CCP domains and increase the density and/or induce exposure of neoepitopes [20] required for antibody binding.

## $\beta_2$ GPI in plasma

$\beta_2$ GPI is produced in the liver and the placenta [9]. The mean serum level of  $\beta_2$ GPI is about 200 mg/l, which makes  $\beta_2$ GPI one of the most abundant proteins in human serum, second only to fibrinogen, among the plasma proteins involved in clotting. Nucleotide polymorphisms of the  $\beta_2$ GPI gene result in varying protein levels between individuals from different races, suggesting an autosomal codominant allelic state with normal and deficient alleles [21]. The heterozygous state does not appear to be thrombophilic, and, interestingly, individuals without detectable  $\beta_2$ GPI have been identified who appear clinically well [22].

$\beta_2$ GPI circulates in plasma mainly free, but also in lipid-bound form. The plasma level of  $\beta_2$ GPI may be increased in chronic infections. In acute infections, it behaves as a negative acute phase reactant, decreasing with albumin. It is also decreased in the clinical setting of disseminated intravascular coagulation which suggests the consumption of this molecule [4]. The level of  $\beta_2$ GPI is positively correlated with smoking, male gender, increasing age and hyperlipidaemia [23].

$\beta_2$ GPI binds to anionic or zwitterionic phospholipids without the need for calcium ions. Binding is achieved through the interactions of the lysine-rich region and the mobile hydrophobic loop in domain V of the protein [14]. Although the exact physiological function of  $\beta_2$ GPI in normal individuals is still unknown, it is believed that, under physiologic conditions, the protein exerts an anticoagulant effect by displacing various coagulation proteins from anionic phospholipid sites [1].

$\beta_2$ GPI does not seem to bind to native LDL, and most plasma  $\beta_2$ GPI is independent of lipoproteins. Nevertheless, oxidative modification of LDL results in the interaction with  $\beta_2$ GPI and the binding of aPLAb, leading to the uptake of ox-LDL particles by macrophages [20]. Not surprisingly, therefore,

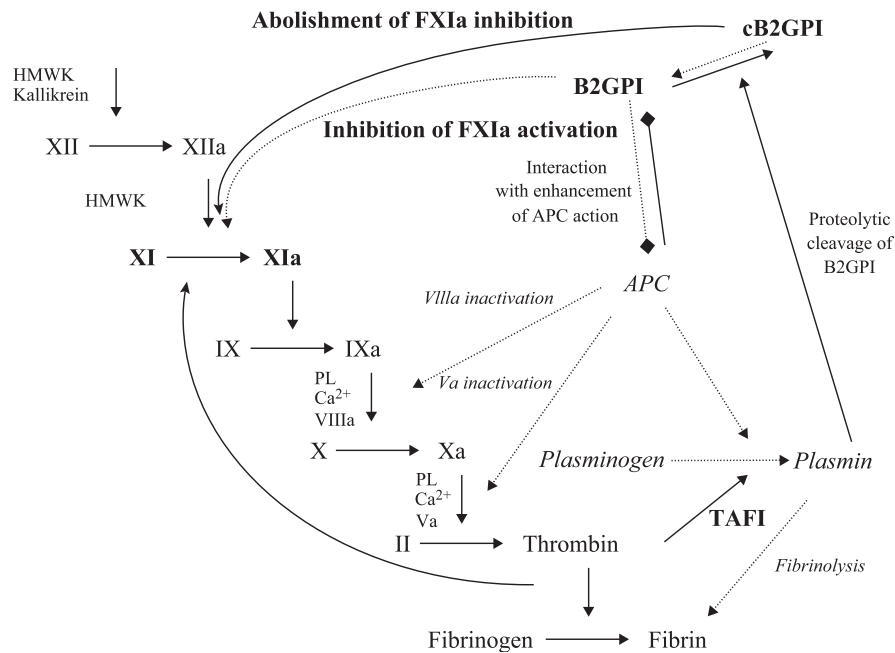
$\beta_2$ GPI is present in atherosclerotic plaques [24]. Furthermore, macrophage phagocytosis is induced by  $\beta_2$ GPI binding to anionic phospholipids, even in the absence of aPLAb [17], which suggests that  $\beta_2$ GPI functions as a marker for apoptotic or necrotic cells.

### $\beta_2$ GPI in the coagulation cascade

$\beta_2$ GPI has been shown to interact with a number of steps of the coagulation and fibrinolytic pathways (Fig. 2). The addition of anti- $\beta_2$ GPI antibodies to normal human plasma has been shown to cause prolongation in thrombin generation [25]. More recently, mice lacking  $\beta_2$ GPI have been produced by gene targeting; plasma from these knockout mice exhibited impaired thrombin generation in vitro. In heterozygous mice, thrombin generation was not as impaired as in the knockouts. This suggests a correlation between thrombin generation and  $\beta_2$ GPI levels [26]. These experiments provide direct evidence of the role of  $\beta_2$ GPI in the direct coagulation cascade, at a stage upstream of thrombin generation [27]. Nevertheless, the exact role of  $\beta_2$ GPI in the clotting process is not fully elucidated. The generation of thrombin, for instance, is important for both thrombus formation

and the initiation of the protein C anticoagulation pathway. Interestingly,  $\beta_2$ GPI-deficient individuals are apparently not at risk of thrombosis [22], and most of their haemostatic and fibrinolytic markers are normal.

The involvement of  $\beta_2$ GPI in the coagulation cascade seems multifaceted because the protein can exert both procoagulant and anticoagulant activities. The binding of  $\beta_2$ GPI to anionic phospholipids on activated platelets and endothelial cells (ECs) possibly results in the competition between  $\beta_2$ GPI and coagulation factors for phospholipid surfaces [1]. On the other hand, as previously mentioned, plasmin and—less efficiently—FXa in vitro can cleave Lys<sup>317</sup>–Thr<sup>318</sup> in the fifth domain of  $\beta_2$ GPI, abolishing its binding to anionic phospholipids [14]. This phenomenon is enhanced by heparin [6]. Proteolytically cleaved  $\beta_2$ GPI at Lys<sup>317</sup>–Thr<sup>318</sup> inhibits plasmin generation by tissue plasminogen activation (tPA; [28]). The conversion of plasminogen to plasmin by tPA is a key event in the fibrinolytic system. As plasmin is a critical protease in this system, the regulation of plasmin generation is important in in vivo haemostasis. Cleaved  $\beta_2$ GPI is found in the plasma of patients with lupus anticoagulant, in patients who have had ischaemic stroke, and in healthy individuals with a history of lacunar infarcts [28].



**Figure 2** The main steps of the intrinsic pathway of the coagulation system and the fibrinolytic mechanism, together with proposed interferences of  $\beta_2$ GPI. Effects promoting clot formation (procoagulant actions) are represented with continuous arrows, whereas effects inhibiting clot formation or promoting fibrinolysis (anticoagulant actions) are represented with dotted arrows. Factors of the intrinsic coagulation pathway are shown with plain characters, and elements of the fibrinolytic mechanism are given in italic characters.

$\beta_2$ GPI cleaved at Lys<sup>317</sup>–Thr<sup>318</sup> is also generated *in vivo* in pathological states of increased fibrinolysis [15] and provides an important feedback mechanism between  $\beta_2$ GPI and the components of the coagulation cascade (Fig. 2). Additionally, the suppression of plasma intrinsic fibrinolytic activity by  $\beta_2$ GPI, reversed by monoclonal anti- $\beta_2$ GPI antibodies in the presence of  $\beta_2$ GPI, has been described [29]. Hence,  $\beta_2$ GPI appears to not only interact with factors of the coagulation cascade, but also participate in the regulation of intrinsic fibrinolysis.

Procoagulant actions of  $\beta_2$ GPI also include the prevention of FVa degradation by activated protein C [30]. This is potentiated in the presence of anticardiolipin antibodies. On the other hand, anticoagulant activities of  $\beta_2$ GPI include the inhibition of the ADP-mediated aggregation of platelets and the impairment of thrombin generation by the prothrombinase complex, also amplified in the presence of aPLab [30]. It must be emphasized, however, that the data acquired *in vitro* regarding the function of  $\beta_2$ GPI needs to be cautiously interpreted. Whether such findings represent a true biological effect needs to always be verified with *in vivo* experiments.

$\beta_2$ GPI inhibits the activation of FXII in the presence of negatively charged phospholipids [31]. This in turn may inhibit the activation of the intrinsic coagulation pathway. Furthermore,  $\beta_2$ GPI also inhibits the generation of FXa in the presence of activated platelets. The presence of aPLab abolishes this inhibitory effect, resulting in protracted, unopposed FXa generation [32].

Recently, it has been demonstrated that  $\beta_2$ GPI binds FXI *in vitro* at concentrations lower than those of  $\beta_2$ GPI in human plasma, and this binding of  $\beta_2$ GPI to FXI inhibits its activation to FXIa by thrombin and FXIIa [16]. The affinity of the interaction between  $\beta_2$ GPI and FXI is equivalent to the known classical interaction between FXI and high molecular weight kininogen (HK). Site-directed mutagenesis indicates that the binding of  $\beta_2$ GPI to FXI is mediated by the terminal C region of the fifth domain. The positively charged residue sites Lys<sup>284</sup>, Lys<sup>286</sup> and Lys<sup>287</sup> in this domain of  $\beta_2$ GPI are essential for the  $\beta_2$ GPI–FXI interaction (Shi et al. unpublished data). Furthermore, proteolytic clipping of  $\beta_2$ GPI by plasmin at Lys<sup>317</sup>–Thr<sup>318</sup> of the fifth domain abolishes  $\beta_2$ GPI inhibition of FXI activation. Cleaved  $\beta_2$ GPI retains its ability to bind FXI [16].

The interaction of  $\beta_2$ GPI with FXI has extended our understanding of the role of  $\beta_2$ GPI in the mechanisms of blood coagulation (Fig. 2). The activation of FXI by thrombin results in additional

thrombin generation inside the fibrin clot. Thrombin formation, in turn, triggers the activation of thrombin activatable fibrinolysis inhibitor (TAFI), which protects the fibrin clot against lysis, by inhibiting the activation of plasminogen to plasmin [33]. The recent association of TAFI levels with levels of FXI and high risk of recurrent thrombosis further emphasizes the importance of this mechanism for blood rheology [34]. On the other hand, it appears that FXI/FXIa binds to activated human platelets *in vivo* in the presence of HK and zinc or prothrombin and calcium ions [35]. Hence, the ability of  $\beta_2$ GPI to bind FXI suggests that  $\beta_2$ GPI may substitute for HK or prothrombin *in vivo* in the interaction of FXI with activated platelets. The interaction of  $\beta_2$ GPI with FXI on the surface of activated platelets could facilitate not only the inhibition of FXI activation, but also the subsequent generation of cleaved  $\beta_2$ GPI by plasmin in a clot. Therefore, the regulation of FXI activation and the plasmin cleavage of  $\beta_2$ GPI in thrombosis may provide a negative feedback loop in FXI activation and the subsequent thrombin burst (Fig. 2). It seems that  $\beta_2$ GPI is implicated in both the coagulation and the fibrinolytic pathways, and that, possibly, one of the main anticoagulant actions of  $\beta_2$ GPI under normal physiologic conditions is the attenuation of the intrinsic coagulation pathway by inhibiting FXI activation by thrombin.

## $\beta_2$ GPI and atherosclerosis

Immunoreactivity against  $\beta_2$ GPI has been detected in atherosclerotic plaque. The interaction of aPLab with the  $\beta_2$ GPI/ox-LDL complex has been described elsewhere in this text.  $\beta_2$ GPI can also bind to lipoprotein (a) [Lp(a)], a molecule conferring a putative risk for atherosclerotic disease, which shares high homology to plasminogen [36]. In comparison, despite great homology between Lp(a) and plasminogen, it is only the proteolytically cleaved  $\beta_2$ GPI that binds plasminogen and not the native molecule, highlighting that cleavage of  $\beta_2$ GPI is a critical process in the regulation of the fibrinolytic pathway.

Taken together, the above data argue for an interaction between  $\beta_2$ GPI and the components of the atherosclerotic plaque. Antiphospholipid antibodies may interfere with this process. The *in vivo* significance of these findings remains unclear because, in contrast with the protean features of APS (thrombosis, miscarriage), atherogenesis in  $\beta_2$ GPI knockout mice has not been extensively studied yet.

## The antiphospholipid syndrome

Antiphospholipid antibody syndrome (APS) is characterised by the occurrence of a constellation of clinical manifestations associated with the presence of antiphospholipid antibodies (aPLab; [13]). Clinical features of APS mainly include recurrent venous and arterial thrombosis, thrombocytopenia, recurrent spontaneous miscarriages, various neurological syndromes, as well as livedo reticularis [13]. The aPLab are directed against either anionic phospholipids or phospholipid-binding protein epitopes. The syndrome may occur alone (in which case, it is referred to as the primary APS) or in association with systemic lupus erythematosus (SLE), other autoimmune diseases, and rarely with infections and drugs (secondary APS; [13]).

The aPLab can be detected either via the Lupus Anticoagulant (LA) *in vitro* coagulation test or via ELISA assays for antibodies interacting with cardiolipin (aCL) and/or  $\beta_2$ GPI [13].  $\beta_2$ GPI constitutes the major antigenic target for aPLab circulating in the plasma of APS patients [3]. Although there is no gold-standard diagnostic test for APS, it is evident that the  $\beta_2$ GPI ELISA has high specificity and positive predictive value for the diagnosis of APS. The presence of anti- $\beta_2$ GPI antibodies significantly correlates with thrombotic manifestations in APS patients [37,38]. Furthermore, most cases of clinically significant LA are associated with the concomitant presence of anti- $\beta_2$ GPI antibodies in the sera of APS patients [39].

The detection of  $\beta_2$ GPI as the major antigenic target of aPLab was a breakthrough in the understanding of the pathophysiology of the APS [3]. The increase in affinity of  $\beta_2$ GPI for anionic phospholipid upon antibody binding further supports the pathogenetic role of this molecule in disorders caused by aPLab, possibly through interference with phospholipid-dependent steps in coagulation, reversible in the presence of excess phospholipid [40]. The precise role, however, of  $\beta_2$ GPI, as well as the exact mechanism of  $\beta_2$ GPI–phospholipid interaction in antibody binding, are still unresolved, and several hypotheses have emerged. In contrast with the phospholipid binding site, which lies in domain V of  $\beta_2$ GPI, the major immunodominant epitope on  $\beta_2$ GPI is localised on domain I [41].

## Platelet activation and $\beta_2$ GPI. The role of the Apolipoprotein E receptor 2

It must be emphasized that, in contrast to mouse anti- $\beta_2$ GPI, human autoantibodies against  $\beta_2$ GPI

generally show low affinity for binding  $\beta_2$ GPI in standard ELISA assays [13]. Therefore, it has been hypothesized that antibody binding to  $\beta_2$ GPI immobilised on a surface is facilitated by the concentration of the protein on the surface, which brings about clustering of antigenic sites, allowing the bivalent binding of otherwise low-affinity antibodies [25,42]. Dimeric  $\beta_2$ GPI has been demonstrated to increase platelet adhesion to collagen in an *in vitro* system that mimicked the effect on platelets produced by the *in vivo* existence of aPLab [42]. Because the binding of dimerized  $\beta_2$ GPI to negatively charged phospholipids is not enough to sensitize platelets, it seems that after the binding of dimeric  $\beta_2$ GPI to the platelet, an interaction of dimeric  $\beta_2$ GPI with a receptor on the platelet surface is involved in further activation [42].

Apolipoprotein E receptor 2 (ApoER2), a member of the LDL receptor family, offers an attractive possible candidate for binding dimeric  $\beta_2$ GPI. Members of the LDL receptor family have specific protein modules of extracellular ligand binding repeats, as well as a cytoplasmic region containing motifs for endocytosis and signal transduction [43]. A splice variant of ApoER2 is the only member of the LDL receptor family found on the platelet membrane. Increased platelet adhesion observed with dimeric  $\beta_2$ GPI disappeared after preincubation with the receptor-associated protein (RAP), which is known to inhibit the interaction of ligands with members of the LDL receptor family [42]. Furthermore, using phage display technology, a peptide region on ApoER2, which specifically interacts with  $\beta_2$ GPI, has recently been identified (Krilis et al., unpublished observations). The coprecipitation of dimeric  $\beta_2$ GPI with ApoER2 suggests that ApoER2 indeed mediates the effects of dimeric  $\beta_2$ GPI on platelets [42].

It is therefore likely that autoantibodies to  $\beta_2$ GPI form complexes with  $\beta_2$ GPI able to bind to activated platelet surface receptors, like ApoER2 (Fig. 3). This binding sensitizes platelets to low concentrations of thrombin, resulting in adherence to collagen [1,42]. It has been demonstrated in this system that dimeric  $\beta_2$ GPI constructs and anti- $\beta_2$ GPI antibodies can induce platelet adhesion, thrombus formation and thromboxane A2 production [42]. Plasmin-mediated cleavage of the carboxy terminus of domain V, potentiated by proteoglycans, would induce the dissociation of the  $\beta_2$ GPI–anti- $\beta_2$ GPI complex from ApoER2 (Fig. 3). This model is proposed for platelet activation and involves a platelet cell surface receptor (likely ApoER2) interacting with the bivalent  $\beta_2$ GPI–anti- $\beta_2$ GPI aPLab complexes. However,



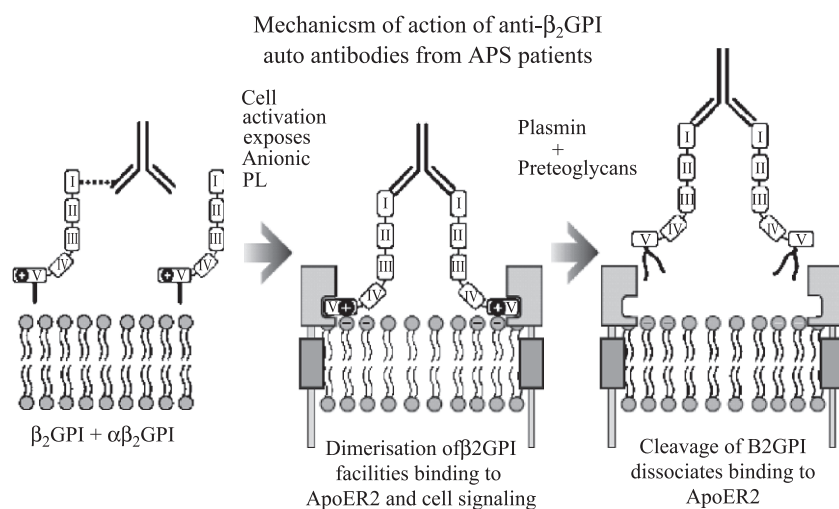
inasmuch as many different cell types express members of the LDL receptor family on their surface, this model might also apply to the activation of other cell types in the APS, possibly involving receptors for  $\beta_2$ GPI other than ApoER2, in the LDL receptor family.

### Recurrent fetal loss in APS. The role of $\beta_2$ GPI

Recurrent miscarriage is one of the main features of APS [13]. Evidence for a pathogenetic role of aPLab in pregnancy first came when a passive immunization of pregnant mice with aPLab from women with APS led to murine pregnancy loss in some, but not all, cases [44]. aPLab reacting with  $\beta_2$ GPI can inhibit the proliferation and secretory activity of human placental trophoblast cells, confirming the involvement of  $\beta_2$ GPI in pregnancy loss associated with APS. The presence in the sera of pregnant women of  $\beta_2$ GPI-dependent aPLab (compared with  $\beta_2$ GPI-independent antibodies) significantly correlates with adverse pregnancy outcomes (miscarriage, preeclampsia and retarded fetal growth), and the detection of anti- $\beta_2$ GPI aPLab has the greatest predictive value for pregnancy complications, among the conventional diagnostic tests for APS [45]. Taken together, these data argue for  $\beta_2$ GPI as the principal antigenic target in APS-related fetal loss.

The exact role of antibodies against  $\beta_2$ GPI in the miscarriage process in APS is not fully elucidated yet. One hypothesis suggests that they prevent implantation *in vivo*, by the binding of aPLab to the trophoctoderm of preimplantation embryos [46]. Another suggested mechanism is that there is impaired placental prostaglandin synthesis, due to the interaction of aPLab with the  $\beta_2$ GPI-mediated activity of lipoprotein lipase on maternal membrane phospholipids [47]. It has also been proposed that there is an increased macrophage uptake of oxidised LDL, secondary to aPLab binding, inducing, thus, placental atherogenesis [20]. The absence of thrombocytopenia despite fetal loss [48] supports the argument that platelet consumption in placental thrombotic events is not the predominant mechanism of recurrent miscarriages in APS. Interestingly, existing *in vivo* data on the influence of either aPLab or  $\beta_2$ GPI on murine pregnancies are, thus far, conflicting [27]. It is evident that the sole existence of aPLab is not sufficient for explaining recurrent fetal loss in human APS because such antibodies are present in only one-tenth of recurrent aborters, while they are present—but without clinical significance—in a high proportions of normal pregnancies [49].

The role of  $\beta_2$ GPI in pregnancy failure has been further elucidated following experiments on genetically modified mice lacking  $\beta_2$ GPI [10,26].  $\beta_2$ GPI knockout mice are able to carry pregnancies to term, with reproductive outcomes indistinguish-



**Figure 3** Autoantibodies to  $\beta_2$ GPI circulate in plasma but do not bind  $\beta_2$ GPI because of low affinity. Once cell activation exposes anionic PL (for example, on platelet surface), the dimerisation of  $\beta_2$ GPI then facilitates binding to  $\beta_2$ GPI cell surface receptor (ApoER2 for platelet cells) and cell signaling. Plasmin cleavage at the site of thrombus formation potentiated by proteoglycans cleaves  $\beta_2$ GPI carboxy terminus and dissociates binding from its receptor. In the absence of  $\beta_2$ GPI, the autoantibodies cannot induce their pathogenic effects. Reproduced with permission from Miyakis et al. [27].

able from that of the control mice [26].  $\beta_2$ GPI deficiency, however, is associated with a 15% reduction in litter size, compared with heterozygote and wild-type pregnancies, along with subtle changes in placental size and structure, indicative of impaired placental efficiency [48]. On the other hand, when  $\beta_2$ GPI heterozygotes on a 129/Sv/C57BL/6 mixed genetic background were intercrossed, only 8.9% of the resulting offspring were homozygous for the deletion, which is significantly lower than the expected 1:2:1 Mendelian ratio, implying that the lack of  $\beta_2$ GPI action confers a selective disadvantage on survival [26]. This is probably the result of a defect at implantation or at even earlier reproductive stages (ovulation, early embryogenesis) because litter sizes were comparable in heterozygote and wild-type pregnancies. Taken together, data from knockout mice indicate that  $\beta_2$ GPI is necessary to achieve the full effect on fetal loss mediated by aPLAb; however, the elimination of  $\beta_2$ GPI action does not seem to be the principal pathogenetic mechanism for APS-related pregnancy loss.

Interestingly, pregnancy in  $\beta_2$ GPI-deficient mice was associated with an increase in mean blood platelet count of 35%, rather than thrombocytopenia, which characterizes APS [26]. Moreover, passive immunization with aPLAb in pregnant  $\beta_2$ GPI null mice resulted in an increased blood platelet content late in gestation, compared with virgin animals or animals during early pregnancy [48]. On the other hand, passive antibody transfer experiments also showed that some antibody preparations elicited more severe pregnancy pathologies in the presence of endogenous  $\beta_2$ GPI than in  $\beta_2$ GPI null mutant animals [48]. The synthesis of the above data to coherently explain the possible pathogenic mechanisms operating in patients with the APS may initially seem difficult. An attractive hypothesis is that aPLAbs may play a pathogenic role by potentiating, rather than abolishing, at least some of the effects of  $\beta_2$ GPI. In this scenario, the procoagulant actions of  $\beta_2$ GPI would be amplified in the presence of aPLAbs, thus explaining the prothrombotic tendency seen in patients with APS.

### The role of $\beta_2$ GPI in the thrombotic events of APS

Recurrent thrombosis affecting arteries, veins or both is a cardinal clinical feature of APS [13]. The direct anti- $\beta_2$ GPI ELISA has high specificity (0.88–0.99) for thrombosis. aPLAb against  $\beta_2$ GPI show the

highest predictive value among aPLAb, especially for arterial thrombosis in APS [50]. Given the multiple interactions of  $\beta_2$ GPI with factors of both the coagulation and fibrinolysis pathways (extensively discussed elsewhere in this text), aPLAb are expected to be involved in the pathogenesis of thrombotic events that characterise APS. Interference of the normal physiological interaction between  $\beta_2$ GPI and the coagulation factors by aPLAb constitutes a possible pathogenic mechanism for APS-related thrombosis. The inhibition of FXa generation on activated platelets by  $\beta_2$ GPI has been shown to be counteracted by aPLAb, leading to an increased, unopposed FXa generation [32]. An alternative explanation for APS-related thrombosis is that aPLAb showing generally low-antigen affinity may efficiently bind platelet-associated  $\beta_2$ GPI, thereby blocking its attenuating effect on FXI activation. The cleavage of  $\beta_2$ GPI by plasmin provides a negative feedback to FXI activation and the subsequent thrombin burst. The cleavage of  $\beta_2$ GPI at Lys<sup>317</sup>–Thr<sup>318</sup> has been demonstrated in patients with LA, independent of plasmin activation, implying that aPLAb may also have an effect on  $\beta_2$ GPI cleavage [51].

Another mechanism by which aPLAb directed against  $\beta_2$ GPI may predispose an individual with APS to thrombosis is by interfering with the fibrinolytic mechanism. The proteolytic degradation of both FVa and FVIIIa is mediated by the protein S-activated protein C (APC) complex, and the activation of protein C is greatly enhanced by phospholipids [52]. Both rabbit and human monoclonal aPLAb can inhibit FVa degradation, the effect being  $\beta_2$ GPI dependent [30]. Moreover, in an *in vitro* system, the LA effect on thrombin generation (and on the subsequent degradation of FVa) was dependent on affinity-purified anti- $\beta_2$ GPI—but not on antiprothrombin—antibodies [53]. Thus, it can be proposed that the underlying mechanism for the inhibition of the APC complex by anti- $\beta_2$ GPI/aPLAb is via competition for anionic phospholipid binding sites (Fig. 2). On the other hand, the recently described interaction between  $\beta_2$ GPI and FXI provides an attractive alternative mechanism for the involvement of  $\beta_2$ GPI in the pathogenesis of APS via interference with the fibrinolytic system:  $\beta_2$ GPI cleavage by plasmin abolishes  $\beta_2$ GPI inhibition of FXI activation. This results in excessive activation of the coagulation pathway and thrombin formation. Thrombin, in turn, inhibits plasmin production by plasminogen (via TAFI; Fig. 2). Therefore, cleaved  $\beta_2$ GPI inhibits the activation of plasminogen to plasmin, thus further attenuating the fibrinolytic system.

Nevertheless, a lot of unresolved issues remain regarding the interaction of  $\beta_2$ GPI with elements of the fibrinolytic system. For instance, the APC complex mediates both procoagulant and anticoagulant effects in  $\beta_2$ GPI-depleted plasma *in vitro*. The clinical significance of this observation remains uncertain [30]. On the other hand, disorders arising from hereditary deficiencies of the fibrinolytic system proteins typically only entail venous thrombosis and are rarely associated with arterial events or miscarriage. Furthermore, apart from anti- $\beta_2$ GPI/aPLAbs, antibodies against prothrombin, protein C, protein S and annexin V have been detected in patients with SLE-associated APS. These antibodies confer independent, significant risk factors for thrombosis and fetal loss [54]. Taken together, the above data make the association between  $\beta_2$ GPI and the fibrinolytic system more complicated and difficult to fully interpret as an isolated mechanism contributing to thrombotic events of APS patients.

The effect of aPLAb in the pathogenesis of APS-associated thrombosis may also be considered in relation to protein Z (PZ). PZ is a vitamin-K-dependent plasma protein that serves as a cofactor for the inactivation of FXa by protein-Z-dependent protease inhibitor (ZPI). Evidence exists that PZ plays an important role in coagulation regulation because of the PZ-dependent inactivation of FXa by ZPI. Recently, it has been demonstrated that FXa inhibition by PZ/ZPI is attenuated, to some extent, by  $\beta_2$ GPI [55]. Most isolated aPLAb were found to further enhance the inhibitory potential of  $\beta_2$ GPI on PZ/ZPI activity. Without  $\beta_2$ GPI, the PZ/ZPI activity was unaffected by the addition of isolated aPLAb. Because the generation of thrombin is closely related to the concentration of FXa, the sum of these aPLAb effects could be inappropriate thrombin generation and an increased risk for thrombosis, possibly via the potentiation of the  $\beta_2$ GPI action on FXa inactivation by PZ/ZPI [55]. The high prevalence of PZ deficiency in association with aPLAb has been detected, as well as significantly lower PZ levels in a definite APS group of patients, but not in the non-APS group, compared with the normal group [55].

Finally, aPLAb against  $\beta_2$ GPI have been shown to exert direct antiendothelial cell activity in APS patients [56]. The binding of aPLAb to  $\beta_2$ GPI on the endothelial cell (EC) surface—mediated through the specific cell surface receptor annexin II—results in EC activation via the up-regulation of adhesion molecules and cytokines. This induces a proinflammatory and procoagulant phenotype in endothelial cells [56]. This effect also implies

that aPLAb, in the presence of  $\beta_2$ GPI, may well bind to healthy ECs without anionic phospholipid involvement.

An alternate hypothesis is that anti- $\beta_2$ GPI antibodies activate ECs through the MyD88-dependent signaling pathway, via involvement of the toll-like receptors (TLR) family [57]. Because TLRs form functional signaling pairs (homodimers or heterodimers) on interaction with the proper ligand, it has been suggested that anti- $\beta_2$ GPI antibodies might cross link  $\beta_2$ GPI molecules together with TLRs, eventually favoring the receptor polymerization and the signaling cascade [57].  $\beta_2$ GPI shares amino acid sequence in common with different microbial pathogens [58], further supporting an association between  $\beta_2$ GPI and TLRs. This recent hypothesis seems very interesting, given also the increase of aPLAb transiently observed during infections [13], the frequent occurrence of APS-related events in patients with chronic viral infections [59] and, perhaps more importantly, the clinical association between the so-called catastrophic variant of APS and infections as triggering events [60].

## Summary and perspectives

$\beta_2$ GPI is one of the most abundant plasma proteins, and this fact implies a significant role in human physiology. Indeed, the structure of  $\beta_2$ GPI confers to the protein the ability to bind phospholipids, as well as to interact with a wide range of other proteins. Evidence thus far indicates that  $\beta_2$ GPI (a) interacts with steps in the coagulation and fibrinolytic pathways, (b) is necessary for placental homeostasis and for optimal implantation, and (c) may be involved in other processes, such as atheromatous plaque formation, endothelial cell activation and the apoptotic mechanism. A more detailed review on apoptosis and antiphospholipid antibodies is published in this volume of *Thrombosis Research*, by Rauch et al.

$\beta_2$ GPI constitutes the main antigenic target of autoantibodies in the APS. It has been proposed that aPL acquire pathogenicity in APS upon the abolishment of at least some of the physiological actions of  $\beta_2$ GPI. The recently demonstrated inhibition of FXI activation [16] gives a characteristic model with multiple interactions and feedback mechanisms, in which  $\beta_2$ GPI holds a key role.  $\beta_2$ GPI may attenuate thrombin generation *in vivo* by inhibition of FXI activation. Plasmin cleavage of  $\beta_2$ GPI provides a negative feedback that counteracts its inhibition of FXI activation. Important

questions, however, have not yet been answered. (1) Although  $\beta_2$ GPI binds FXI via its fifth domain, the exact mechanism that leads to inhibition of FXI activation is not known. It is likely that the binding of  $\beta_2$ GPI to FXI causes a conformational change such that FXI does not bind to anionic phospholipids or the proteases thrombin and FXIIa. This hypothesis needs to be proved. (2) What is the exact nature of the interference of aPLab with the  $\beta_2$ GPI actions? Although it was classically believed that antibodies abolish  $\beta_2$ GPI effect, recent data suggest that—at least in some cases—the enhancement of  $\beta_2$ GPI activation by the autoantibodies in vivo is a more likely possibility. This apparent inconsistency between in vitro and in vivo data must be examined in light of the fact that  $\beta_2$ GPI in vivo may bind surface receptors, such as ApoER2, annexin II and proteoglycans, on surfaces of cells, in addition to negatively charged phospholipids.

Not surprisingly, thus, in vitro data so far suggests that  $\beta_2$ GPI exerts multiple actions, some of them procoagulant and some anticoagulant. Which of these actions are of clinical importance and how exactly their balance is disrupted in the APS remains to be examined in vivo. It has been hypothesized that, despite multiple in vitro interactions of  $\beta_2$ GPI with the coagulation and fibrinolytic pathways, exerting both procoagulant and anticoagulant functions, the net in vivo effect of  $\beta_2$ GPI must be anticoagulant. The neutralization of this effect by anti- $\beta_2$ GPI aPLab confers the prothrombotic status that characterizes APS. Even if such simplicity is to be accepted, a net anticoagulant in vivo effect for  $\beta_2$ GPI has not been demonstrated thus far. This could mean that (1) such a simplified explanation for the role of  $\beta_2$ GPI is not valid, (2)  $\beta_2$ GPI is not pathogenic for APS, and/or (3) the protein exerts a net procoagulant in vivo effect. If the latter were correct, then related antibodies must act synergistically, enhancing the  $\beta_2$ GPI effect in APS, and evidence towards this hypothesis has been provided from recent data. An alternative hypothesis is that anti- $\beta_2$ GPI aPLab indeed neutralize such a procoagulant action of  $\beta_2$ GPI, initially conferring an inappropriate anticoagulant subclinical phenotype in APS. The maintenance of such a phenotype would promote the consumption of elements of the coagulation cascade, as well as the exhaustion of fibrinolytic factors, so that thrombotic episodes eventually manifest in the vasculature of sites so predisposed for anatomical reasons. The latter hypothesis can be supported by the fact that thrombosis tends to recur at the same part of the vasculature (arterial vs. venous) in a given APS patient. In any case, it is

likely that the net effect of the assumed opposing  $\beta_2$ GPI actions would depend on the microenvironment at a particular stage.

Experiments performed so far in  $\beta_2$ GPI knockout mice have suggested a role for  $\beta_2$ GPI in embryonic implantation and the optimal functioning of the placenta [48]. Important and still unsettled questions arise regarding the exact stage of early pregnancy that is compromised in the absence of  $\beta_2$ GPI. Hence, the exact function of  $\beta_2$ GPI, even in the undisputed areas of its involvement in APS (that is, recurrent thrombosis and miscarriage), remains to be elucidated. The proposed model for platelet activation upon the interaction of the bivalent  $\beta_2$ GPI-antibody complexes with the platelet receptor ApoER2 could also apply to the activation of other cell types in the APS. The possibility also exists that the  $\beta_2$ GPI–autoantibodies interaction confers the predisposing background conditions for APS, and that the clinical manifestations are triggered by another critical event. Despite the existence of data supporting this, the validity of this hypothesis, as well as the exact candidate triggering event (infection, surgery, pregnancy, long immobilisation, etc.), needs to be further explored.

Another important issue that needs to be elucidated is the precise role of  $\beta_2$ GPI in atherogenesis and its possible clinical implications for APS. Interesting data may emerge from the examination of the in vivo atherogenic potential of mammals deprived of  $\beta_2$ GPI, as well as their behaviour under different dietary conditions. Recently, the promoter of the human  $\beta_2$ GPI gene has been cloned and characterized [8]. This is a step towards the identification of the genetic parameters determining  $\beta_2$ GPI tissue expression and its association with other molecules and relevant interacting genes. These findings, coupled with the new data on the direct interaction between  $\beta_2$ GPI, FXI and plasmin in the coagulation and fibrinolysis cascades, have given impetus to the ongoing research directed towards the illumination of the role of  $\beta_2$ GPI in normal health and in the pathogenesis of APS.

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