X-ray and solution structures of human beta-2 glycoprotein I reveal a new mechanism of autoantibody recognition

Eliza Ruben¹, William Planer¹, Mathivanan Chinnaraj¹, Zhiwei Chen¹, Xiaobing Zuo², Vittorio Pengo³, Vincenzo De Filippis⁴, Ravi K. Alluri⁵, Keith R. McCrae⁶, Paolo Macor⁷, Francesco Tedesco⁷, and Nicola Pozzi¹.

¹Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO 63104, ²X-Ray Science Division, Argonne National Laboratory, Argonne, Illinois 60439; ³Division of Cardiac, Thoracic, and Vascular Sciences, University of Padova, Padova, Italy. ⁴Department of Pharmaceutical & Pharmacological Sciences, University of Padua, via F. Marzolo 5, 35131 Padua; ⁵Department of Cellular and Molecular Medicine, Cleveland Clinic, Cleveland, OH, United States; ⁶Department of Life Sciences, University of Trieste, Italy; ⁷Istituto Auxologico Italiano, IRCCS, Laboratory of Immuno-Rheumatology, Milan, Italy.

These authors contributed equally.

*Corresponding author:
Nicola Pozzi
Department of Biochemistry and Molecular Biology
Saint Louis University School of Medicine
St. Louis, MO 63104
Tel: (314) 977-9241, Fax: (314) 977-9206
E-mail: nicola.pozzi@health.slu.edu

Running title: How anti-DI antibodies recognize β₂GPI
Abstract

Venous and arterial thromboses in patients suffering from the autoimmune disorder Antiphospholipid Syndrome (APS) are caused by the presence of antiphospholipid antibodies (aPL). Emerging evidence indicates that autoantibodies targeting the epitope R39-R43 in the N-terminal domain, Domain I (DI), of β2-glycoprotein I (β2GPI) are among the most pathogenic aPL in patients with APS. How such autoantibodies engage β2GPI at the molecular level remains incompletely understood. Here, we have used X-ray crystallography, single-molecule FRET, and small-angle X-ray scattering to demonstrate that, in the free form, under physiological pH and salt concentrations, human recombinant β2GPI adopts an elongated, flexible conformation in which DI is exposed to the solvent, thus available for autoantibody recognition. Consistent with this structural model, binding and mutagenesis studies revealed that the elongated form interacts with a pathogenic anti-DI antibody in solution, without the need of phospholipids. Furthermore, complex formation was affected neither by the neighboring domains, nor by the presence of the linkers, nor by the glycosylations. Since the pathogenic autoantibody requires residues R39 and R43 for optimal binding, these findings challenge longstanding postulates in the field envisioning β2GPI adopting immunologic inert conformations featuring inaccessibility of the epitope R39-R43 in DI and support an alternative model whereby the preferential binding of anti-DI antibodies towards phospholipid-bound β2GPI arises from the ability of the pre-existing elongated form to bind to the membranes and then oligomerize, processes that are likely to be supported by protein conformational changes. Interfering with these steps may limit the pathogenic effects of anti-DI antibodies in APS patients.
Significance

In the autoimmune disorder called Antiphospholipid Syndrome (APS), the presence of autoantibodies targeting the plasma glycoprotein beta-2 glycoprotein I (\(\beta_2\)GPI) is associated with arterial and venous thrombosis as well as pregnancy complications. Understanding how \(\beta_2\)GPI becomes immunogenic and how autoantibodies in complex with \(\beta_2\)GPI cause the blood to clot remains a top priority in the field. By elucidating the structural architecture of \(\beta_2\)GPI free in solution, our studies challenge longstanding postulates in the field and shed new light on the pathogenic mechanisms of APS that may help the development of new diagnostics and therapeutic approaches.
Introduction

β₂GPI is a 50-kDa multi-domain glycoprotein that circulates in the plasma at a concentration of 0.2 mg/ml(1, 2)(Fig 1A). It acquired centerstage in hematology in 1990 when it was recognized by two independent studies as the dominant antigen of antiphospholipid antibodies (aPL) in the Antiphospholipid Syndrome (APS)(3-5), a life-threatening blood clotting disorder characterized by vascular thrombosis and pregnancy morbidity(6). Autoantibodies against β₂GPI (anti-β₂GPI) are indeed frequently found in young patients with a history of thrombosis(7, 8); they are often associated with lupus anticoagulant, a laboratory test that indicates predisposition for blood clots(9); they induce(10) and potentiate thrombus formation in vivo (11, 12) and cause pregnancy complications resulting in fetal loss(13).

While significant efforts have been made over the past decade to identify the cellular targets and signaling pathways triggered by anti-β₂GPI antibodies(6, 14-17), our knowledge of the structural properties of β₂GPI and the structural determinants for antigen-antibody recognition has lagged behind. Only two major advances came in recent years. First, it was discovered that anti-β₂GPI antibodies in strong correlation with thrombosis recognize a conformational epitope comprising residues R39-R43 in the N-terminal domain, Domain I (DI), of β₂GPI(18-22). Importantly this epitope was also proposed to be cryptic based on the evidence that anti-DI antibodies showed no reactivity against β₂GPI in solution but did react well when β₂GPI was immobilized onto hydrophilic surfaces or plastic plates pre-coated with negatively charged phospholipids(18, 23). Second, using X-ray crystallography(24, 25), small-angle X-ray scattering (SAXS)(26), negative stain electron microscopy (EM)(27), and atomic force microscopy (AFM)(28), it was found that β₂GPI can adopt O-circular, S-twisted and J-elongated conformations according to its ligation status. (Fig. 1B). Given the poor reactivity of β₂GPI toward anti-DI antibodies in solution as compared to β₂GPI bound to negatively charged surfaces(19), the high salt conditions used in the crystallization experiments(24, 25) and the harsh purification method used to purify the protein used in the X-ray and SAXS studies, the O-circular form is currently regarded as the predominant conformation that the protein adopts under physiological pH and salt concentrations, which is immunologically inert, incapable of reacting against anti-DI antibodies(27). In contrast, the J-elongated form is believed to represent the immunogenic conformation of the protein, which appears when β₂GPI is bound to the membranes. Dimeric complexes of the elongated form assembled on the activated endothelium(29) and stabilized by anti-β₂GPI antibodies would then engage cellular receptors, such as Annexin A2, TLR4, ApoER2, and Gplbα, to induce a procoagulant and proinflammatory state(17, 30-34). Since the S-twisted conformation of the protein inferred by SAXS was detected neither by EM, nor by AFM, nor by X-ray crystallography, the current model also predicts that the S-twisted form represents a transient, unreactive intermediate state that the protein populates while transitioning between the J- and O-forms.

Although very popular in the APS field, it is important to acknowledge that the structural features of the O- and S- conformations are poorly defined due to the low-resolution models generated by EM, AFM
and SAXS. Consequently, it remains unclear under what circumstances and how these forms interconvert, what is their physiological role, and how they participate in the mechanism of autoantibody recognition. Encouraged by our recent results with prothrombin,(35-37), the second most common antigen of aPL in APS, this work was initiated to investigate the structural and conformational properties of $\beta_2$GPI under conditions relevant to physiology and provide new insights into the mechanism of autoantibody recognition. Our results based on X-ray crystallography, single-molecule FRET (smFRET), SAXS, binding kinetics, and mutational studies unexpectedly reveal that human recombinant $\beta_2$GPI adopts an elongated, flexible conformation, not circular, in which DI is exposed to the solvent and therefore available for autoantibody recognition. Based on this new evidence and previous findings, an alternative mechanism to explain how negatively charged phospholipids may enhance the affinity toward anti-DI autoantibodies without requiring opening of the protein structure or relocation of the glycosylations away from DI is proposed, and its implication to our understanding of APS discussed.

**Results**

**Expression, purification, and functional characterization of human recombinant beta-2 glycoprotein I.** Like beads on a string, CCP-domains are known to adopt a variety of orientations depending on the length of the linker connecting two adjacent domains and electrostatic properties(38). To get a better grasp of the structural architecture of $\beta_2$GPI under conditions relevant to physiology, we set out to perform structural and biophysical studies of fully glycosylated human recombinant $\beta_2$GPI. Two versions of the proteins were successfully expressed and purified under native conditions at high yield and purity. The first version, called LT-$\beta_2$GPI, contained a long multifunctional cleavable tag at the N-terminus, located right before the natural N-terminal sequence $^1$GRTC$^4$ (**Fig. 2A**). The tag was then cleaved with enterokinase to generate the intact, mature protein (hr$\beta_2$GPI). Removal of the tag was confirmed by N-terminal sequencing (**Fig. 2B**). The second version, called ST-$\beta_2$GPI, contains a shorter, non-cleavable purification tag at the N-terminus that, based on our previous work(36), is expected not to affect the conformational properties of the protein (**Fig. 2B**). ST-$\beta_2$GPI was made to eliminate the enterokinase cleavage step that was very laborious and not as efficient as expected. The presence of the short tag was confirmed by N-terminal sequencing and accounted for the different electrophoretic mobility observed between recombinant and plasma purified protein before and after enzymatic removal of the N-glycosylations (**Fig. 2B**).

To evaluate the functional integrity of the recombinant proteins, LT-$\beta_2$GPI, hr$\beta_2$GPI and ST-$\beta_2$GPI were tested side by side with plasma purified $\beta_2$GPI (p$\beta_2$GPI) in several biochemical assays. Using surface plasmon resonance (SPR), we found that all variants interacted avidly with liposomes containing negatively charged phospholipids such as phosphatidylserine, yet they failed to interact with phospholipids entirely made of phosphatidylcholine (**Fig. 2C-D**). Importantly, the values of the affinity constants were similar for all the constructs and consistent with published data(39), and so was the
inhibitory effect of physiological concentrations of calcium chloride. These results document structural
integrity of the hydrophobic loop in DV and also prove that the phospholipid binding activity of β₂GPI is
not perturbed by the presence or removal of the purification tags.

In addition to properly interacting with phospholipids, the recombinant proteins were also successfully
recognized in ELISA assays by aPL isolated from four triple positive APS patients, which contain anti-DI
antibodies(22, 35, 40) (Fig. 2E). In this case, however, LT-β₂GPI exhibited significantly lower values of
OD₄₅₀ nm compared to the other variants and plasma purified protein, suggesting that the presence of
the long tag may mask some epitopes or, more likely, change the preferential orientation of the molecule
that is adsorbed onto the plastic surface. Taken together, these studies validate recombinantly made
β₂GPI as a proxy for plasma-purified β₂GPI. They also demonstrate that, under physiological conditions,
β₂GPI is primed for phospholipid and heparin binding.

**X-ray crystal structure of human recombinant beta-2 glycoprotein I.** To investigate the structural
properties of the recombinant proteins, crystallization experiments were performed for all the protein
constructs. While it was not possible to crystallize LT-β₂GPI, we solved the X-ray crystal structures of
hrβ₂GPI, ST-β₂GPI and pβ₂GPI at 2.6, 3.0 and 2.4 Å resolution, respectively (Fig. 3A-C). Diffraction
quality crystals were obtained after two weeks at 4°C using ammonium sulfate as a precipitating agent.
The crystals belong to the orthorhombic space group C222₁ (Table 1). Notably, ST-β₂GPI, for which extra
electron density was observed at the N-terminus (Fig. 3D), crystallized under similar conditions and in
the same space group compared to hrβ₂GPI and pβ₂GPI, confirming minimal structural perturbation
introduced by the artificial tag. β₂GPI contains 22 cysteine residues. In our structures, regardless of the
biological source and method of purification, all of them are engaged in 11 disulfide bridges (Fig. 3A-C).
Given that purification of the recombinant proteins occurs under native conditions, this result indicates
that, right after cell secretion, β₂GPI does not contain free thiols.

All three independently solved X-ray crystal structures depicted β₂GPI featuring an elongated
conformation spanning ~140 Å in length, from the N- to the C-terminus. The first three domains, DI-DIII,
are aligned along the vertical axis of the molecule, whereas DIV and DV bend, forcing the molecule to
adopt a characteristic J-shaped elongated form resembling a hockey stick. DI and DV are located >100
Å apart and both of them are exposed to the solvent. Interestingly, however, the side chain of residue
R43, which is part of the cryptic epitope recognized by anti-DI antibodies, is not exposed to the solvent
and is part of a hydrogen bond network made up by residues R39, G41 and T57 (Fig. 3E).

Overall, the three new structures are superimposable and similar to the published ones(24, 25), yet
they are not identical (Fig. 4A). One significant difference regards the conformation the hydrophobic loop
in DV (residues 308-319), which, given its flexibility and exposure to the solvent, varies in every structure.
Another difference is the significant extra electron density after molecular replacement in the datasets at
a higher resolution (i.e., 2.4 and 2.6Å), suggesting that the input structural model used to solve the
structures (1C1Z(25)) was incomplete (Fig. 4B). We attributed this density to the N-linked glycosylations (Fig. 4C). Modeling of the glycans provided a more complete view of the glycoprotein and offered new important clues on how β2GPI may adopt multiple conformational changes in solution and become immunogenic. Our new structural model predicts that epitopes in DII, DIII and DIV are mostly buried by the presence of the glycosylations, whereas DI and DV are not. It also suggests that a conformational change may be required to expose R43 to the solvent. Due to the presence of sialic acid, N-glycosylations typically are negatively charged. DI and DV, in contrast, are positively charged (Fig. 4D). It is therefore reasonable to admit that the presence of glycosylations affects the conformational landscape of the molecule: 1) by limiting the number of spatial arrangements that the protein can adopt in solution because of steric hindrance, 2) by facilitating looping of the molecule by neutralizing the repulsive positive electrostatic potential of DI and DV and 3) together with DIV, by preserving an elongated conformation of the protein when the protein is anchored to the lipids via DV.

Solution structure of human recombinant beta-2 glycoprotein I. It is generally believed that the J-elongated conformation trapped in the X-ray studies is not a genuine representation of the protein structure in solution (2, 26). The high ionic strength used in the crystallization buffers may destabilize hydrogen bonds and favor hydrophobic interactions thus forcing the protein to assume a non-native conformation (2). To address this concern, we applied smFRET to β2GPI (37, 41, 42). By recording the energy that is transferred from an excited molecule (Donor) to a second molecule with spectral overlap (Acceptor) at the single molecule level, smFRET measures distances on a nanometer scale thus serving as a molecular ruler.

Guided by our new structures, we generated four FRET pairs in the ST background, S13C/S112C, S13C/S312C, S112C/S312C and S190C/S312C (Fig. 5A-B), by substituting the natural serine residues with isosteric cysteines and then reacting those newly engineered cysteines with Alexa Fluor 555 (AF555) maleimide as FRET donor and Alexa Fluor 647 (AF647) maleimide as FRET acceptor. Residue 13 is located in DI, residue 112 is located in DII, residue 190 is located in DIV and residue 312 is located in DV. Labeling occurred only at the engineered sites as no fluorescence was observed for β2GPI wild type (Fig. 5C). This result is consistent with our structural data and previous findings (43) documenting that β2GPI wild type does not contain free thiols. Given the Förster radius $R_0 = 50 \, \text{Å}$ of the 555/647 FRET couple, the crystal structure predicts no FRET for the mutants S112C/S312C and S13C/S312C. In contrast, high FRET and low but measurable FRET values are expected for the FRET pair S13C/S112C and S190C/S312C, respectively. This is because residues 13 and 112 are located ~24 Å apart while the $C\alpha-C\alpha$ distance between residues 190 and 312 is ~46Å. Remarkably, the experimental results were fully consistent with our structure-based calculations, thus validating the elongated conformation in solution and unequivocally proving that this conformation predominates (>90%) under physiological pH and salt concentrations (Fig. 5D). Specifically, probes located at positions C13 and C312 and C112 and C312
reported a negligible FRET signal, whereas probes attached to the S13C/S112C and S190C/S312C mutants reported high ($E_{\text{FRET}}=0.92$) and low ($E_{\text{FRET}}=0.26$) FRET values, respectively. Interestingly, the construct 190/312 displayed a FRET distribution wider than the theoretical distribution predicted by shot noise (44) documenting the existence of multiple conformations at equilibrium brokered by the flexibility of Lnk4. Importantly, no significant FRET differences were observed neither in the presence of high (1.5 M) or low (25 mM) concentrations of sodium chloride nor under acidic (pH 3.4) or alkaline (pH 11.0) pH, suggesting that, in contrast to previous findings, variation of the ionic strength and pH produces minor conformational changes that could not be detected by our FRET pairs.

To rule out potential artifacts arising from the substitution of natural amino acids with cysteine and incorporation of fluorescent dyes, we collected SAXS data for the ST-$\beta_2$GPI and p$\beta_2$GPI, under physiological conditions (Fig. 6A-B). SAXS is a biophysical method that is particularly useful to assess the overall shape of biological macromolecules in solution (45), i.e., linear vs. globular, and, similar to smFRET, is therefore ideal to detect large conformational changes in $\beta_2$GPI. Both the radius of gyration ($R_g$) and the computed pair distance distribution functions $p(r)$ were fully consistent with interpretation provided by the smFRET experiments and previous SAXS measurements (26) suggesting an extended, flexible protein conformation in solution. Furthermore, the scattering profile of the recombinant and plasma proteins were very similar, confirming structural equivalency between the two proteins.

**Autoantibody binding studies.** In addition to demonstrating that $\beta_2$GPI adopts an elongated conformation in solution, our structural analysis predicts that this form may be primed for autoantibodies binding, especially anti-DI antibodies. To test this hypothesis, we took advantage of the reactivity of MBB2 (13), a newly developed recombinant monoclonal antibody raised against DI that, upon complement fixation, recapitulates, in vivo, most of the clinical characteristics assigned to pathogenic aPL. The binding of $\beta_2$GPI to MBB2 was followed using SPR (Fig. 7), a technique that allows to measure association (on) and dissociation (off) rate constants in real-time thus enabling a deeper understanding of the chemical nature of the molecular interaction.

To retain the native conformation of $\beta_2$GPI in solution, we immobilized MBB2 to the chip’s surface and injected $\beta_2$GPI in the fluid phase. Binding between MBB2 and $\beta_2$GPI should occur only if DI is exposed to the solvent. This experimental setup is different from previously reported interaction data between MBB2 and $\beta_2$GPI in which $\beta_2$GPI was covalently immobilized on a dextran-based chip and the antibody was used in the fluid-phase to mimic binding of MBB2 to $\beta_2$GPI bound to negatively charged phospholipids (13). The results of the experiments shown in Fig. 7A demonstrate that MBB2 interacts with $\beta_2$GPI in solution with a modest but measurable affinity, characterized by a dissociation constant $K_d=2.2\pm0.2\ \mu\text{M}$ (Fig. 7B). Remarkably, the value of $K_d$ determined for MBB2 is similar to the value of $K_d$ obtained by Dienava-Verdoodl et al. for patient-derived monoclonal antibodies targeting Domain I (46), yet it is 200-fold weaker than the affinity previously determined for MBB2 towards immobilized $\beta_2$GPI (i.e.,
Kd=11 nM) (13). Such a dramatic difference is expected for aPL found in APS patients, which prefer immobilized β₂GPI, thus making MBB2 a highly relevant tool for biochemical investigations.

Interestingly, the interaction between MBB2 and soluble β₂GPI was characterized by very fast on and off rates, suggesting that electrostatic interactions may dominate the binding interface. This was demonstrated by systematic experiments in which we varied the ionic strength of the running buffer from 300 to 15 mM NaCl. As expected, the affinity constant of MBB2 for β₂GPI (Kₘ) increased ~700-fold at low salt concentrations, from 4.4*10⁴ M⁻¹ at 300 mM NaCl to 3.2*10⁷ M⁻¹ at 15 mM NaCl (Fig. 7C). Importantly, the higher affinity of MBBS for β₂GPI at low salt originated from a substantial reduction of the off-rate whereas the on-rate remained mostly unaffected. A significant ~30-fold reduction of the affinity under physiological conditions was also detected after mutating the positively charged residues R₃⁹, R₄³ and K₄⁴ in DI with the neutral amino acid alanine (Fig. 7D-E), confirming the electrostatic nature of such interaction and the ability of MBB2 to interact with an epitope of DI that is targeted by pathogenic aPL.

Previous studies have proposed that the epitope R₃⁹-R₄³ is cryptic because it is buried by DV in the O-circular form (27) or by the N-linked glycosylations in the S-twisted form (26). To test these hypotheses, we measured the affinity of three new constructs, isolated DI (residues 1-60), β₂GPI deletion linker 2 (β₂GPI Δ120-122) and recombinantly deglycosylated β₂GPI (degβ₂GPI, T130S/N143Q/N164Q/N174Q/N234Q/) toward immobilized MBB2. Our binding data shown in Fig. 7F indicates that all three constructs interact with immobilized MBB2 with micromolar affinity comparable to full-length β₂GPI wild-type thus ruling out a significant contribution of the neighboring domains and the glycosylations in shielding the R₃⁹-R₄³ epitope when the protein is free in solution. These constructs were indeed designed based on previous knowledge to force the exposure of DI to the solvent and, in principle, should have had higher affinity for MBB2.

Discussion

Owing to its flexibility, the structural architecture of β₂GPI has remained controversial, and so is the mechanism of autoantibody recognition. A first major conclusion emerging from this study is that human recombinant β₂GPI expressed in mammalian cells and purified under native conditions adopts an elongated, flexible conformation in which DV and DI are exposed to the solvent. In the free form, under physiological pH and salt concentrations, β₂GPI is therefore primed for phospholipid binding and autoantibody recognition. A second major conclusion is that the recombinant protein is structurally and functionally identical to β₂GPI purified from plasma using the perchloric acid method. Hence, the elongated conformation of β₂GPI is not an artifact caused by the harsh purification methods or crystallization conditions but a genuine conformation of the protein in solution.

The recognition that the elongated form of β₂GPI exists and, according to our smFRET experiments, perhaps predominates in human plasma bears important implications in our understanding of the APS
pathology. It also provides new ideas for the development of APS-focused diagnostics and therapeutics.

Regarding the mechanism of anti-DI antibody recognition, our structural and binding data indicates that opening of the protein structure and relocation of DI away from the glycosylations are neither necessary nor sufficient to explain how $\beta_2$GPI becomes a better antigen for anti-DI antibodies upon binding to the membranes. They instead strongly suggest that, in agreement with previous models(39, 47, 48), binding of the pre-existing elongated conformation of $\beta_2$GPI to the membranes gives rise to an ideal surface in which $\beta_2$GPI has a sufficiently high density and adopts a favorable orientation that promotes bivalent binding. In this context, rotation or bending of the CCP domains relative to the plane of the membrane such as those documented here by the FRET couple 190/312 may be important for proper packing of $\beta_2$GPI onto the lipids and, in agreement with previous studies(23, 49), they may even promote oligomerization. A contribution of local conformational changes, such as exposure of R43 upon binding to the lipids, is also possible, yet, considering the intrinsic low affinity of such autoantibodies for their targets, the modest effect caused by mutations in isolated DI(50), and the key role of bivalency documented before(48, 51), the energetic contribution of this process is expected to be minor. The fact that immunocomplexes are very difficult to detect in patients’ plasma(52) even though $\beta_2$GPI is primed for autoantibody binding, is a consequence of the low affinity, fast binding kinetics and low abundance of such autoantibodies in APS patients (0.5% of the total IgG or less). Since the low affinity arises from a very fast dissociation rate constant, the immunogenic complexes are unstable in the fluid phase as they dissociate very rapidly. In this context, a possible role of the negatively charged surfaces suggested by our SPR binding experiments performed at low ionic strength would be to stimulate the binding of anti-DI antibodies to $\beta_2$GPI by slowing down the dissociation rate, thus resulting in complex stabilization. Such a mechanism is fully consistent with recent and previous data(35, 48), and might be conserved among other aPL.

Another important aspect of the pathogenesis of APS is the interaction of $\beta_2$GPI/aPL complexes with cell receptors, mainly via DV, resulting in amplification of pro-inflammatory and pro-thrombotic responses(6, 17, 30, 33, 53, 54). Our structural model predicts that interaction of $\beta_2$GPI with cell receptors may occur without the need of phospholipids, yet the signaling cascade may be triggered by receptor dimerization, which is induced by aPL(17, 33, 55). The need for $\beta_2$GPI, aPL, and suitable receptors explain why the clustering of $\beta_2$GPI onto negatively charged phospholipids is not sufficient to trigger cell activation(29) and why binding of $\beta_2$GPI to cell receptors and anionic phospholipids is mutually exclusive(54).

Regarding the development of new diagnostics and therapeutics, if the main role of the membranes is to increase the local concentration of the elongated form, we speculate that immobilization of human recombinant $\beta_2$GPI produced in this work at the desired density and with a defined orientation should provide a novel, efficient, robust and cost-effective method to detect anti-$\beta_2$GPI antibodies. On the other
side, blocking the binding of the elongated form of β₂GPI to cell receptors and phospholipids should limit
the pathogenic effects of anti-DI antibodies. This approach could complement current strategies aimed
at competing with anti-DI antibodies in solution (13, 56-58) as it would theoretically block other potentially
pathogenic aPL, in addition to those targeting DI. Consistent with this premise, antibodies against DV
found in APS patients do not induce thrombosis but are protective instead (59), and a novel dimeric
molecule, A1-A1, protects mice from aPL-induced thrombosis by interfering with ApoER2 and
phospholipid binding (60).

It is important to acknowledge that, even though all the structural and biochemical experiments were
performed using highly purified proteins solubilized in physiological buffers, β₂GPI has never been
exposed to endothelial or circulating blood cells. Hence, it remains possible, although unlikely, that the
O-circular form of β₂GPI previously documented by EM and AFM studies but not detected by our
structural studies, unless the result of an experimental artifact, may arise from chemical and/or
posttranslational modifications catalyzed by membrane-bound proteins. Future studies will be needed to
clarify this matter.

Materials and Methods

Protein production and purification. β₂GPI wild-type and mutants were expressed in BHK and HEK293
mammalian cells and purified to homogeneity by immunopurification, heparin and size exclusion
chromatography (SEC) after swapping the signal peptide of β₂GPI with the one of the coagulation factor
X to boost expression and adding a furin specific recognition motif RRKR for quantitative post-
translational processing. The purity and chemical identity of each fragment were verified by SDS-PAGE
and N-terminal sequencing. Domain I (1-60) was chemically synthesized and refolded as done
before (57). Plasma-derived β₂GPI (pβ₂GPI) was purified using the perchloric acid method, as described
previously (57). MBB2 was produced as described before (13). Liposomes composed of
phosphatidylcholine (PC) or phosphatidylcholine and phosphatidylserine (PS) in a 4:1 molar ratio
(PC:PS) were prepared by extrusion using 100 nm polycarbonate membranes (Avanti Polar Lipids,
Alabaster, AL), kept a 4°C and used within 7 days. ELISA assays were performed as described before (22,
35, 57). Protein concentrations were determined by reading at 280 nm with molar extinction coefficients
adjusted according to the amino acid sequence. All other chemicals were purchased from Sigma-Aldrich.

Surface Plasmon Resonance (SPR) experiments. Binding affinities for liposomes were measured as
done before (35) using L1 sensor chip in which liposomes were immobilized at 1600 RU. Titrations were
performed by injecting increasing concentrations (0-2 µM) of β₂GPI and its variants in running buffer (20
mM Tris pH 7.4, 150 mM NaCl, 0.1% w/w BSA) at a flow rate of 25 µl/min at 25°C. Binding affinities for
MBB2 were measured using CM5 sensor chip in which MBB2 was immobilized at 6000 RU using
NHS/EDC chemistry. Titrations were performed by injecting increasing concentrations (0-20 µM) of β₂GPI
and its variants in running buffer (20 mM Tris pH 7.4, 25-300 mM NaCl, 0.01% w/w Tween20) at a flow rate of 25 μl/min at 25°C. All experiments were carried out using a BIAcore-S200 instrument (GE-Healthcare). The dissociation constants (Kd) were obtained as a fitting parameter by plotting the value of the response units (RU) at the steady state for each concentration using the BIAevaluation software and Origin Pro 2015.

X-ray studies. Crystallization of human recombinant (hr β2GPI and ST- β2GPI) and pβ2GPI was achieved at 4°C by the vapor diffusion technique, using the Art Robbins Instruments PhoenixTM liquid handling robot with 10 mg/ml protein 0.3 μl mixed with an equal volume reservoir solution. Optimization of crystal growth was achieved by the hanging drop vapor diffusion method mixing 3 μl of protein (10 mg/ml) with equal volumes of reservoir solution at 4°C. After 7-10 days at 4°C, crystals were frozen with 25% glycerol from original mother liquid. X-ray diffraction data were collected at 100° K with a home source (Rigaku 1.2 kw MMX007 generator with VHF optics) Rigaku Raxis IV++ detector for pβ2GPI and ST-β2GPI, and with detector Pilatus of Beamline IDD23, at the Advanced Photon Source, Argonne, IL for hrβ2GPI. Datasets were indexed, integrated and scaled with the HKL2000 software package(61). All structures were solved by molecular replacement using PHASER from the CCP4 suite(62) and the structure of pβ2GPI (PDB ID code 1C1Z) as starting model. Refinement and electron density generation were performed with REFMAC5 from CCP4 package. 5% of the reflections were randomly selected as a test set for cross-validation for four structures. Model building and analysis of the structures were carried out using COOT(63). Ramachandran plots were calculated using PROCHECK. Statistics for data collection and refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in Protein Data Bank (accession codes: 6V06 for pβ2GPI at 2.4Å, 6V08 for hrβ2GPI at 2.6Å, and 6V09 for ST-β2GPI at 3.0Å).

Single-molecule FRET. Selective labeling of the unpaired Cys residues with Alexa Fluor 555-C2-maleimide as the donor and Alexa Fluor 647-C2-maleimide as the acceptor was achieved as described recently for prothrombin(36, 37). FRET measurements of freely diffusing single molecules were performed with a confocal microscope MicroTime 200 (PicoQuant, Berlin, Germany), as detailed elsewhere(36, 37).

Small Angle X-ray Scattering Measurements. SAXS data were collected at the beamline 12-ID-B of the Advanced Photon Source at Argonne National Laboratory (Argonne, IL) on ST-β2GPI and pβ2GPI at concentrations of 0.5, 1, 2, and 5 mg/ml. The radius of gyration, Rg, was determined using the Guinier approximation in the low q region (qRg<1.3), and its linearity served as an initial assessment of data and sample quality. Maximum particle dimension, D_{max}, and distance distribution function, P(r), were calculated using GNOM.
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Table 1. Crystallographic data for the structures of human beta-2 glycoprotein I

| Names          | pβ2GPI | hrβ2GPI | ST-β2GPI                  |
|----------------|--------|---------|---------------------------|
| Buffer/salt    | 100 mM HEPES, pH 7.5/1.5 M AmSO₄, 20 mM CaCl₂, 2% glycerol | 100 mM MES, pH 6.0/1.6 M AmSO₄, 20 mM CaCl₂, 2% glycerol | 100 mM HEPES, pH 7.5/1.5 M AmSO₄, 20 mM CaCl₂, 2% glycerol |
| PDB ID         | 6V06   | 6V08    | 6V09                      |
| Data collection: |        |         |                           |
| Wavelength (Å) | 1.54   | 1.033   | 1.54                      |
| Space group    | C222₁  | C222₁   | C222₁                     |
| Unit cell dimensions (Å) | a=160.8, b=166.9, c=114.0 | a=159.3, b=173.2, c=115.2 | a=160.2, b=171.2, c=113.4 |
| Molecules/asymmetric unit | 1      | 1       | 1                         |
| Resolution range (Å) | 40-2.4 | 40-2.6  | 40-3.0                    |
| Observations   | 513296 | 363697  | 158386                    |
| Unique observations | 59497  | 48503   | 31543                     |
| Completeness (%) | 98.8 (97.1) | 97.1 (77.1) | 98.8 (97.7) |
| Rsym (%)       | 7.9 (50.6) | 14.3 (64.7) | 9.5 (46.1) |
| l' (l)         | 21.1 (2.4) | 10.0 (1.5)  | 13.7 (2.4) |
| Refinement:    |        |         |                           |
| Resolution (Å) | 40-2.4 | 40-2.6  | 40-3.0                    |
| R cryst, Rfree | 0.201, 0.236 | 0.200, 0.232 | 0.223, 0.246 |
| Reflections (working/test) | 56556/2935 | 45862/2599 | 29924/1611 |
| Protein atoms  | 2540   | 2510    | 2517                      |
| Solvent molecules | 431    | 377     | 15                       |
| Rmsd bond lengths (Å) | 0.013  | 0.010   | 0.011                     |
| Rmsd angles (°) | 2.0    | 2.0     | 1.7                       |
| Rmsd B (Å²) (mm/ms/ss)² | 5.12/5.28/6.93 | 4.54/5.13/5.85 | 3.97/3.58/4.38 |
| B> p<rotein (Å²) | 63.8   | 68.5    | 71.0                      |
| <B> solvent (Å²) | 64.4   | 62.8    | 49.6                      |
| Ramachandran plot: |        |         |                           |
| Most favored (%) | 98.9   | 100.0   | 99.6                      |
| Generously allowed (%) | 1.1    | 0.0     | 0.4                       |
| Disallowed (%)  | 0.0    | 0.0     | 0.0                       |

*Root-mean-squared deviation (Rmsd) from ideal bond lengths and angles and Rmsd in B-factors of bonded atoms. \(^{2}\)mm, main chain-main chain; ms, main chain-side chain; ss, side chain-side chain.
Figure 1. Structure of human β2GPI and current mechanism of antigen-antibody recognition for pathogenic anti-DI antibodies in APS (A) Color-coded domain structure of human β2GPI (pβ2GPI). β2GPI consists of 326 amino acids organized into five domains (DI-V) connected by four short linkers, Lnk1 (residues 61-64); Lnk2 (residues 119-122), Lnk3 (residues 182-185) and Lnk4 (residues 242-244) (64). Domains I-IV are canonical complement control protein (CCP) domains, each containing two disulfide bonds. In contrast, DV is aberrant, consisting of one extra disulfide bond and a 19-residue hydrophobic loop that is responsible for anchoring the protein to negatively charged phospholipids (47, 65). Similar to other CCP-containing proteins, β2GPI is also heavily glycosylated, bearing four N-linked and one O-linked glycosylations located at positions T130, N143, N164, N174, and N234 that account for ~20% of the total protein mass. The position of the O- and N-linked glycosylations is shown as triangles (▲) and stars (*), respectively. (B) Based on previous studies, β2GPI is believed to adopt an O-circular (27, 28), an S-twisted (26) and a J-elongated conformation (24, 25). The J-open conformation results upon interaction of DV with the phospholipids exposing the cryptic epitope R39-R43 (purple) to the solvent. The O-circular form features an intramolecular interaction between DI (blue) and DV (yellow) with amino acids K19, R39 and R43 in DI potentially making contact with K305 and K317 in DV. The S-twisted conformation features a rotation of the DI/DII module, brokered by Lnk2, relative to the rest of the protein resulting in the blockade of the immunogenic region R39-R43 by the N-linked glycosylation (orange line).

Figure 2. Functional characterization of human recombinant β2GPI. (A) Color-coded domain structure of the recombinant variants used in this work (i.e., LT-β2GPI, hrβ2GPI and ST-β2GPI) highlighting the position and chemical composition of the N-terminal tag. The long tag (LT, yellow) is composed of three parts: i) a calcium-dependent epitope for the monoclonal antibody HPC4 (EDQVDPRLLIDGK); ii) a site-specific biotinylation sequence (AviTag); and iii) a conventional enterokinase recognition site (DDDDK). Two flexible linkers (i.e., GGGS) were introduced to separate the three functional units of the tag to avoid the formation of secondary structure and ensure exposure of the tag to solvent. Removal of the LT with enterokinase generates hrβ2GPI. The short tag version of β2GPI contains only the HPC4 purification tag (purple). (B) SDS-PAGE analysis of the recombinant proteins (sample 1=LT-β2GPI; sample 2=hrβ2GPI; sample 3=ST-β2GPI) and plasma purified β2GPI (sample 4) before (left) and after (right) the removal of the glycosylations. Protein Deglycosylation Mix II from NEB was used to remove O-linked and N-linked glycosylations under denaturing conditions. Chemical identity was verified by N-terminal sequencing and results are as follows: band 1=EDQVD; band 2=GRT; band 3=EDQVD; band 4=GRTC. (C) Representative sensograms of the interaction
between LT-β2GPI and liposomes (PC:PS 80:20) monitored by SPR. Liposomes were immobilized on a L1 chip and soluble β2GPI (0-2 μM) was used in the fluid phase (D) Dose-dependent curves quantifying the interaction of LT-β2GPI (red circles), hrβ2GPI (blue circles) and ST-β2GPI (green circles) and pβ2GPI (gray circles) with liposomes monitored by SPR. Affinity values (K\text{d}) are 0.19±0.05 μM for LT-β2GPI, 0.33±0.08 μM for hrβ2GPI; 0.22±0.08 μM for ST-β2GPI and 0.32±0.09 μM for pβ2GPI. No significant binding was observed with liposomes entirely made of PC (light gray circles). Each experiment was repeated at least three times, using two distinct batches of proteins. (E) Reactivity of immobilized LT-β2GPI (red bars), hrβ2GPI (blue bars) and ST-β2GPI (green bars) and pβ2GPI (gray bars) against IgG anti-β2GPI antibodies followed by ELISA. Comparisons between 2 groups were performed using a Two-sample t-Test. Results were considered significant at p<.05 (*).

**Figure 3. X-ray crystal structures of human recombinant β2GPI.** X-ray crystal structures of (A) hrβ2GPI (blue), (B) pβ2GPI (green) and (C) ST-β2GPI (green) solved at 2.6 Å, 3.0 and 2.4 Å resolution. All three structures document similar elongated conformations of the protein spanning ~140 Å in length. Disulfide bonds are highlighted in yellow. (D) Zoom-in of three extra residues (DGK) belonging to the N-terminal tag preceding the natural sequence of β2GPI (\text{GRTC}) were exclusively found in the structure of ST-β2GPI, as expected. The electron density 2F\text{o}-Fc map is countered at 1.0σ. (E) Structural architecture of the epitope R39-R43 in DI highlighting the position and interactions of R43 (magenta stick) with the nearby residues R39, G41 and T57. Hydrogen bonds between the guanidinium group of R43 and neighboring residues are shown in black. Of note, the conformation of this segment is not involved in crystal contacts and is conserved in all the available crystal structures of β2GPI solved thus far, despite the high salt concentrations in which the crystals grow, suggesting that this is a genuine structural feature of β2-GPI.

**Figure 4. Location and structural role of the N-linked glycosylations.** (A) Superposition of five X-ray crystal structures of β2GPI highlights structural similarities yet diversity of the phospholipid binding loop in DV (residues 308-319). (B) Extra electron density detected in the structure of pβ2GPI solved at 2.4Å resolution attributed to the N-linked glycosylations. The domains of β2GPI are color coded as shown in Figure 1. Guided by mass spectrometry analyses(66), we modeled the following sugar sequences: Gal\text{2}GlcNAc\text{2}Man\text{3}GlcNAc\text{2} at N143, Gal\text{2}GlcNAc\text{2}Man\text{3}GlcNAc\text{2} at N164, GlcNAc\text{2} at N174 and Gal\text{3}GlcNAc\text{2} at N234. The presence of a putative O-linked glycosylation at T130 could not be confirmed because of weak density. (C) Side and top (D) views of the N-glycosylations surrounding DIII. The electron density 2F\text{o}-Fc map is countered at 0.8σ. (E) Asymmetric distribution of the electrostatic potential displaying positive (blue) and negative (red) clusters with a -2.0-2.0-intensity scale. The N-glycosylations are shown as magenta sticks.
**Figure 5. smFRET measurements of β2GPI in solution.** (A) Structure-based design of the FRET constructs S13C/S112C, S13C/S312C, S112C/S312C, and S190C/S312C used in this study. Ser residues mutated to Cys for conjugation with the thiol-reactive dyes AF555 and AF647 used in smFRET measurements are indicated by red spheres. (B) FRET couples are listed with their respective domains. Ca-Ca distances obtained from the crystal structure of hrβ2GPI. (C) After labeling and gel filtration, selective incorporation of the fluorescence dyes was verified by loading the proteins (samples 2-5) alongside β2GPI WT (sample 1), into a gradient 4–12% polyacrylamide gel in the presence of SDS and visualized by Coomassie Brilliant Blue R-250 (black and white) or fluorescence intensity by exciting donor at 532 nm (red panel) and acceptor at 640 nm (blue panel). (C) smFRET histograms of the mutants S13C/S312C, S112C/S312C, S13C/S112C, and S190C/S312C labeled with AF555/647 measured in Tris 20 mM pH 7.4, 145 mM NaCl, 0.1% Tween 20 for 1 hour at room temperature at a concentration of 100 pM. Populations were fit to a single Gaussian distribution (black lines). FRET efficiency values and calculated distances are indicated. The theoretical shot noise peak highlighting conformational heterogeneity for the FRET pair 190/312 is shown as dotted line.

**Figure 6. Elongated conformation of β2GPI revealed by SAXS.** Scattering profiles (A) and pair distribution functions (B) for pβ2GPI (black) and ST-β2GPI (magenta) collected at 2 mg/ml under physiological conditions (Tris 20 mM pH 7.4, 145 mM NaCl). The calculated values of the radius of gyration, Rg, are very similar for pβ2GPI and ST-β2GPI. The blue curve in panel B, which is significantly different from the experimental scattering profiles, represents the theoretical pair distribution function for a hypothetical circular conformation.

**Figure 7. Exposure of DI revealed by MBB2 binding and mutagenesis studies.** (A) Interaction of MBB2 and LT-β2GPI monitored by SPR. MBB2 was immobilized onto a CM5 chip using NHS/EDC chemistry to a final density of 6000 RU. A solution of LT-β2GPI (0-20 µM) in Tris 20 mM pH 7.4, 145 mM NaCl 0.01% Tween 20 was injected at 30 µl/min for 60 sec to observe binding followed by 60 sec dissociation in running buffer. (B) Dose-dependent curves quantifying the interaction of LT-β2GPI (red circles), hrβ2GPI (blue circles) and ST-β2GPI (green circles) and pβ2GPI (gray circles) with MBB2 monitored by SPR. Affinity values (Kd) are 2.2±0.5 µM for LT-β2GPI, 2.1±0.6 µM for hrβ2GPI; 1.8 ±0.5 µM for ST-β2GPI and 1.4±0.6 µM for pβ2GPI. Each experiment was repeated at least three times, using two distinct batches of proteins. (C) Effect of the ionic strength. SPR binding experiments were performed at 15, 75, 150 and 300 mM NaCl. Analysis of the slope of the linear fit of the association constant vs Na+ reveals a strong dependency of the ionic strength and the presence of at least 2.0 ionic contacts (salt bridges) in the complex(66, 67). (D) Location of residues R39, R43 and K44 targeted by site-directed
mutagenesis. (E) SPR analysis of $\beta_2$GPI WT and mutant R39A/R43A/RK44A reveal that residues R39 and R43 are critical for MBB2 binding. (F) SPR analysis of $\beta_2$GPI WT (black circles), D1 (green circles), $\beta_2$GPIΔ(120-122) (magenta circles) and deg$\beta_2$GPI (blue circles). Removal of DII-DV, perturbation of Lnk2 and removal of the glycosylations do not affect the binding affinity of MBB2 towards $\beta_2$GPI. This indicates that 1) D1 is primed for autoantibody recognition in solution 2) the exposure of residues R39 and R43 in D1 is independent of the conformation of Lnk2 and 3) Lnk2 is not an epitope of MBB2. Given the different molecular weight between the constructs, the binding curves are reported as normalized fraction bound vs analyte to facilitate comparison. Each experiment was repeated at least three times, using two distinct batches of proteins.
Figure 1

A

B

O-Circular form
S-twisted form
J-elongated form

Di, DII, DIII, DIV, DV

R39-R43 epitope
Hidden
Negatively charged phospholipids

Available
Figure 2

A diagram showing the structure and reactions of different GPI (GM1) fragments labeled as LT-β₂GPI, hrβ₂GPI, and ST-β₂GPI.

B) A gel electrophoresis image with arrows indicating the bands for N-term seq 1, 2, 3, and 4 with and without sugars.

C) A graph depicting the response over time with LT-β₂GPI and hrβ₂GPI concentrations indicated.

D) A graph showing a dose-response curve for LT-β₂GPI, hrβ₂GPI, ST-β₂GPI, and pβ₂GPI.

E) A bar graph with time (sec) on the x-axis and OD at 450 nm on the y-axis, comparing different samples labeled P1 through P4.
Figure 3

Figure 3 shows the structures of hrβ₂GPI (2.6Å), pβ₂GPI (2.4Å), and ST-β₂GPI (3.0Å) with labeled amino acid regions. The structures are color-coded and labeled to highlight specific regions such as the N-terminus, DI (1-60), DII (65-118), DIII (123-181), DIV (186-241), and DV (245-236). The images also include close-up views of specific amino acids and interactions, such as Gly, Asp, Lys, Arg, Thr, and Cys.
Figure 5

| FRET pair       | Domains | Distance (Å) |
|-----------------|---------|--------------|
| S13C/S112C      | DI-DII  | 24           |
| S13C/S312C      | DI-DV   | 119          |
| S112C/S312C     | DII-DV  | 109          |
| S190C/S312C     | DIV-DV  | 46           |

Counts

|                | FRET Efficiency |
|----------------|-----------------|
| 13/312         | E_{FRET} = 0.05 |
|                | (>80 Å)         |
| 112/312        | E_{FRET} = 0.05 |
|                | (>80 Å)         |
| 13/112         | E_{FRET} = 0.92 |
|                | (<35 Å)         |
| 190/312        | E_{FRET} = 0.26 |
|                | (~60 Å)         |
Figure 6
Figure 7

A. Response vs. Time (sec) for LT-β₂GPI

B. RU vs. β₂GPI (μM) for LT-β₂GPI, hrβ₂GPI, ST-β₂GPI, pβ₂GPI

C. Log Kₐ vs. Log [Na⁺] (M) for LT-β₂GPI

D. Molecular structure showing Lys-44, Arg-43, and Arg-39

E. RU vs. β₂GPI (μM) for WT (Kₐ=2.2 μM) and R39A/R43A/K44A (Kₐ=60.5 μM)

F. Normalized Fraction Bound vs. β₂GPI (μM) for β₂GPI WT, DI, β₂GPI Δ(120-122), degβ₂GPI