

Chapter 2

Natural Proteins Involved in Antiphospholipid Syndrome

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Introduction

The antiphospholipid syndrome (APS) is characterized by antiphospholipid antibodies (aPL) in the plasma or serum of patients with thrombosis or pregnancy complications [1]. The APS is a misnomer, because the so-called aPL are directed not against phospholipids but against plasma proteins with affinity for anionic phospholipids. Autoantibodies against many different plasma proteins have been described. In this chapter we will enumerate these proteins, discuss the arguments why they are linked to the syndrome and discuss why these proteins become prothrombotic in the presence of autoantibodies.

Plasma Proteins Involved in Antiphospholipid Syndrome

β_2 -Glycoprotein I

β_2 -glycoprotein I (β_2 GPI) is a 50 kDa plasma protein with increasing evidence that it has important roles in innate immunity and coagulation [2–4]. Many studies show that anti- β_2 GPI antibodies (a β_2 GPI), either mouse monoclonal or patient derived, induce a prothrombotic phenotype in mice that have been primed either with lipopolysaccharide (LPS) or an injury to the vessel wall [5, 6]. Studies that separate a β_2 GPI-associated aPL from those in which a β_2 GPI are removed by affinity chromatography show that the prothrombotic effect of aPL is present only in the a β_2 GPI containing fraction [7].

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Further studies, characterizing correlations of the individual domains of β_2 GPI with thrombosis and fetal loss, show correlation for both manifestations only with anti-domain I antibodies [8, 9]. Studies using $\alpha\beta_2$ GPI depleted of anti-domain I antibodies further demonstrate that domain I antibodies are pathogenic, while antibodies against the other domains are not [10]. The major epitope for the autoantibodies is located within the region of amino acids arginine 39 and arginine 43 and a minor epitope involving lysine19 [11]. Exogenous human domain I can inhibit the prothrombotic phenotype in a mouse model of APS [12]. When arginine 39 is replaced by serine, the inhibitory potential of domain I is lost. These experiments in mouse models show conclusively that β_2 GPI, in particular its first domain, is central to the pathogenesis of APS.

Prothrombin

Antiprothrombin antibodies are commonly found in patients with APS. Prothrombin is one of the major coagulation factors in blood. However, antiprothrombin antibodies do not correlate with thrombosis. A recently developed assay that measures autoantibodies against prothrombin complexed with phosphatidylserine (PS) shows better correlation of these antibodies with thrombosis [13]. Two studies show that antiprothrombin antibodies are prothrombotic in mouse models of APS [14, 15]. The thrombotic response to an induced vascular injury was much stronger with antiprothrombin than with control antibody. However, the antibodies used in these studies were not well characterized. In rare cases antiprothrombin antibodies can cause bleeding due to decreased levels of prothrombin because, in contrast to autoantibodies against β_2 GPI, antiprothrombin antibodies enhance clearance of prothrombin from the circulation [16].

Annexin A2

The annexins constitute a family of highly conserved, Ca^{2+} -regulated, phospholipid-binding proteins that have many functions related to membrane-mediated processes [17]. Annexin A2 influences haemostasis as it is a receptor for tissue plasminogen activator (tPA) on endothelial cells and for β_2 GPI. Annexin A2 knockout ($-/-$) mice show deposition of fibrin in the lungs, spleen, liver, and kidney as they age consistent with decreased fibrinolysis [18]. Annexin A2 autoantibodies develop in patients with APS; high titers of anti-annexin A2 autoantibodies correlate with thrombosis [19]. Annexin A2 ($-/-$) mice, in a model of APS, reduce the prothrombotic effect of injected aPL, suggesting that the mechanism by which annexin A2 is involved in APS is due to disruption of its fibrinolytic function by the autoantibodies [20].

Annexin A5

Annexin A5 is another member of the annexin family of calcium-dependent phospholipid-binding proteins. The anticoagulant properties of this protein result from its rapidly forming two-dimensional crystal arrays over the polar heads of the anionically charged membrane phospholipids [21]. Anionic phospholipids are required cofactors for the four critical phospholipid-dependent coagulation reactions: the IXa-mediated tenase reaction, the tissue factor-VIIa-mediated tenase, the tissue factor-VIIa-mediated IXase reactions, and the prothrombinase reaction. Assembly of the annexin A5 array shields phospholipids from contributing to the enzymatic reactions. Annexin A5 knockout mice have increased placental thrombosis and infarction but no increased propensity for systemic thrombosis [22].

Several studies explore the possibility that anti-annexin A5 antibodies correlate with clinical manifestations of APS [23]. IgG anti-annexin A5 antibodies occur in patients with pregnancy complications but not in those with venous or arterial thrombosis.

An alternative research path asks whether aPL antibody-mediated disruption of annexin A5 crystallization, on activated platelets and on phospholipid vesicles, leading to reduction of anticoagulant activity (called “A5 resistance”) correlates with adverse clinical outcomes. Recent data suggest that A5 resistance does correlate with increased risk of thrombosis and pregnancy complications. A recent paper [24] correlates A5 resistance with increased prevalence of thrombosis in a “real-world” retrospective population and in a group of prospectively observed asymptomatic patients. In both the retrospective and prospective groups, A5 resistance correlates with positivity for multiple criteria-based aPL assays.

In summary, A5 resistance correlates with an increased risk for thrombosis. The resistance is specifically mediated by anti-domain one of β_2 GPI [25] and potentially other aPL cofactor proteins, but it is not mediated by antibodies to annexin A5. To date, although anti-annexin A5 antibody assays may be associated with an APS process, it is not clear that they have a causal relationship to the disease.

Platelet Factor 4 (CXCL4) and Other Platelet-Derived Chemokines

Platelet factor 4 (PF4) or CXCL4 is an 8kD molecule belonging to the CXC chemokine family. It circulates as a tetramer. It was first recognized to play a role in APS when platelet membrane protein extracts, from three healthy donors and seven APS patients, were passed through a β_2 GPI affinity column and analysed by mass spectrometry [26]. Experiments using in silico molecular docking models indicated that a tetramer of PF4 act as a scaffold to which two molecules of β_2 GPI bound. According to this model, domain I of β_2 GPI became accessible for recognition by a β_2 GPI, while domain V was available to interact with other proteins on the platelet membrane [26]. The multimeric

complex $(PF4)_4/(\beta_2GPI)_2/a\beta_2GPI$ can exist in solution. Furthermore, platelets from healthy individuals, primed with very small amounts of thrombin, were activated only when PF4, β_2GPI and $a\beta_2GPI$ were present and were associated with the phosphorylation of p38 MAP kinase. Natural dimerization of β_2GPI is necessary for more effective recognition by $a\beta_2GPI$, the whole complex being a powerful platelet activator [27]. The interaction of β_2GPI with PF4 induces β_2GPI dimers in a completely natural way and facilitates antibody binding and platelet activation, which itself is important for enhanced activation of endothelium and fibrinogen in a mouse thrombosis model. Plasma levels of platelet-derived chemokines such as PF4, PF4var (PF4 variant, also known as CXCL4L1), CXCL7, and CCL5 are elevated in patients with APS but not in patients with systemic lupus erythematosus (SLE), coronary artery disease (CAD), or healthy controls [28], indicating marked platelet activation in APS patients. These data support the notions that platelet activation in APS is induced by the complex $(PF4)_4/(\beta_2GPI)_2/a\beta_2GPI$.

Other Proteins

A recent study suggests that true anticardiolipin antibodies (those that recognize cardiolipin without the support of a plasma protein) may also induce a prothrombotic state in mice [28]. It is difficult to prove that these antibodies function in the absence of a natural protein because, in in vivo models, many candidate molecules are obligatorily present. Anticardiolipin antibodies, as measured with currently available assays, correlate weakly with thrombosis compared to lupus anticoagulant (LA); moreover, syphilis and leprosy patients have these autoantibodies without a clear increased risk of thrombosis. Cardiolipin is likely too small to elicit an immune response on its own without a carrier protein.

Autoantibodies to a number of other coagulation-relevant proteins, such as protein S, protein C, tissue factor pathway inhibitor, factor X, XI and XII, are found in a small subgroup of patients with APS [29]. Some correlate with clinical manifestations, and mechanisms regarding how these autoantibodies might induce a prothrombotic state have been proposed, but none of the autoantibodies have been tested in in vivo models. There is no convincing evidence that they play a role in thrombosis or pregnancy complications in APS.

Why Do Plasma Proteins Become Prothrombotic in the Presence of Autoantibodies?

Based on animal models of APS, three natural proteins, β_2GPI , prothrombin, and annexin A2, are identified as important antigens in APS. Although inhibition of annexin A2 can inhibit fibrinolysis, the absence of plasminogen does not cause high risk for thrombosis in humans, suggesting a minor role of annexin A2.

Autoantibodies against prothrombin differ from autoantibodies against a prothrombin- phosphatidylserine (PS) complex. There are autoantibodies that recognize prothrombin only when it is bound to an anionic phospholipid, suggesting that prothrombin undergoes a conformational change when it is bound to PS, exposing a cryptic epitope. Based on human studies, antibodies directed against this cryptic epitope correlates better with thrombosis than do antibodies against the rest of the prothrombin molecule. Further analysis of the specific antigenic epitope will help us understand how these autoantibodies might be prothrombotic.

It is not immediately clear why antibodies against β_2 GPI or prothrombin could induce thrombosis. Inhibition of prothrombin would result in bleeding, and no physiological function has been described for β_2 GPI to explain its role in a prothrombotic risk. To become prothrombotic, the autoantibodies should induce a new property in their target proteins. Here are a few possible mechanisms: (a) increased affinity due to dimerization by antibodies [27], (b) conformational changes and expression of a hidden epitopes [30], and (c) reshuffling of disulphide bridges within proteins [31].

Conformational Changes of β_2 -Glycoprotein I

The first demonstration that a plasma cofactor was required for aPL to bind cardiolipin was made by McNeil et al. in 1989 [32]. In the following year, this cofactor was identified as β_2 GPI by peptide sequencing which itself was later identified as the major autoantigen for aPL [33, 34]. β_2 GPI consists of 326 amino acid residues organized in five CCP (complement control protein) domains [35] (DI-DV), which function as protein-protein interaction modules in many proteins. DI-DIV have evolutionary conserved sequences; DV contains a six-residue insertion, a 19-residue C-terminal extension and an additional disulphide bond that includes a C-terminal cysteine residue. DV also harbours a large, positively charged patch that determines affinity for anionic phospholipids. The crystal structure of β_2 GPI, solved in 1999, [36, 37] suggests a stretched arrangement of the DI-IV, with DV lying at a right angle to the other domains, in the shape of a J. The phospholipid-binding site is located at the bottom of DV and consists of 14 charged amino acid residues and a flexible and hydrophobic loop. This crystal structure predicts that, when β_2 GPI is bound to a lipid layer, DI to IV point away from the lipid layer and that the potential binding site for a β_2 GPI autoantibodies in DI is fully exposed [38].

There are no circulating nor tissue deposition of β_2 GPI-antibody immune complexes in patients with APS. A logic interpretation of this observation is that the antibodies directed against β_2 GPI do not recognize β_2 GPI in the circulation. The antibodies recognize a cryptic epitope in the molecule. It has been shown that β_2 GPI expose the autoantibody binding site when it binds to anionic phospholipids [39].

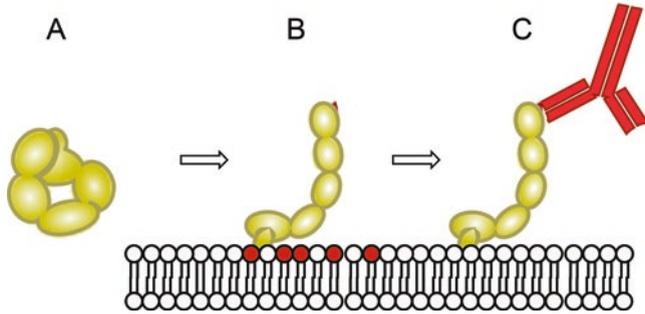


Fig. 2.1 Conformational change within β_2 GPI. (A) β_2 GPI as it circulates in plasma. (B) Binding to negatively charged phospholipids opens up β_2 GPI. (C) Binding of autoantibodies stabilizes β_2 GPI in its stretched conformation

Electron microscopy studies show that β_2 GPI exists in two different conformations (Fig. 2.1). In plasma, it is present as a circular protein in which DI interacts with DV. On binding to anionic surfaces, the protein opens up and expresses the #hockey-stick conformation of the crystal structure [40]. The circular conformation predicts shielded epitopes within DI and DV [40], and, indeed, autoantibodies against DI recognize β_2 GPI only when it is bound to anionic surfaces, not when it is present in the circulation. Since β_2 GPI binds to cell receptors via its DV, and since binding is enhanced by autoantibodies, it must be mediated by a cryptic epitope in DV that is expressed after the molecule has opened.

Small-angle X-ray scattering (SAXS) experiments suggested that in solution, β_2 GPI adopts an S-shaped conformation with an additional buckle between DII and DIII [41]. Additional SAXS experiments show that β_2 GPI adopts different conformations, depending on pH, ionic strength, and certain cations. β_2 GPI turns out to be a flexible molecule, not constrained to a single, specific conformation; its conformation depends on interactions with its surroundings. Apparently β_2 GPI can adapt a number of different structural conformations that in vitro can coexist in a dynamic equilibrium. Factor H, a complement factor built up of 20 CCP domains, also adopts different domain orientations in solution with consequences for its functional activity [42, 43]. Proteins consisting of CCP domains vary their conformations, depending not only on the length and flexibility of the linker sequences between domains but also, predominantly, on interactions with their surroundings.

Redox Balance

Antiphospholipid syndrome is characterized by oxidative stress and systemic inflammation [44, 45]. The overproduction of reactive oxygen species (ROS) results in an oxidative microenvironment that exacerbates inflammation, inducing cell death and tissue damage, compromising antioxidant defence mechanisms [46]. Patients with APS have high levels of circulating pro-inflammatory cytokines interleukin-2 (IL-2), interleukin-6 (IL-6), and tumour necrosis factor (TNF), together with markers of oxidative stress and inflammation such as serum amyloid A (SAA), C-reactive protein (CRP), 8-isoprostane, and prostaglandin E2 (PGE2) [47, 48].

In vivo and under normal physiological conditions, β_2 GPI is produced in the liver and exists predominately in circulation in its free thiol form, which is less immunogenic than the oxidized form (Fig. 2.2). The precise role of β_2 GPI and its different forms are complex [49]; it is thought to act as a natural anticoagulant that mediates a range of functions including the clearance of liposomes, apoptotic bodies and microparticles [49–52].

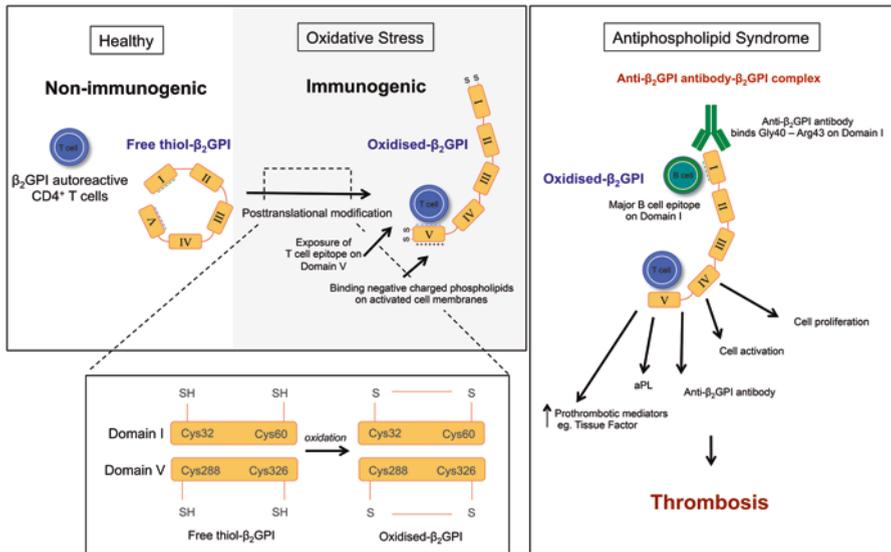


Fig. 2.2 How does oxidized β_2 GPI participate in the formation of thrombotic APS? During oxidative stress, free thiol β_2 GPI can undergo post-translational modification to form the immunogenic form, oxidized β_2 GPI after binding phospholipids. β_2 GPI autoreactive CD4⁺ T cells recognize newly exposed epitopes located on Domain V but not on free thiol β_2 GPI. A complex is formed between a β_2 GPI, autoreactive CD4⁺ T cells and oxidized β_2 GPI triggering the production of aPL, specifically a β_2 GPI, cell proliferation and the release of pro-inflammatory cytokines which are key events in the pathophysiology of thrombotic APS

Quantitation of Oxidized β_2 GPI as a Biomarker for Antiphospholipid Syndrome

Oxidized β_2 GPI level in APS has been proposed as a biomarker of thrombotic risk for APS. Levels of oxidized β_2 GPI in patients with APS are higher than in healthy subjects and patients with other autoimmune disease or non-aPL disease controls with thrombosis [53]. Free thiol β_2 GPI may play a protective role in APS, since free thiol β_2 GPI protects human umbilical vein endothelial cells (HUVEC) against hydrogen peroxide-induced cell injury [54]. Decreased plasma free thiol β_2 GPI may thus lower the physiological buffer against oxidative stress-induced injury. Free thiol β_2 GPI also protects human retinal pigment epithelium and the subretinal endothelial cell against oxidative, hydrogen peroxide stress-induced, cell death [55].

A multicentre, cross-sectional, international study using prospectively acquired samples has demonstrated that the redox status of β_2 GPI differs between healthy individuals and patients with thrombotic APS [53]. In the former it exists predominately with free thiols; APS patients have higher levels of total and oxidized β_2 GPI compared to both healthy subjects and patients with other autoimmune disease [53, 56].

Diagnostic and Prognostic Implications of the Oxidized β_2 GPI ELISA

Anticardiolipin antibodies, $a\beta_2$ GPI, and LA test serve as diagnostic markers in APS. The predominant isotypes of aPL in APS patients are IgG aCL and IgG $a\beta_2$ GPI [57, 58]. Although, non-criteria or non-classical aPL such as antiphosphatidylserine, antiphosphatidylethanolamine, and antiphosphatidylglycerol have been reported, only the three classical aPL tests are used in diagnosis of APS [59]. The LA assay identifies autoantibodies against either prothrombin and/or β_2 GPI, whereas the aCL assay detects the aCL and/or $a\beta_2$ GPI antibodies. The $a\beta_2$ GPI assay detects only antibodies against β_2 GPI. Concomitant triple positivity for aCL, β_2 GPI and LA may indicate severe APS and high recurrence risk [60], a point that is still controversial [61]. Lupus anticoagulant correlates much better with the clinical manifestations of APS than the detection of the autoantibodies with an ELISA [62, 63], and a positive LA assay due to $a\beta_2$ GPI has a stronger correlation for thrombotic risk than due to antiprothrombin autoantibodies [64].

In a clinical setting, it is important to stratify risk for development of clinical events in APS and in asymptomatic, aPL-positive individuals. Delayed or inadequate treatment can result in damage and impaired quality of life [65, 66]. Although β_2 GPI levels are not routinely measured in patients with APS, considering the specificity of high levels of oxidized β_2 GPI, measuring their levels may assist in diagnosis and prognosis. The level of oxidized β_2 GPI is calculated by subtracting the concentration of free thiol from total β_2 GPI. Using an ELISA to measure post-translational redox modifications of β_2 GPI (including total and free thiol β_2 GPI) [53] (Fig. 2.3) and β_2 GPI

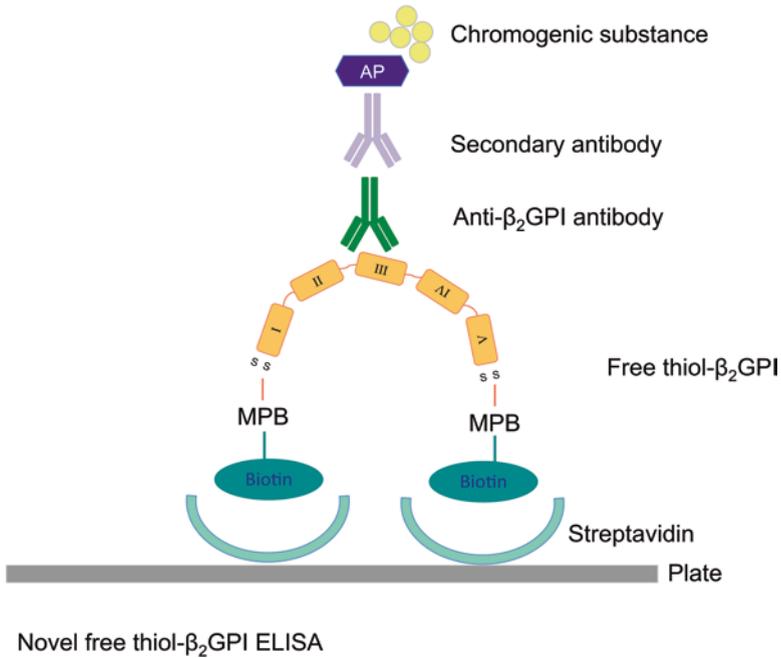


Fig. 2.3 Schema of enzyme-linked immunosorbent assay (*ELISA*) to measure free thiol β_2 glycoprotein I (β_2 GPI). Free thiol β_2 GPI bind streptavidin via biotin-conjugated maleimidyl-propionyl biocytin (*MPB*) and become immobilized. Acetone precipitation removes unbound *MPB*, and bound free thiol β_2 GPI is quantified with $\alpha\beta_2$ GPI and detected using a secondary antibody, alkaline phosphatase (*AP*) and chromogenic substance para-nitrophenylphosphate (PnPP)

plasma levels in 359 patients (identified through an international multicentre initiative) who had either APS or other autoimmune diseases or non-APS vascular thrombosis, Ioannou et al. found that the redox state of β_2 GPI and its concentration in APS patients had a profile distinct from that in the various control groups.

Group Conclusion

Evidence from both clinical and animal studies supports the concept that β_2 GPI is the main autoantigen in APS. Understanding of the pathophysiology of APS and the involvement of β_2 GPI and its post-translational modified forms remains incomplete; understanding the relevance of oxidized β_2 GPI in APS will be important. Although $\#aPL$ are a defining, hallmark feature of APS, their presence does not exclusively indicate APS nor do they stratify individuals for risk of thrombosis.

Current methods for diagnosing APS patients do not incorporate quantitation of total and free thiol β_2 GPI. Specific ELISAs for quantifying these parameters may enhance our diagnostic and prognostic capabilities. Prospective studies may validate

measurement of oxidatively modified forms of β_2 GPI as biomarkers. The AntiPhospholipid Syndrome Alliance For Clinical Trials and InternatiOnal Networking (APS ACTION), an international research network that collects patient samples from 25 centres around the world [67], may allow a longitudinal study that measures oxidized β_2 GPI.

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