Curr Rheumatol Rep (2011) 13:70–76
DOI 10.1007/s11926-010-0144-8

Autoantibodies Directed Against Domain I of Beta2-Glycoprotein I

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Published online: 3 November 2010

Abstract Patients diagnosed with the antiphospholipid syndrome typically suffer from vascular thrombosis, pregnancy morbidity, or a combination of the two. Due to the high prevalence of these clinical symptoms, the diagnosis of antiphospholipid syndrome is almost completely dependent on the detection of antiphospholipid antibodies in patient plasma. However, not every individual with antiphospholipid antibodies in his or her plasma suffers from thrombosis and/or pregnancy morbidity, which suggests the existence of different populations of antiphospholipid antibodies. Although many antigens have been identified in relation to the antiphospholipid syndrome, β2-glycoprotein I is regarded as clinically most significant. During the past decade, evidence has accumulated to suggest the presence of a dominant epitope on the first domain of β2-glycoprotein I. Several studies have detected a specific population of antibodies recognizing a cryptic epitope on domain I, at least comprising arginine 39 to arginine 43. In contrast to antibodies recognizing other domains of β2-glycoprotein I, anti-domain I antibodies are found to be highly associated with clinical symptoms. This review discusses several studies that have investigated a role for domain I within the antiphospholipid syndrome on a predominantly diagnostic level.

Keywords Antiphospholipid syndrome · β2-glycoprotein I · Domain I

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Introduction

Many autoimmune diseases share clinical symptoms, which makes it hard to distinguish them from one another based solely on clinical manifestations. In those cases, the diagnosis depends heavily on other diagnostic criteria (eg, the detection of the presence of antibodies against self-proteins). This is especially important for diagnosing a patient with the antiphospholipid syndrome [1]. A diagnosis of the antiphospholipid syndrome is made on the basis of a history of vascular thrombosis and/or pregnancy morbidity in combination with the detection of antiphospholipid antibodies as described in the official guidelines of the International Society of Thrombosis and Haemostasis [2]. Although the diagnosis is made clinically on the basis of both thrombosis and pregnancy morbidity, many other clinical symptoms have been described as being associated with the antiphospholipid syndrome, but they are not part of the criteria that define the disease. Due to the high prevalence of thrombosis and pregnancy morbidity in the general population, a heavy burden rests on the specificity of the assays to detect the presence of antiphospholipid antibodies.

Three assays that detect antiphospholipid antibodies are included in the serologic criteria for the antiphospholipid syndrome: prolongation of phospholipid-dependent coagu-

lation assays, also known as *lupus anticoagulant*; anti-cardiolipin enzyme-linked immunosorbent assay (ELISA) to detect antiphospholipid antibodies binding to the complex cardiolipin-β2-glycoprotein I (β2GPI); and anti-β2GPI ELISA to detect antibodies that recognize β2GPI [2]. Several studies have shown that the results of all three assays are very sensitive for external factors, making them extremely difficult to standardize [3]. Of these assays, the one that detects lupus anticoagulant is regarded as best correlated with thrombosis, but the proper detection of lupus anticoagulant depends heavily on the proper processing of the blood and the quality of the plasma [4]. The anticardiolipin ELISA is less sensitive to differences in handling of the blood, as it does not depend on the functionality of the antibodies (prolonging coagulation assays) but rather simply on the binding of antibodies to the cardiolipin-β2GPI complex coated to an ELISA plate. The downside of this assay is the large variability between the results obtained with assays from different manufacturers and the relatively large number of false-positive patients, which is probably due to direct binding to cardiolipin rather than to the complex cardiolipin-β2GPI [5].

Therefore, the anti-β2GPI ELISA seems to be the best choice. It is less sensitive to differences in processing of the blood compared with lupus anticoagulant, as it measures the binding of antibodies to β2GPI directly to the plate and not functional activity [6]. There is no need for cardiolipin, thereby eradicating aspecific binding of antibodies directly to cardiolipin (which are thought not to be associated with the antiphospholipid syndrome). Although it seems promising, the assay is far from perfect. Several problems with the anti-β2GPI ELISA need to be resolved to reduce false positivity, interassay variability, and reproducibility [6]. In this review, we discuss an important cause of these problems: the heterogeneity of the anti-β2GPI antibodies. We advocate that a specific subpopulation of these anti-β2GPI antibodies directed toward domain I are the important antibodies to measure.

Specificity of Antiphospholipid Antibodies

Many antigens have been proposed to be involved in binding antiphospholipid antibodies, including β2GPI, prothrombin, annexin A5, protein S, protein C, factor XI, and factor XII [7, 8]. β2GPI is generally regarded as the most important antigen within the antiphospholipid syndrome [9]. Several groups have studied the fine specificity of anti-β2GPI antibodies, and every domain of β2GPI has been described to bind antibodies [10]. From an immunologic point of view, it is difficult to imagine a self-protein containing many immunodominant epitopes. Many studies have been initiated to identify this epitope, and most evidence points to the first domain of β2GPI, also known as *domain I*, as the main epitope. Iverson et al. [11] were among the first to show that most anti-β2GPI antibodies reacted with domain I by using domain-deletion mutants of the protein. They continued their research by making point mutations within domain I of β2GPI. Interestingly, they found that most anti-β2GPI antibodies lost their reactivity to domain I when glycine 40 or arginine 43 (which together form a positive-charged epitope) was mutated [12]. This led to the assumption that charge was involved in the interaction between antibody and antigen. In 2005, we also investigated the specificity of anti-β2GPI antibodies and their relation to clinical significance [13]. At first, we could not detect any binding of anti-β2GPI to domain I when domain I was directly coated to the plate. Studying in detail the biochemistry of domain I, we hypothesized that domain I was coated onto the negatively hydrophilic ELISA plate, with its positively charged epitope arginine 39-arginine 43 downward. Therefore, we tested the reactivity of anti-β2GPI antibodies toward domain I when coated onto a neutral hydrophobic plate. After changing ELISA plates, we were able to detect anti-domain I antibodies. Ioannou et al. [14] showed that not only is arginine 39-glycine 43 important for the binding of antibodies, but the epitope comprises a much larger region on both domain I and II. They proposed that the epitope is built up out of epitope arginine 39-arginine 43, aspartic acid 8-aspartic acid 9, and the interlinker region between domain I and II [14].

Binding of Anti-domain I Antibodies to β2GPI Is Conformation Dependent

Although most evidence is directed toward domain I of β2GPI, the question remains as to why the epitope arginine 39-glycine 43 of domain I is immunodominant. Several theories have been published describing the induction of autoantibodies, two of which have been extensively investigated with respect to the antiphospholipid syndrome: molecular mimicry and cryptic epitope exposure.

Molecular mimicry is the possibility that sequence similarities exist between a foreign protein/peptide and a self-protein/peptide. The presence of the foreign protein/peptide will result in activation of autoreactive T and B cells [15]. This can result in a loss of immunologic tolerance toward self-proteins, thereby inducing autoimmunity. Several groups have shown that an infection such as cytomegalovirus or rubella precedes the diagnosis of the antiphospholipid syndrome [16, 17]. In addition, it was shown that some viruses and bacteria share amino acid sequences and that peptides derived from viruses induced

antibodies with an affinity for β2GPI [18]. However, based on the results of site-directed mutagenesis, it is now believed that the epitope on domain I of β2GPI is not linear, but rather three dimensional. This does not mean that molecular mimicry cannot be involved in the induction of antiphospholipid antibodies, but that amino acid homology between foreign invaders and β2GPI cannot be automatically related to the induction of antiphospholipid antibodies.

The conformation of β2GPI has been and still is of major interest to many groups, and it is thought that the conformation of β2GPI has consequences not only for the binding of antiphospholipid antibodies but also for its physiologic function in the human body [19]. Two groups almost simultaneously published the crystal structure of β2GPI [20, 21]. Both studies displayed β2GPI as a fishhook shape and indicated that domain V was responsible for binding to a phospholipid surface, that domain I was erected from the phospholipid surface into the solution. In the crystal structure, epitope arginine 39-glycine 43 is completely exposed and therefore available for antibodies to react with it. Although some groups have shown fluid-phase binding of anti-β2GPI antibodies to its antigen, no research group has been able to isolate β2GPI-antibody complexes from patients, indicating that epitope arginine 39-glycine 43 is unavailable to react with antiphospholipid antibodies in the fluid phase [22]. That fluid-phase binding has been shown might be because the β2GPI used in these studies was of a different conformation than in plasma. We hypothesized that the epitope on domain I is cryptic and becomes exposed after interaction of domain V with a phospholipid surface. Indeed, when studying the structure in solution in detail by applying small x-ray scattering, β2GPI showed an S-shaped conformation, with a carbohydrate chain positioned on top of domain I covering epitope arginine 39-arginine 43 [23]. This led to the hypothesis that the structure solved by crystallization was β2GPI in its phospholipid-binding conformation, and the structure solved by small-angle x-ray structure was the conformation as present in plasma. Binding of β2GPI to a phospholipid surface would induce a conformational change in β2GPI from an S-shaped conformation to a J-shaped conformation. When we removed the carbohydrate chains from β2GPI, antiphospholipid antibodies with reactivity toward epitope arginine 39-arginine 43 were able to bind to β2GPI in solution, which is in contrast to fully intact β2GPI [19].

Another recently published study described a different conformation of β2GPI by making use of electronic microscopy [24••]. Although the authors also found that β2GPI was folded into a J shape when bound to phospholipids, plasma-purified β2GPI appeared to have a circular conformation in a phospholipid-free environment. This was in contrast to the S shape described by Hammel et al. [23]. However, as in the S-shape conformation, epitope arginine 39-arginine 43 was also shown to be covered, preventing antibodies from binding β2GPI in solution. In this circular conformation, it was shown that domain V of β2GPI was positioned on top of the interface of domain I and II. Affinity of domain V for domains I and II looks difficult, as both domain V and the interface of domains I and II are positively charged. External factors may play a role in keeping β2GPI in this circular conformation. However, Hammel et al. [23] showed an intermediate conformation of β2GPI between the circular conformation and the J shape. One might hypothesize that the carbohydrate, which is negatively charged, is positioned on domains I and II, reversing the charge of this part of the molecule and making it favorable for domain V to be positioned on top of the interface of domains I and II. Binding of β2GPI to negatively charged phospholipids would push the carbohydrate chain away from domain V, resulting in the J shape, with the S shape as a possible intermediate (Fig. 1).

Based on these studies, it can be assumed that β2GPI can adapt to different conformations and that the conformation of β2GPI determines whether or not antibodies against domain I can bind. In addition, β2GPI, as in the anti-β2GPI assays, should adsorb in the right conformation on the plate. Differences in conformation of β2GPI preparations due to different purification methods or coating procedures might be a factor in the relatively large variability between assays of different manufacturers [25•].

Association Between Anti-domain I Antibodies and Clinical Symptoms

As shown by several groups, anti-β2GPI antibodies are associated with thrombosis and to a lesser extent with pregnancy morbidity. The detection of anti-β2GPI antibodies was recently included in the official criteria for diagnosing a patient with the antiphospholipid syndrome [2]. Still, a significant number of individuals who tested positive for these antibodies never developed thrombosis or pregnancy morbidity. Iverson et al. [11] showed that a certain subpopulation of anti-β2GPI antibodies reacted with domain I. We hypothesized that only a specific population of anti-β2GPI antibodies was associated with thrombosis. Therefore, we expanded the study by Iverson et al. [11] by including the clinical significance. We conducted a single-center study of 198 patients with underlying autoimmune diseases [13]. We found that about half of the patients with anti-β2GPI antibodies showed

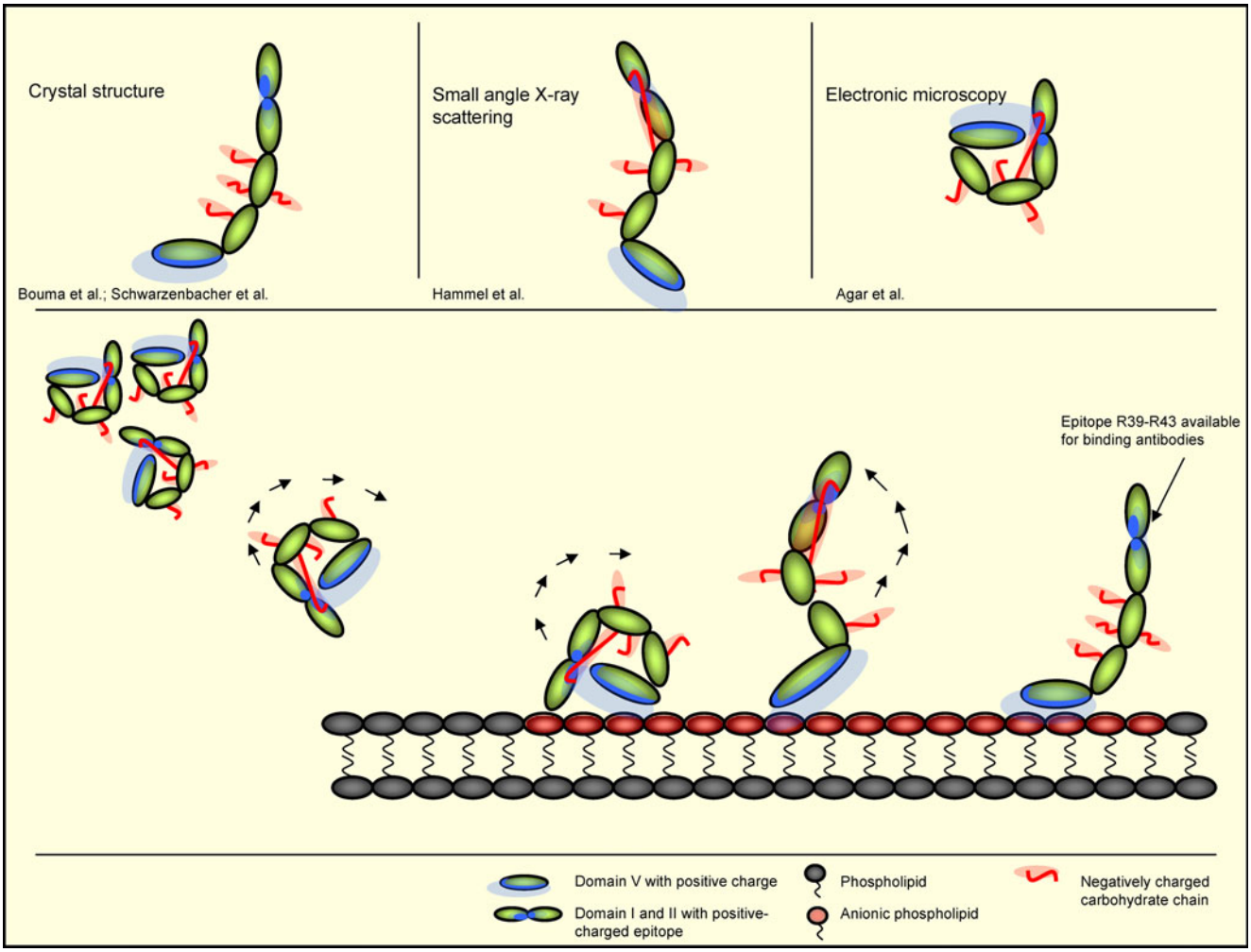

Fig. 1 Model for conformational change in different published β2-glycoprotein I (β2GPI)-based structures. The crystal structure of β2GPI was first found and showed β2GPI in a fishhook-like shape [20, 21]. Interestingly, using small x-ray scattering, β2GPI was found to be in an S-shape conformation, with a carbohydrate chain on top of the interface between domains I and II [22]. Agar et al. [24••] recently found a circular shape of β2GPI in the absence of anionic phospholipids when applying electron microscopy. This circular conformation could be transformed into a fishhook-like shape by adding anionic phospholipids to the β2GPI preparation. It can be hypothesized that all three conformations exist in the human body. Based on this hypothesis, a model can be designed in which β2GPI exists in a circular conformation in solution. Although domain V and domains I and II have a predominantly positively charged surface, they can interact due to the fact that a negative carbohydrate chain lies in between and serves as a sort of glue. Upon binding to phospholipids, β2GPI transitions from a circular conformation into an S-shaped conformation. This conformation is based on the fact that both the surface (anionic phospholipids) and the carbohydrate on top of domains I and II are negatively charged, thereby causing domains I and II to dissociate from domain V, which has a higher affinity for phospholipids. Subsequently, the whole molecule erects, making epitope arginine 39-arginine 43 available to react with anti-domain I antibodies

reactivity toward domain I. In addition, the presence of anti-domain I antibodies was better associated with (predominantly venous) thrombosis (OR, 18.9; 95% CI, 6.8–53.2), compared with anti-β2GPI antibodies with reactivity toward other domains (OR, 1.1; 95% CI, 0.4–2.8). To confirm this result, we conducted a multicenter study including only patients with anti-β2GPI antibodies. As in the previous study, we found that anti-β2GPI antibodies with reactivity toward domain I were better correlated with thrombosis as compared with antibodies recognizing other domains of β2GPI. Interestingly, for anti-domain I antibodies, we also found a better association with pregnancy morbidity in the multicenter study (OR, 2.4; 95% CI, 1.4–4.3).

Domain I as Clinical Drug

Treatment of the antiphospholipid syndrome is complicated and simple at the same time. The simplicity lies in the fact that there is only one proven method to treat patients suffering from antiphospholipid syndrome-related thrombosis: anticoagulation [26]. The difficulty is in the period

and the level of anticoagulation. There is no guideline for or direct evidence of any time point for discontinuation of treatment, and rethrombosis can occur at any time, but especially during the first 6 months after discontinuation. In addition, patients might still suffer from thrombosis despite treatment, which would indicate deeper anticoagulation. However, no evidence is available to justify high-intensity treatment and is merely based on eminence [27]. Furthermore, anticoagulation has many side effects that worsen with increased intensity of treatment.

Therefore, the idea came up not to treat thrombosis itself but to stay one step ahead and prevent the formation or the actions of the antibodies. Nearly a decade ago, Jones et al. [28] published a method for eradicating anti-domain I antibodies from the circulation. A tetramer of domain I was constructed with an ethylene glycol-based linker named LJP 993. Multivalent presentation of antigens in the absence of T-cell epitopes has been described to tolerize autoreactive B cells, meaning that a tetramer of domain I could silence an anti-domain I antibody-producing B cell. The same company applied this method to reduce anti-double-stranded DNA antibodies to treat lupus nephritis [29]. Despite big hopes, none of these products are on the market now, all for different reasons.

Despite silencing anti-domain I-producing B cells, domain I could also be used to capture and neutralize antiphospholipid antibodies. Ioannou et al. [30••] recently conducted a study in which they investigated this method of treatment. Mice were injected with IgG purified from patients diagnosed with the antiphospholipid syndrome or from healthy controls. In addition, domain I containing a mutation (D8S/D9G), thereby enhancing fluid-phase binding of antibodies, was injected into the mice. After standardized vessel injury, mice injected with antiphospholipid-related IgG displayed increased thrombus size, which could be inhibited by the domain I mutant.

One of the hesitations when conducting studies in which the antigen itself is injected is the possibility of further activating the immune system, resulting in increased antibody levels. It has been shown that one of the T-cell epitopes is present on domain V, possibly decreasing the risk of potentiating the disease, but it is not known whether domain I itself contains a T-cell epitope [31]. We recently showed at the Biannual Congress of the International Society of Thrombosis and Haemostasis that injecting mice with domain I in the absence of Freund's adjuvant does result in anti-domain I antibodies [32••]. Therefore, one should be extremely cautious in extrapolating these data to treatment options in clinical practice.

Conclusions

The presence of antibodies toward β2GPI does not automatically mean that an individual will suffer from antiphospholipid syndrome-related symptoms. The assay to detect the antibodies has many pitfalls; thus, we do not know when we should consider a result positive. We know now that there are different subpopulations of antibodies that recognize β2GPI. Together with the high prevalence of thrombosis and pregnancy morbidity, it is difficult to make a diagnosis of antiphospholipid syndrome for certain. In this respect, the detection of anti-domain I antibodies could be an addition to the current serologic criteria, as it has a

Table 1 Differences between the anti-domain I assay and the anti-β2GPI ELISA

	Anti-domain I assay	Anti-β2GPI ELISA
Conformation of β2GPI	Conformation independent, as only domain I is coated	Binding of antibodies to β2GPI depends on whether epitope R39–R43 is exposed
Charge of the ELISA plate	A neutral plate is needed to prevent epitope R39–R43 from being coated downward to the plate	A negative plate is needed, which is thought to induce a conformational change and enables 1 antibody to bind 2 molecules
Specificity and sensitivity	High specificity, but it is not known whether other pathogenic antibody populations are missed	High sensitivity, but a mediocre specificity, as a significant number of positive patients do not develop APS-related clinical symptoms
Source of protein	Domain I is produced and thereby recombinant	β2GPI can be purified from human or bovine plasma, or produced recombinant. It is not known whether there are differences in conformation between the different sources
Purification	Domain I contains a his-tag and is purified via nickel-Sepharose[a]	β2GPI can be purified by applying different techniques; the influence of the different techniques on the conformation of β2GPI is not known

APS antiphospholipid syndrome, *ELISA* enzyme-linked immunosorbent assay, *GPI* glycoprotein I

[a] Sigma-Aldrich (St. Louis, MO)

much higher specificity for the clinical manifestations than the standard anti-β2GPI ELISA [13].

As mentioned previously, domain I may also be of value in standardizing the anti-β2GPI ELISA. Many attempts have been made to standardize the anti-β2GPI ELISA without success [5, 33, 34]. One of the reasons may be that binding of anti-domain I antibodies is dependent on the conformation of β2GPI. Therefore, the preparation and coating of β2GPI to ELISA trays is of major importance to the results of the assay. In addition, β2GPI needs to be coated onto a negatively charged plate in order to unfold. These problems can be overcome when using domain I as a coating, as it does not need a conformational change (Table 1). Therefore, it is less sensitive for in vitro artifacts.

It is too soon to replace the anti-β2GPI ELISA with the anti-domain I assay, as there is still the possibility that other populations of thrombosis-related antibodies are present. Moreover, the results obtained should be confirmed in larger cohorts. In fact, some authors suggest that in addition to domain I, domain IV is also a candidate for binding thrombosis-related antibodies [35]. It is possible that certain symptoms are associated with certain subpopulations of antibodies. We have shown that anti-domain I antibodies highly associate with predominantly venous thrombosis. However, it is hard to believe that this population of antibodies causes all the thrombotic complications observed in patients diagnosed with the antiphospholipid syndrome. It is tempting to further investigate whether one population of antibodies can explain all the clinical manifestations or whether different subpopulations are responsible for the various events present in the antiphospholipid syndrome [36].

Acknowledgments Dr. de Laat is a postdoctoral research fellow of the Netherlands Heart Foundation (2006T053) and has received grant support from the Netherlands Heart Foundation and the Netherlands Thrombosis Foundation.

Dr. de Groot has received grant support from the Rheuma Foundation.

Disclosure Sanquin, with which Dr. de Laat is affiliated, is the owner of a patent regarding diagnostic and therapeutic purposes of domain I. Dr. de Groot reported no potential conflicts of interest relevant to this article.

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- • Of importance
- •• Of major importance

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