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Immune Responses Against Domain I of β_2 -Glycoprotein I Are Driven by Conformational Changes

Domain I of β_2 -Glycoprotein I Harbors a Cryptic Immunogenic Epitope

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Objective. The presence of autoantibodies against a cryptic epitope in domain I of β_2 -glycoprotein I (β_2 GPI) is strongly associated with thrombotic events in patients with the antiphospholipid syndrome. We hypothesized that a conformational change could be a trigger for the formation of antibodies against domain I of β_2 GPI. Therefore, we investigated whether immune responses against β_2 GPI are related to its conformation.

Methods. Conformational changes in β_2 GPI were studied using various techniques, either upon binding to cardiolipin or after disruption of the internal disulfide bonds. The immunogenicity of β_2 GPI in different conformations as well as the individual domains of β_2 GPI were studied in vivo by monitoring the generation of antibodies after intravenous administration of β_2 GPI to mice. Furthermore, plasma samples from these mice were assessed for lupus anticoagulant activity and thrombin–antithrombin complex levels.

Results. We observed that the interaction of β_2 GPI with cardiolipin induced a conformational change in β_2 GPI: electron microscopy revealed that β_2 GPI assembled into polymeric meshworks. We next investigated the immunogenicity of both human and murine β_2 GPI in mice. Both human and murine β_2 GPI combined with cardiolipin and misfolded β_2 GPI triggered antibody formation against the native protein as well as against domain I of β_2 GPI, while native β_2 GPI was not immunogenic. In addition, we observed that anti–domain I antibodies developed in mice injected with domain I of β_2 GPI, and that antibodies did not develop in mice injected with domains II–V. The induced anti–domain I antibodies prolonged the dilute Russell’s viper venom plasma clotting time. The plasma of mice with anti–domain I antibodies had increased levels of circulating thrombin–antithrombin complexes.

Conclusion. The results of our studies indicate that the exposure of cryptic epitopes due to conformational changes in β_2 GPI can induce autoantibody formation.

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The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease characterized by thrombosis that can occur in the arteries and veins and/or in the setting of recurrent pregnancy loss (1). The diagnosis of APS depends on both clinical features and laboratory parameters. Laboratory criteria include the persistent presence in plasma of circulating autoantibodies (anti- β_2 -glycoprotein I [anti- β_2 GPI] and anticardiolipin, as measured using standardized enzyme-linked immunosorbent assays [ELISAs]) and a phospholipid-dependent prolongation of the clotting time in plasma, as determined by the detection of lupus anticoagulant (LAC) (2). Currently, the treatment of APS is based on

oral anticoagulation, because there is no proper way to neutralize or eliminate the pathologic autoantibodies that are responsible for the syndrome.

Although the name "antiphospholipid syndrome" suggests that the disease is caused by antibodies directed against phospholipids, it has become clear that immune responses against phospholipid-binding proteins are essential for the development of APS-related thrombotic pathology (3). Many autoantigens have been described in APS, but antibodies against β_2 GPI correlate best with the incidence of thrombosis (4). β_2 GPI is a 44-kD plasma protein with a plasma concentration of $\sim 200 \mu\text{g/ml}$.

In patients with APS, antibodies against a number of epitopes within β_2 GPI develop, but predominantly antibodies directed against a single epitope in domain I of β_2 GPI are associated with a strong risk of developing thrombosis (5). This epitope is situated in a region around a glycine residue on position 40 (G40) and an arginine residue on position 43 (R43) within the first domain of β_2 GPI. The pathologic antibodies can bind to this epitope only when β_2 GPI is immobilized on a negatively charged surface (e.g., a microtiter plate), when the carbohydrate side chains are removed, or when the protein is crosslinked, but also when β_2 GPI binds to negatively charged phospholipid vesicles (6). Apparently, a conformational change leads to exposure of a cryptic epitope in domain I. The binding of antibodies to this epitope enhances the affinity of β_2 GPI for negatively charged phospholipids, which results in LAC activity *in vitro* and thrombosis *in vivo* (5).

At present, not much is known about the mechanisms that cause the generation of autoantibodies against β_2 GPI. Immunization studies in mice have been helpful by showing that human β_2 GPI, when incubated with phospholipids, becomes immunogenic (7). Similarly, β_2 GPI becomes immunogenic in combination with apoptotic cells (8,9). Earlier work from our group identified protein misfolding as a risk factor for immune responses against biopharmaceuticals (10). In those studies, conformational changes in a therapeutic protein led to increased immunogenicity and tolerance-breaking in a transgenic mouse model. In a similar manner, we hypothesized that the immune responses against β_2 GPI are the consequence of a conformational change resulting in the induction of autoantibodies against β_2 GPI recognizing the cryptic domain G40–R43 on domain I.

MATERIALS AND METHODS

β_2 -glycoprotein I purification. β_2 GPI was purified from fresh plasma as described previously, with minor modifi-

cations (11). Briefly, plasma was added to a DEAE-Sephadex column. The flow-through was collected, pooled, and added to a protein G-sepharose column to eliminate IgG content. Then, the effluent pool was added to a MonoS Sepharose column. The bound β_2 GPI was eluted by a linear salt gradient (138–550 mM NaCl) and added to a heparin-sepharose column to increase the purity and concentration. The eluted fractions were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining and contained a single 44-kD protein band. The identity of the band was confirmed as β_2 GPI by ELISA, using an in-house-produced monoclonal antibody against β_2 GPI, as well as by Western blot analysis. The protein preparation was $>98\%$ pure. Additionally, the amount of proteolytically cleaved β_2 GPI was 1–2% of the total protein, as detected by a previously described ELISA using an in-house-produced anti-body specific for cleaved β_2 GPI (clone 13A10) (12).

Structural modifications of β_2 GPI. β_2 GPI was misfolded by reduction in 4M urea and 10 mM dithiothreitol in an atmosphere of N_2 gas for 3 hours. Subsequently, alkylation with 0.5 mM iodoacetamide was used to prevent the free cysteine residues from reforming disulfide bridges. The mixture was dialyzed for 2 hours in H_2O and overnight in phosphate buffered saline (PBS), pH 7.2, at 4°C . This method induces controlled, irreversible misfolding of proteins (13), including β_2 GPI (14), leading to a uniform species of β_2 GPI that is stable (i.e., not degraded or aggregated/precipitated) as determined by gel electrophoresis and silver staining (data not shown).

Cardiolipin vesicles were prepared according to the method described by Pengo et al (15). Briefly, multilamellar cardiolipin dissolved in ethanol was transferred to a glass tube. The ethanol was evaporated with N_2 , resuspended in Tris buffered saline (TBS; 50 mM Tris, 100 mM NaCl, pH 7.4) and vortexed thoroughly to obtain vesicles. Subsequently, β_2 GPI and cardiolipin were diluted in TBS to 300 $\mu\text{g/ml}$ and 50 μM , respectively, mixed at a 1:1 ratio, and allowed to incubate for 10 minutes prior to performance of the experiments.

Recombinant protein production. DNA encoding full-length murine β_2 GPI (and murine domain I) was obtained from NCBI and cloned into pcDNA3.1+ (Invitrogen) after introducing a C-terminal His tag. β_2 GPI with carbohydrate chains was transiently expressed in 293 cells, using the Free-Style 293 Expression System (Invitrogen) according to the manufacturer's instructions. His-tagged murine domain I was produced using a BD BaculoGold Baculovirus Expression Vector System (BD Biosciences), as previously described (16). Purification of murine β_2 GPI was performed by using a 1-ml Talon column (Clontech) according to the manufacturer's instructions. Protein fractions were checked for purity by SDS-PAGE followed by silver staining and contained a single 44-kD protein band. Domain deletion mutants of human β_2 GPI (DI and DII–V) were kindly provided by Dr. M. Iverson (La Jolla Pharmaceutical) (16).

Thioflavin T (ThT) fluorescence. Fluorescence of the amyloidophylic dye ThT (Sigma) was measured on a Hitachi F-4500 spectrophotometer at an excitation wavelength of 435 nm and emission wavelength of 485 nm. All samples were prepared in TBS. Fluorescence was measured in triplicate with an integration time of 5 seconds per reading. The background fluorescence of both protein in buffer and the fluorescence

values of the dye alone were subtracted from the total fluorescence signal. Heat-denatured ovalbumin (100 $\mu\text{g/ml}$) was used as a positive control.

Tissue-type plasminogen activator (tPA) binding assay. The binding of tPA and an N-terminally truncated deletion mutant (reteplase) to $\beta_2\text{GPI}$ was determined as described previously (10). Immobilizer plates (Exiqon) were coated with 10 $\mu\text{g/ml}$ $\beta_2\text{GPI}$ in 100 mM NaHCO_3 , pH 9.6, for 1 hour and blocked with 1% (volume/volume) TBS–Tween 20 (TBST) for 1 hour. The wells were incubated with a concentration series of tPA in the presence of 10 mM ϵ -aminocaproic acid to prevent lysine-dependent interactions. Subsequently, tPA binding was detected by antibody 374b (American Diagnostica), followed by peroxidase-labeled rabbit anti-mouse immunoglobulin (RAMPO; DakoCytomation). Between incubations, the plates were washed repeatedly with TBST. Finally, the plates were stained with tetramethylbenzidine (TMB) substrate (BioSource Europe) according to the manufacturer's instructions. Plates were read on a SpectraMax 340 microplate reader at 450 nm. Binding curves were fitted with a 1-site binding model (GraphPad Prism version 4.02 for Windows).

Transmission electron microscopy (TEM). Formvar/carbon-coated 100-mesh copper grids were placed on top of 5- μl drops of $\beta_2\text{GPI}$ in TBS or TBS only for 5 minutes. The grids were washed by placing them on a 100- μl drop of PBS and 3 drops of H_2O , with incubation for 2 minutes at each step. The grids were stained with 2% (mass/volume) methylcellulose, 0.4% (m/v) uranyl acetate, pH 4.0, for 2 minutes. The grids were analyzed using a JEOL 1200EX transmission electron microscope, and photomicrographs (10,000 \times magnification) were obtained.

Immunizations. All animal experiments were performed in compliance with institutional guidelines and were approved by the institutional animal care and ethics committee. BALB/c mice (7–9 weeks old) (The Jackson Laboratory) were injected intravenously with 15 μg of $\beta_2\text{GPI}$ in TBS on days 1, 5, 22, and 26. On day 36, either serum or plasma was collected. For serum preparation, whole blood was drawn from the vena saphena and collected in tubes with Z Serum Clot Activator (BD Biosciences). Serum was isolated by centrifugation at 16,000 relative centrifugal force (RCF) for 15 minutes. For plasma collection, whole blood was obtained by cardiac puncture and was anticoagulated using 3.8% sodium citrate. Plasma was prepared from the supernatant of 2 consecutive centrifugation steps at 16,000 RCF for 15 minutes to remove cells and platelets.

Analyses in murine plasma and serum. *Detection of anti- $\beta_2\text{GPI}$ antibodies.* Serum samples were analyzed for antibodies against human/murine recombinant $\beta_2\text{GPI}$. Microtiter high-binding microtiter plates (Greiner) were coated with 5 $\mu\text{g/ml}$ $\beta_2\text{GPI}$ in 100 mM NaHCO_3 for 1 hour and blocked with Roche Blocking Reagent (200 $\mu\text{l/well}$) for 1 hour. Murine sera were serially diluted in TBST and incubated on the microtiter plates for 1 hour. Antibody binding was detected by incubation of the plates with RAMPO in TBST. The plates were washed repeatedly with TBST after each incubation step. The plates were stained with TMB substrate according to the manufacturer's instructions and read at 450 nm on a SpectraMax 340 microplate reader. Alternatively, the plates were stained using 100 $\mu\text{l/well}$ orthophenylenediamine (OPD) substrate (4 mg/ml OPD in 100 mM Na_2HPO_4 buffer, pH 7.4) and 1M H_2SO_4 (50

$\mu\text{l/well}$), after which the absorbance was read at 490 nm. Antibody titers were defined as the dilution factor of the sample that leads to half of the signal intensity that is reached with the undiluted sample.

Detection of anti-domain I antibodies. Antibodies against domain I of $\beta_2\text{GPI}$ were determined essentially as previously described (5). Briefly, human or murine domain I of $\beta_2\text{GPI}$ was coated onto a hydrophobic ELISA plate (catalog no. 2595; Costar) followed by blocking of the plate with 2% bovine serum albumin in PBS and 0.1% Tween 20. Antibody binding in serum was determined as described above.

Detection of thrombin–antithrombin (TAT) complexes. The TAT complex concentration in plasma was measured at the last time point (36 days) using a commercially available ELISA kit designed for human TAT (Enzygnost TAT; Dade Behring), which also cross-reacts with murine TAT. The content of murine TAT in plasma was calculated according to the human TAT standard curve (17).

Determination of lupus anticoagulant activity. LAC activity was determined in murine plasma at the last time point of the immunization protocol (36 days), using a dilute Russell's viper venom time (dRVVT)–based coagulation assay (Gradipore). The assay was done according to the manufacturer's instructions provided for human plasma.

RESULTS

$\beta_2\text{GPI}$ conformational change upon cardiolipin binding. Conformational changes in proteins can be monitored in a number of ways. The misfolding of proteins is often accompanied by the adoption of amyloid-like properties (18,19). This can be detected by binding of small-molecular dye compounds, such as ThT, that are commonly used to stain pathologic amyloid deposits for the diagnosis of amyloid diseases. ThT binds amyloid fibrils and misfolded protein aggregates, which changes its fluorescence behavior. This change can be used to qualitatively track conformational changes of proteins in solution and has been used for determining semiquantitative correlations between changes in protein structure and immunogenicity (10). Figure 1A shows that ThT fluorescence increased when $\beta_2\text{GPI}$ was bound to cardiolipin (or was chemically misfolded) compared with the ThT fluorescence associated with native $\beta_2\text{GPI}$.

Like ThT fluorescence, the binding of tPA can be used as a qualitative marker for changes in protein conformation that relate to immunogenicity (10). Binding experiments showed that full-length tPA bound to $\beta_2\text{GPI}$ that had been incubated with cardiolipin (Figure 1B). The binding of tPA to phospholipid-bound $\beta_2\text{GPI}$ was mediated by the N-terminal domains of tPA, which are not present in the deletion mutant K2P; this region is held responsible for the binding of misfolded protein aggregates (20). We next used electron microscopy to

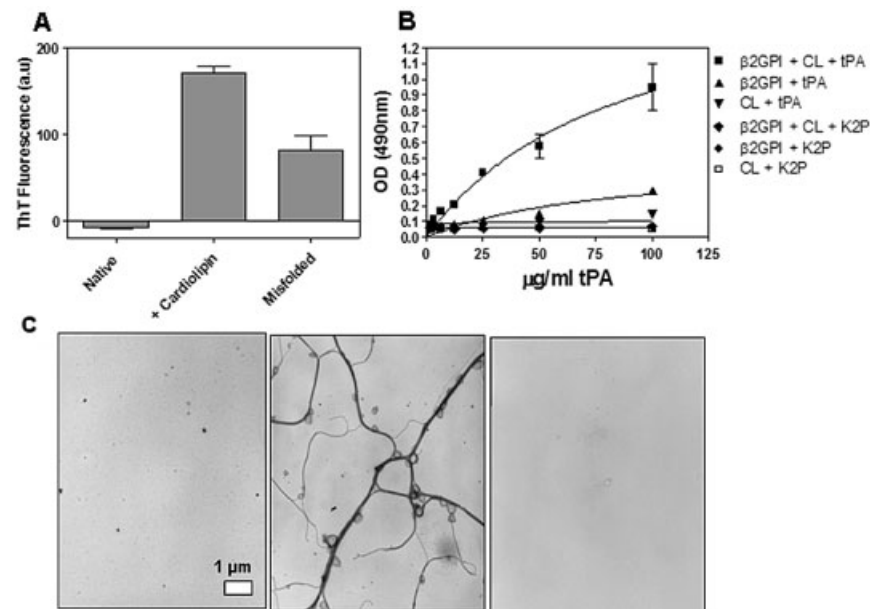


Figure 1. Cardiolipin (CL) binding induces conformational changes in β_2 -glycoprotein I (β_2 GPI). **A**, Thioflavin T (ThT) fluorescence increased when β_2 GPI was incubated with cardiolipin vesicles. **B**, Incubation of β_2 GPI with cardiolipin led to the exposure of a binding site for tissue-type plasminogen activator (tPA). **C**, Electron microscopic analysis revealed that β_2 GPI assembles into polymeric meshworks upon incubation with cardiolipin vesicles (middle), compared to that in the absence of cardiolipin (left). This formation of a polymeric meshwork was inhibited when β_2 GPI was pretreated with plasmin (right). Values in **A** and **B** are the mean \pm SEM. AU = arbitrary units; OD = optical density; K2P = deletion mutant of tPA.

investigate the structure of β_2 GPI in the presence of cardiolipin. In contrast to β_2 GPI without cardiolipin (Figure 1C, left), β_2 GPI with cardiolipin vesicles assembled into a meshwork of branching fibrils (Figure 1C, middle). The fibrils appeared flexible and self-associated to form thicker fibrils. These fibrils remained associated with vesicles that presumably consist of cardiolipin.

The cleavage of β_2 GPI impairs its phospholipid-binding capacity (12). When β_2 GPI was pretreated with plasmin, cardiolipin-induced polymerization was completely abrogated (Figure 1C, right). From these experiments, we concluded that β_2 GPI aggregates as a consequence of binding to phospholipid vesicles, which helps to explain how phospholipid vesicles are precipitated by β_2 GPI (21).

Influence of β_2 GPI structure on its immunogenicity. Simultaneous administration of β_2 GPI with apoptotic cells and phospholipid vesicles leads to the formation of anti- β_2 GPI antibodies in mice (7–9). These immune responses could arise either because of a potential adjuvant effect of cells/vesicles, or, alternatively, the immunogenicity could result from the exposure of

neoepitopes by β_2 GPI as a consequence of a conformational change. To investigate whether conformational changes in β_2 GPI (in the absence of phospholipids) enhance the immunogenicity of β_2 GPI via neoepitope exposure, we artificially changed the conformation of β_2 GPI. To this end, we irreversibly disrupted the internal disulfide bridges in the molecule (13,14), of which 11 are present within β_2 GPI (22). This modification of β_2 GPI led to increased ThT binding (Figure 1A) and binding by tPA (Figure 2A).

For the first immunizations, groups of mice ($n = 5$) were intravenously injected with β_2 GPI with or without cardiolipin, or alternatively, with misfolded β_2 GPI. All proteins were administered without adjuvant. After the administration of 4 injections over a period of 36 days, antibodies against the native protein developed in mice that had received a mixture of β_2 GPI and cardiolipin (Figures 2B and C), whereas antibodies did not develop in mice that received native β_2 GPI alone. Similarly, injection of misfolded β_2 GPI in the absence of cardiolipin vesicles induced the generation of antibodies against native β_2 GPI (Figures 2B and C). When sera

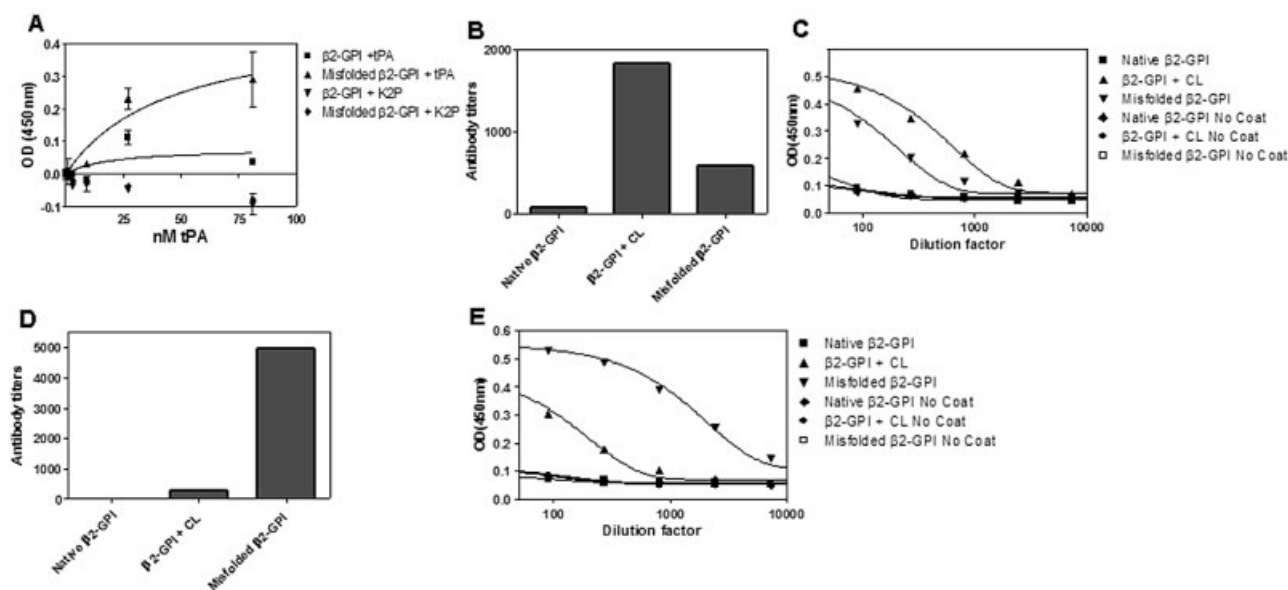


Figure 2. Conformational change in β_2 GPI induces immune response. **A**, Tissue-type plasminogen activator had an increased affinity for misfolded β_2 GPI. Mice were repeatedly injected with native β_2 GPI, β_2 GPI combined with cardiolipin, or misfolded β_2 GPI. After 36 days, sera were pooled ($n = 5$ per group) and analyzed for antibodies against native and misfolded β_2 GPI. **B** and **C**, In mice injected with cardiolipin-bound β_2 GPI or misfolded β_2 GPI, antibodies that recognized the immobilized native protein were generated. No such antibodies arose when mice were injected with native β_2 GPI. **D** and **E**, Antibodies against the misfolded β_2 GPI were mainly present in the pooled sera of mice that were injected with misfolded β_2 GPI. This indicates that misfolded β_2 GPI exposed immunogenic epitopes that are not present in cardiolipin-bound β_2 GPI. The results shown are representative of at least 4 individual experiments. Values in **A** are the mean \pm SEM. See Figure 1 for definitions.

obtained from these same mice were analyzed for the presence of antibodies against misfolded β_2 GPI, significantly increased antibody titers were observed only in mice that had been injected with misfolded β_2 GPI (Figures 2D and E). This result indicated that misfolded β_2 GPI exposed a number of additional immunogenic epitopes besides the one presented by cardiolipin-bound β_2 GPI. This was most likely attributable to differences in the extent of conformational changes that follow the disruption of disulfide bridges within β_2 GPI (22) compared with the more subtle changes that follow phospholipid binding.

The risk of thrombosis in patients with APS is related to the presence of high-affinity antibodies against a cryptic epitope domain I of β_2 GPI (5,23,24). Therefore, we sought to determine whether sera obtained after the first immunizations contained antibodies against this epitope. As shown in Figure 3, few to no antibodies against the cryptic epitope on domain I formed in mice injected with native β_2 GPI. Sera obtained from the 2 other groups of mice (those immunized with cardiolipin-bound β_2 GPI and those immunized with misfolded β_2 GPI alone) displayed antibody

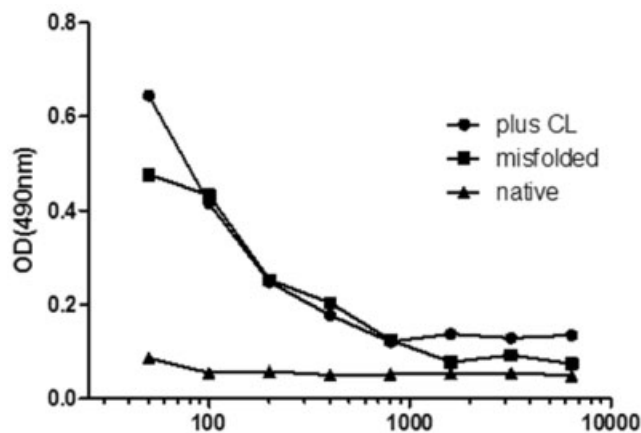


Figure 3. Immunization with β_2 -glycoprotein I (β_2 GPI) induces anti-domain I antibodies. The sera of mice immunized with cardiolipin (CL)-bound β_2 GPI or misfolded β_2 GPI contain antibodies directed against a cryptic epitope of domain I. The pooled sera of mice in each group were diluted 50-, 100-, 200-, 400-, 800-, 1,600-, 3,200-, and 6,400-fold and tested for the binding of domain I of β_2 GPI coated on a hydrophobic microtiter plate. The pathology-associated cryptic epitope is exposed when domain I is coated on a hydrophobic plate. OD = optical density.

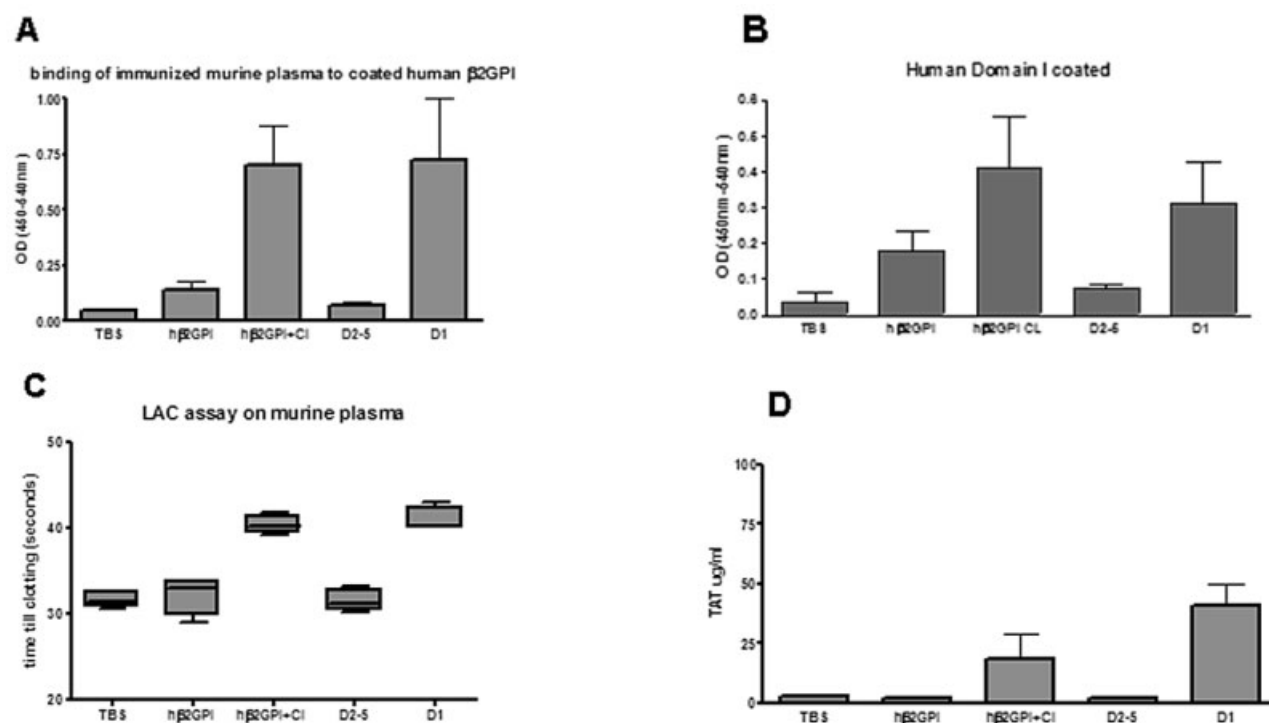


Figure 4. Domain I of β_2 -glycoprotein I (β_2 GPI) harbors an immunogenic epitope of β_2 GPI. Mice were injected with either Tris buffered saline (TBS; control), human native β_2 GPI (h β_2 GPI) with or without cardiolipin (CL), β_2 GPI mutant containing domains II–V, or β_2 GPI mutant containing domain I. Antibodies from the plasma of mice injected with human β_2 GPI plus cardiolipin or with domain I showed increased reactivity against human β_2 GPI coated on a hydrophilic enzyme-linked immunosorbent assay plate (A), increased reactivity against domain I (B), an increased coagulation time as determined using a dilute Russell's viper venom time-based assay (C), and increased thrombin–antithrombin (TAT) levels (D) compared with controls. All data are derived from measurements obtained at the last time point (36 days). Values in A, B, and D are the mean \pm SEM. Data in C are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. OD = optical density; LAC = lupus anticoagulant.

binding to domain I of β_2 GPI coated on an ELISA plate. This suggests that during immunization of mice with cardiolipin-bound β_2 GPI or misfolded β_2 GPI alone, antibodies are generated that recognize a specific pathology-associated epitope on domain I of β_2 GPI (5).

Because immunization with cardiolipin-bound β_2 GPI induced antibodies against domain I of β_2 GPI, we tested the immunogenicity of human domain I itself. Therefore, mice were injected with native human β_2 GPI, native human β_2 GPI with cardiolipin, a β_2 GPI domain I deletion mutant (human domains II–V), or human domain I alone. The same immunization scheme as that described above was used. Antibodies in the plasma of mice injected with native β_2 GPI in the presence of cardiolipin or domain I showed strong reactivity toward β_2 GPI coated to a hydrophilic plate (Figure 4A) and strong reactivity against domain I of β_2 GPI (Figure 4B). In contrast, such antibodies were absent in plasma from

mice injected with native β_2 GPI or β_2 GPI mutant domains II–V. In addition, these plasma samples were tested using a dRVVT-based coagulation assay (Figure 4C). Prolongation of the clotting time was observed in mice injected with either domain I (mean \pm SEM 40.9 ± 0.8 seconds) or cardiolipin-bound β_2 GPI (40.4 ± 0.6 seconds) compared with control (31.7 ± 0.4 seconds; $P < 0.05$). Interestingly, increased TAT levels were observed in both mice injected with cardiolipin-bound β_2 GPI and those injected with domain I (18.6 ± 10.5 μ g/ml and 40.5 ± 9.6 μ g/ml, respectively) compared with TBS or native β_2 GPI (3.0 ± 0.2 μ g/ml; $P < 0.05$) (Figure 4D), which suggests a prothrombotic state (25).

A third round of immunizations was performed to investigate the immunogenicity of murine β_2 GPI. Four groups of mice were injected with TBS, native murine β_2 GPI, native murine β_2 GPI in the presence of cardiolipin, or murine domain I. Antibody titers against

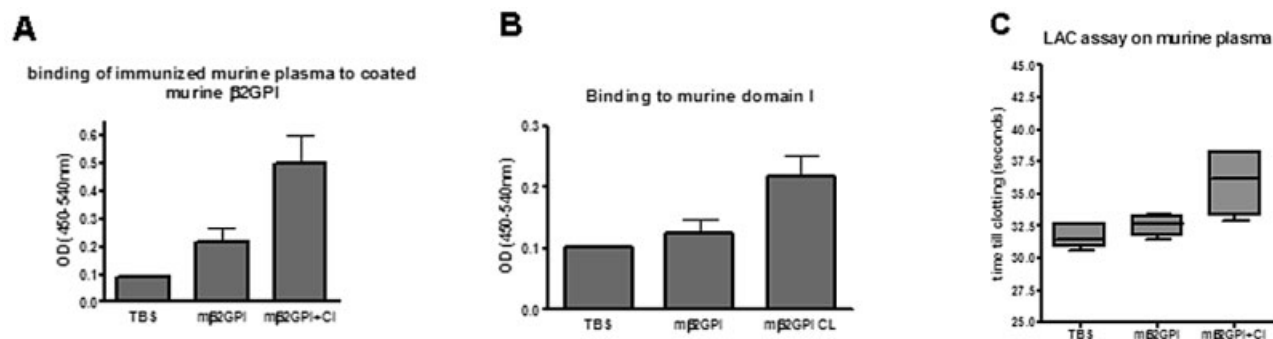


Figure 5. Immunization with murine β_2 GPI induces anti-murine domain I antibodies. Plasma from mice injected with either TBS, murine β_2 GPI (m β_2 GPI), or murine β_2 GPI combined with cardiolipin were tested for reactivity against murine β_2 GPI (A) and murine domain I (B). In addition, plasma from mice injected with cardiolipin-bound murine β_2 GPI also showed an increased coagulation time as determined using a dilute Russell's viper venom time-based assay compared with mice injected with either TBS or murine β_2 GPI alone (C). All data are derived from measurements obtained at the last time point (36 days). Values in A and B are the mean \pm SEM. Data in C are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. See Figure 4 for definitions.

murine β_2 GPI were the highest in plasma from mice injected with the combination of murine β_2 GPI and cardiolipin, although some reactivity could be observed in plasma from mice injected with murine β_2 GPI only (Figure 5A). Based on the optical density (OD) cut-off value of 0.12 that applies to human samples analyzed in this ELISA, significantly elevated levels of anti-domain I antibodies developed only in mice given murine β_2 GPI in combination with cardiolipin (OD 0.21 versus OD 0.12 in mice given murine β_2 GPI only [$P = 0.04$] and OD 0.10 in mice given TBS [$P = 0.03$]) (Figure 5B). When LAC activity was assessed using a dRVVT assay, plasma from mice injected with murine β_2 GPI plus cardiolipin displayed an increased coagulation time compared with control mice treated with TBS (mean \pm SEM 35.9 ± 1.3 versus 31.7 ± 0.4 seconds; $P < 0.05$) (Figure 5C). The group of mice injected with murine β_2 GPI alone did not show a significantly increased coagulation time compared with control mice (mean \pm SEM 32.58 ± 0.41 versus 31.7 ± 0.4 seconds; $P = 0.20$) (Figure 5C). No differences in TAT levels were observed between the 3 groups (data not shown).

DISCUSSION

In previous studies, we have shown that immune responses against therapeutic proteins can be a consequence of protein misfolding (10). Here, we addressed the possibility that the endogenous plasma protein β_2 GPI can become immunogenic as a consequence of a conformational change. β_2 GPI is the major antigen for

autoantibodies that cause APS, an autoimmune disease characterized by an increased risk of thrombosis and recurrent fetal loss. Although studies in animal models have shown that autoantibodies against β_2 GPI are causative for the clinical complications observed in this syndrome, the presence of these autoantibodies, as measured with an ELISA, hardly correlates with the clinical manifestations in case-control studies (26,27). A possible explanation could be that only subsets of autoantibodies are pathogenic. Indeed, autoantibodies against domain I of β_2 GPI correlate much better with thrombosis than do autoantibodies against other parts of this protein (5,16,24). It has also been shown that autoantibodies against domain I recognize a cryptic epitope in β_2 GPI that is exposed only when the protein is bound to an anionic surface (6).

β_2 GPI changes its conformation when it binds to negatively charged phospholipids such as cardiolipin or the surface of apoptotic cells. In the circulation, β_2 GPI has a circular conformation. This conformation is held together by an interaction between domain I and domain V (28,29). When domain V of β_2 GPI binds a negatively charged phospholipid surface, this interaction is disrupted, allowing β_2 GPI to acquire an open linear structure, exposing the G40-R43 epitope on domain I. Also, under conditions of molecular stress in the absence of phospholipids, β_2 GPI can change its conformation from a circular shape to an open, elongated structure (29).

In the current study, we addressed the question

of whether conformational changes drive immune responses against β_2 GPI. To this end, we irreversibly misfolded human β_2 GPI and studied its immunogenicity in the absence of phospholipids. Moreover, we compared the immunogenicity of domain I of human β_2 GPI with that of domains II–V and studied the effects of cardiolipin on the immunogenicity of human and murine β_2 GPI.

Mice injected with human or murine β_2 GPI in the presence of cardiolipin developed (auto)antibodies against domain I of β_2 GPI, while these antibodies did not develop in mice injected with native β_2 GPI. The importance of structural changes becomes apparent from the response that misfolded human β_2 GPI evokes in the absence of phospholipids. We observed that domain I of human β_2 GPI can trigger antibody generation by itself, whereas a construct of human β_2 GPI domains II–V cannot, indicating that exposure of the cryptic epitope on domain I is sufficient to induce antibody formation. Plasma specimens from mice in which antibody responses against β_2 GPI and domain I of this protein developed also showed a prolongation of phospholipid-dependent clotting, which is suggestive for the presence of LAC.

In the present study, we did not subject mice to an *in vivo* thrombosis model. However, we observed increased levels of TAT complexes in the plasma of mice that had been injected with human β_2 GPI with cardiolipin or with domain I alone. Because increased TAT complex levels are associated with thrombotic disease, these experiments suggest that the generation of antibodies against β_2 GPI could have consequences in terms of thrombogenesis. However, *in vivo* studies are needed to confirm whether this assumption holds true.

Our experiments demonstrate that extensive conformational changes (as were present in misfolded β_2 GPI) can trigger immune responses in the absence of phospholipids. However, we presume that the immune responses against β_2 GPI in APS are caused by a more subtle conformational change that exposes a neoepitope only on domain I, which takes place during cardiolipin binding. Although phospholipids can induce the exposure of this epitope *in vitro*, the endogenous trigger(s) for the conformational change that drives immune responses against β_2 GPI remains elusive. Apoptotic cells may present a negatively charged surface that may trigger a conformational change in β_2 GPI, but the question remains whether these cells are endogenously present long enough and in sufficient amounts to trigger immune responses against β_2 GPI. Recently, an alternative hypothesis was proposed: that β_2 GPI represents a

class of pathogen-opsonizing proteins that direct reactions of the adaptive immune system toward these pathogens (30). This hypothesis suggests that antibody generation against β_2 GPI, which originally was known because of its role in disease, may actually have a beneficial role in host defense. However, it is presently unclear how β_2 GPI could interact with the bacterial outer surface.

Several groups of investigators have provided data on immunization studies with β_2 GPI (7,31). Some of these studies showed data in favor of other immunogenic domains besides domain I (i.e., domains IV and V). This discrepancy may be caused by the absence of adjuvants in our immunization experiments, which were present in these earlier studies. It should be noted that mice injected with murine β_2 GPI alone, which is completely “self” to mice, triggered the generation of low antibody titers. Although this preparation and the human protein were purified in the same manner, we cannot exclude the possibility that the purified murine β_2 GPI is partly folded in a way that exposes an immunogenic epitope on domain I of β_2 GPI.

At present, 3 studies have shown a conformational change in β_2 GPI upon binding to phospholipid vesicles, by examination of its biophysical properties (7,21,32). Our experiments confirm that cardiolipin-bound β_2 GPI undergoes a conformational change by adopting an increased affinity for the amyloidophilic dye ThT (Figure 1). Additionally, the protein can assemble into large polymers when it is incubated with cardiolipin vesicles *in vitro*. This process can take place in the absence of pathologic antibodies. However, the possibility exists that this is a reversible process that is stabilized by the presence of antibodies. It was recently reported that phospholipid vesicles precipitate *in vitro* when full-length human β_2 GPI is present (21), and that phospholipids induce the aggregation of β_2 GPI (32), which may correspond to the polymerization of β_2 GPI on cardiolipin vesicles that we observed. This leads to the question of whether β_2 GPI can also polymerize in a physiologic environment, and, if so, whether there are physiologic benefits for this aggregation behavior.

In conclusion, not only is the interaction of pathologic antibodies with β_2 GPI related to its conformation, but the immunogenicity of this protein also seems to relate to its structure. These findings may contribute to further understanding of the mechanisms that underlie antibody generation in APS as well as other autoimmune diseases.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. de Laat had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. de Laat, de Groot, Gebbink, Maas.

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ROLE OF THE STUDY SPONSOR

Neither Synapse BV nor Sanquin Blood Foundation had any influence on the study design, data collection, data analysis, or writing of the manuscript. Both institutes agreed to publish the results regardless of the outcome, and publication was not contingent on their approval.

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