Site-Directed Mutagenesis of Recombinant Human β_2-Glycoprotein I Identifies a Cluster of Lysine Residues That Are Critical for Phospholipid Binding and Anti-Cardiolipin Antibody Activity[1]

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β_2-Glycoprotein I (β_2GPI) is a phospholipid-binding serum protein with anticoagulant properties. It plays a vital role in the binding of anti-cardiolipin Abs purified from patients with autoimmune disease when assayed in a cardiolipin (CL) ELISA. Based on a three-dimensional model of β_2GPI, electrostatic calculations, and earlier peptide studies, a highly positively charged amino acid sequence, Lys^{282}-Asn-Lys-Glu-Lys-Lys^{287}, located in the fifth domain of β_2GPI, has been predicted to be the phospholipid binding site. We tested this hypothesis by site-directed mutagenesis of residues in the predicted phospholipid binding site and by assessing the mutants for phospholipid binding and anti-β_2GPI activity. A single amino acid change from Lys^{286} to Glu significantly decreased the binding of β_2GPI to CL. Double and triple mutants 2k (from $Lys^{286, 287}$ to $Glu^{286, 287}$), 2ka (from $Lys^{284, 287}$ to $Glu^{284, 287}$), and 3k (from $Lys^{284, 286, 287}$ to $Glu^{284, 286, 287}$) possessed no binding of Ab to β_2GPI in a CL ELISA, as well as no inhibitory activity on the binding of iodinated native β_2GPI to CL. These results indicate that the residues Lys^{284}, Lys^{286}, and Lys^{287} in the fifth domain of β_2GPI are critical for its binding to anionic phospholipids and its subsequent capture for binding of anti-β_2GPI Abs. *The Journal of Immunology,* **1996, 157: 3744–3751.**

Human β_2-glycoprotein I (β_2GPI)[3] is a phospholipid-binding plasma protein that is essential for the binding of anti-cardiolipin (aCL) Abs purified from patients with autoimmune disease to β_2GPI complexed to cardiolipin (CL) (1–4). This has provoked much interest, as it is in this group of patients that thromboembolic complications have been reported (5–8). Although there has been considerable controversy as to the exact nature of the antigenic epitope to which anti-phospholipid (aPL) Abs bind, it has become clear that β_2GPI is the target Ag (2, 9–12). These Abs preferentially bind β_2GPI that has been immobilized on anionic phospholipid surfaces (13, 14). Binding in the fluid phase is weak and requires high concentrations of β_2GPI (14).

β_2GPI with a molecular mass of 50 kDa, as estimated by SDS-PAGE, was first purified by Schultze et al. (15). The complete amino acid sequence was published by Lozier et al. (16), and the complete nucleotide sequence, by cDNA cloning, has also been reported (17–19). These sequencing analyses show that the mature β_2GPI protein is composed of 326 amino acids preceded by a putative leader sequence of 19 amino acids. This protein also has five potential *N*-glycosylation sites, abundant proline residues, and 11 internal disulphide bonds.

Although its physiologic role remains unclear, β_2GPI binds to negatively charged substances, such as phospholipids (20), heparin (21), lipoprotein (22), and activated platelets (23), and inhibits the intrinsic blood coagulation pathway (24) and ADP-dependent platelet aggregation (25).

It has been predicted on the basis of homology modeling that the amino acid sequence Lys^{282}-Asn-Lys-Glu-Lys-Lys^{287}, located in the fifth domain of β_2GPI, is involved in interaction with CL (17, 26). Recently, binding and inhibition studies using synthetic peptides spanning the fifth domain of β_2GPI provided some experimental evidence for this prediction (27).

To study the interaction between native β_2GPI and anionic phospholipids such as CL in more detail, we first calculated (28) a three-dimensional model of the fifth domain of β_2GPI (β_2GPI-5), relying on its similarity to the 15th module from human factor H, whose three-dimensional structure has been determined by NMR (29). The electrostatic calculations (30) with this model confirmed that the loop Lys^{282}-Lys^{287} is likely to be part of the CL binding site. To test this prediction, the cDNA for human β_2GPI was inserted into the baculovirus viral DNA BacPAK 6 for expression in insect cells (*Spodoptera frugiperda* (Sf)). Site-directed mutagenesis was then performed to assess the role of the individual amino acids in the Lys^{282}-Lys^{287} loop in the phospholipid binding and anti-β_2GPI activity. It was found that residues Lys^{284}, Lys^{286}, and Lys^{287} were indeed critical for β_2GPI binding to anionic phospholipids, but not crucial for direct binding of β_2GPI by anti-β_2GPI Abs.

Materials and Methods

The three-dimensional model and electrostatic potential of the fifth domain of β_2GPI (β_2GPI-5)

A detailed comparative molecular modeling study of β_2GPI-5 was combined with electrostatic calculations to obtain specific predictions as to which residues do and do not participate in CL binding. The template structure for comparative modeling was that of the 15th domain of the human factor H (H15), whose conformation has been determined by NMR

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[3] Abbreviations used in this paper: β_2GPI, β_2-glycoprotein I; aCL, anti-cardiolipin; aPL, anti-phospholipid; CL, cardiolipin; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; Sf, *Spodoptera frugiperda*.

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(29) (Brookhaven Protein Data Bank code 1HFH). Due to the low sequence similarity between β_2GPI-5 and H15, the alignment of the sequences of β_2GPI-5 (84 residues)[4] and H15 (62 residues) was derived by hand, strongly relying on the equivalence of the two disulfide bonds, bonds 3–54 and 39–64 in β_2GPI-5 to disulfides 5–50 and 36–61 in H15, as discussed previously (26). The alignment between β_2GPI-5 and H15 was used as input for MODELLER-1[5] (28) to produce a model of β_2GPI-5 containing all main chain and side chain nonhydrogen atoms.

The electrostatic potential on the surface of β_2GPI-5 and its mutants were calculated with GRASP (30), a computer program that uses the finite difference method to solve the linearized Poisson-Boltzmann equation. A net charge of −1 was assigned to each Asp and Glu residue, and a net charge of +1 to each Lys and Arg residue. Each His was assigned a neutral charge because β_2GPI is active in plasma at pH of ~7.2. The models with all hydrogen atoms and the partial charges from the CHARMM-22 force field (M. Karplus et al., manuscript in preparation) were used for electrostatic calculations.

Bacterial strains and plasmid DNA

The *Escherichia coli* strains used for this study were JM109 (endA1, recA1, gyrA96, thi, hsdR17, relA1, supE44, D (lac-proAB), (F', traD36, ProAB, LacIZ D M15)) and ES1301 mutants (lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC, IN(rrnD-rrn-E)).

Plasmid dsDNA was isolated using the Wizard maxipreps DNA purification system (Promega Corp., Madison, WI).

Construction of transfer vectors

A *Xba*I-*Pst*I fragment containing a full-length human β_2GPI cDNA was obtained from a pβ_2I-1 plasmid (17) and inserted into the same restriction sites on the baculovirus transfer vector pBacPAK 9 (Clontech Laboratories, Palo Alto, CA). This recombinant plasmid is referred to as pBac β.

Cells and virus

Sf insect cells were maintained in serum-free medium, Sf-900II (Life Technologies, Gaithersburg, MD). A BacPAK 6 viral DNA (Clontech Laboratories) that was modified from AcMNPV DNA has been digested with Bsu361 to remove part of an essential viral gene, forcing recombination with cotransfected transfer vectors. Nearly 100% of the virus recovered contained the target gene.

Generation and purification of recombinant viruses

A quantity amounting to 2×10^6 Sf cells was cotransfected with 100 ng of BacPAK 6 viral DNA and 500 ng of the transfer vectors pBac β by the Lipofectin method in a 60-cm^2 tissue culture dish. A pure clone of a recombinant virus was obtained by diluting the cotransfection supernatant (collecting 4 days after the infection) containing progeny viruses and performing a plaque assay to produce individual plaques.

Expression of β_2GPI in Sf cells

Sf cells (5×10^6) grown in a monolayer were infected by recombinant virus, with multiplicity of infection of ~10 in 100-cm^2 tissue culture dishes. The infected cells were cultured in 10 ml of serum-free medium, Sf-900II, for 3 to 5 days at 27°C. Ten microliters of culture supernatant were collected for electrophoresis, and subjected to SDS-PAGE and Western blots.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot were performed as previously described (31).

Site-directed mutagenesis

Reactions were performed using an oligonucleotide-directed in vitro mutagenesis kit, Altered Sites in vitro mutagenesis system (Promega Corp.). Four mutagenic oligonucleotide primers were the following: 1k, 5'-AGGAAGAG AAGTGTAGCTATACAGA-3' (for mutation of Lys^{286} to Glu^{286}); 2k, 5'-A GGAAGAGGAGTGTAGCTATACAGA-3' (for mutation of $Lys^{286, 287}$ to $Glu^{286, 287}$); 2ka, 5'-TGCAAAAATGAGGAAAAGGAGTGTAGCTATAC-3' (for mutation of $Lys^{284, 287}$ to $Glu^{284, 287}$); and 3k, 5'-CTGCAAAAATGA GGAAGAGGAGTGTAGCTATAC-3' (for mutation of $Lys^{284, 286, 287}$, to $Glu^{284, 286, 287}$). The mutation in each primer is underlined. The mutants were identified by dideoxy sequencing from double-stranded, alkali-denatured templates, utilizing the T7 sequencing kit (Pharmacia, Uppsala, Sweden) with an internal primer. The mutant recombinant fragments were released from pALTER vector and subcloned to pBacPAK 9 transfer vector following the strategy described for construction of pBac β.

Preparation of recombinant wild-type and mutants of β_2GPI

Recombinant wild-type and mutants of β_2GPI were purified from the culture supernatant of β_2GPI-transfected Sf21 cells by affinity chromatography using polyclonal anti-β_2GPI Ab (31, 32). Culture supernatant was diluted with 10 mM sodium phosphate (PBS), pH 7.4, containing 150 mM NaCl, and was applied to the anti-β_2GPI affinity column. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5. The eluted fractions were neutralized immediately with 2 M Tris, pH 8.0, and the eluted protein was concentrated with a Centricon 10 ultrafilter (Amicon, Beverly, MA). The concentrated eluants were dialyzed against PBS. The purity of the sample was assessed by SDS-PAGE.

N-terminal amino acid sequencing

Wild-type and mutants of β_2GPI (200 pmol) were subjected to Automated Edman sequencing using an Applied Biosystems (Foster City, CA) sequencer (model 470A). The generated phenylthiohydantoin (PTH) amino acids were identified on an on-line HPLC system, Applied Biosystems model 120A, using conditions as specified by Applied Biosystems.

Iodination of protein

Five micrograms of native β_2GPI and recombinant β_2GPI were radiolabeled with 1 mCi of $Na^{125}I$ (Ansto, Sydney, Australia) using the lactoperoxidase method (33). The radiolabeled proteins were separated from free ^{125}I on a Bio-Gel P-6 DG spherical polyacrylamide gel column. The sp. act. of labeled proteins was ~6×10^7 cpm/μg.

Binding of native ^{125}I-labeled β_2GPI, recombinant wild-type ^{125}I-β_2GPI, and ^{125}I mutants of β_2GPI to CL and PC

For binding experiments, Immulon II microtiter wells (Dynatech Labs., Chantilly, VA) were coated with 30 μl of CL at 30 μg/ml in ethanol and dried under vacuum; PC at the same concentration was used as a control. Nonspecific binding sites were blocked with 200 μl of 1% milk/0.3 gelatin/PBS for 1 h at room temperature. The wells were washed three times with PBS. Fifty microliters (6×10^4 cpm) of native ^{125}I-β_2GPI, wild-type ^{125}I-β_2GPI, and ^{125}I mutants of β_2GPI in 0.3% gelatin/PBS were added to each well, respectively (in four replicates). The samples were incubated at room temperature for 3 h, and removed by aspiration. Finally, the wells were washed three times with PBS, separated, and placed individually into disposable test tubes for counting in a gamma counter. The difference in binding of the different preparations of ^{125}I-β_2GPI to CL was assessed by Student's t test.

Native ^{125}I-β_2GPI binding to CL: competitive inhibition assay using recombinant wild-type and mutants of β_2GPI

Experiments were performed to determine whether the recombinant wild-type and mutants of β_2GPI could competitively inhibit the binding of native ^{125}I-β_2GPI to CL. A fixed amount of native ^{125}I-β_2GPI (115,000 cpm) was added to CL-coated plates, in either buffer alone (0.3% gelatin/PBS) or with increasing concentrations of unlabeled recombinant wild-type and mutants of β_2GPI (8.75, 17.5, 35, 70, 140, and 280 μg/ml). The total volume of the reaction mixture was 50 μl/well (triplicates per sample). The plate was then processed as described above for the direct binding study.

Binding of anti-β_2GPI Abs to native, wild-type, and mutants of β_2GPI in a phospholipid-free system

An anti-β_2GPI ELISA was performed as described by Wang et al. (12). Microtiter plates were irradiated (10 kGy) by the Australian Nuclear Science and Technology Organization (Sydney, Australia). The wells were coated with 50 μl of native, wild-type, and mutants of β_2GPI or haptoglobin (40 μg/ml in carbonate buffer, pH 9.6) overnight at 4°C, and processed as previously described (12).

Modified CL ELISA for anti-β_2GPI activity

To assay the anti-β_2GPI activity of recombinant β_2GPI, the modified CL ELISA system was used, as previously described (27).

The anti-β_2GPI Abs used in this assay were purified by sequential CL-affinity (34) and cation-exchange (1) chromatography from serum obtained from two 30-yr-old female patients, one with a history of deep venous

[4] The residue numbers 1–84 in β_2GPI-5 correspond to residues 243–326 in β_2GPI.

[5] MODELLER is available by anonymous file transfer protocol from http://guitar.rockefeller.edu, and also as part of QUANTA and INSIGHT (MSI, San Diego, CA).

FIGURE 1. Amino acid alignment of β_2GPI-5 and the 15th domain of the human factor H. The numbers in the bottom line refer to the residues in β_2GPI-5.[4] The stars indicate the Lys residues that were mutated to Glu residues.

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1HFH-15   EKIPCSQPPQIEHGTINSSRSSQ-----ESYAHGTKLSYTCEGGFR-ISEENETTCYM-GKWSS-PPQCE------------------
β2 GPI-5  --ASCKLPVKKATVVYQGERVKIQEKFKNGMLHGDKVSFFCKNKEKKCSYTEDAQCID-GTIE--VPKCFKEHSSLAFWKTDASDVKPC
                    10        20        30        40 * **   50        60        70        80
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thrombosis and recurrent miscarriage, and the other with both arterial and venous thrombosis. The Abs were of the type that required β_2GPI to bind in a modified CL ELISA (1).

Results

Three-dimensional model of β_2GPI-5 and its electrostatic properties

A three-dimensional model of the fifth domain of β_2GPI has been constructed with a comparative protein modeling technique based on satisfaction of spatial restraints (28). The NMR structure of H15 (29) was used as a template (Fig. 1). The fold of the β_2GPI-5 model consists of eight strands, organized in two distorted β-sheets with long coiled regions connecting the strands (Fig. 2). There are no helices. The model for the last 19 residues in the module is speculative because there is no equivalent region in the H15 template structure. Most of the positively charged side chains (14 of 16) are located on the surface of two regions. The first of these regions is defined by segments 40–46, 63–66, and 81–84 (*top face* of the module in Fig. 2). The second region is defined by one long and wide omega loop 3–28 (*left face* in Fig. 2). Most of the negatively charged residues (8 of 11) are located in segments 33, 50–62, and 67–80 (*right face* in Fig. 2). Both positively charged faces are likely to attract negatively charged ligands such as CL (Fig. 3*A*). However, since the top positively charged face contains peptide 284–288, that is known to bind CL (27), the three central charges in this particular region are predicted to be part of the binding site for CL in the intact β_2GPI-5 domain. Moreover, the mutation of these three Lys residues to Glu residues is predicted to prevent the interaction between CL and β_2GPI-5 (Fig. 3*B*).

Expression of human β_2GPI in insect cells

In the present studies, the cDNA for human β_2GPI (1.15 kb) containing 978 bp of the coding region, 57 bp of the putative signal sequence, and 5′- and 3′-untranslated regions was inserted into the BacPAK 6 viral DNA. Sf21 cells infected with recombinant virus and wild-type virus were cultured in a serum-free medium, Sf-900II. β_2GPI was first detected at ~24 h after infection, and reached maximal expression levels at ~72 h. SDS-PAGE analysis of culture supernatants showed that cells infected by recombinant virus produced a 40-kDa protein, which was absent in mock-infected cells or cells infected by wild-type virus. The amount of β_2GPI obtained from the culture supernatant was ~10 μg/ml.

Site-directed mutagenesis

By the Altered Site in vitro mutagenesis system, four mutants were generated, mutant 1k (from Lys^{286} to Glu^{286}), 2k (from $Lys^{286, 287}$ to $Glu^{286, 287}$), 2ka (from $Lys^{284, 287}$ to $Glu^{284, 287}$), and 3k (from $Lys^{284, 286, 287}$ to $Glu^{284, 286, 287}$). Each was expressed in insect cells.

Purification of recombinant wild-type and mutants of β_2GPI

The recombinant β_2GPI secreted into the serum-free culture medium was purified from culture supernatant by immunoaffinity chromatography. The recovery of eluted β_2GPI from the affinity column was ~50%. Purified recombinant β_2GPIs were subjected to SDS-PAGE and were analyzed by staining with Coomassie brilliant blue R-250 (Fig. 4*A*).

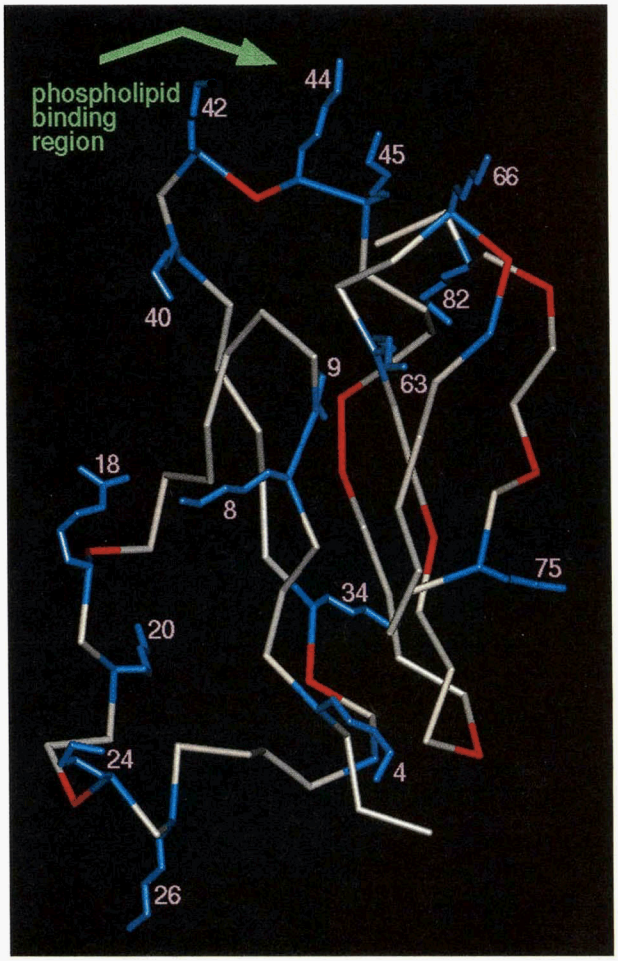

FIGURE 2. Main chain trace of the three-dimensional model of β_2GPI-5. The positively charged side chains (Lys, Arg) are shown in blue. The His side chains are not shown, but their main chain is colored blue. The main chains of the negatively charged residues (Asp, Glu) are shown in red. The numbering of the Lys and Arg side chains corresponds to that in Figure 1. The phospholipid binding site is indicated by an arrow. The figure was prepared by program QUANTA (MSI, San Diego, CA).

SDS-PAGE indicated that all of the mutants were of the same size as the wild-type β_2GPI with a band at 40 kDa (Fig. 4*A*). Western blot analysis of gels with an affinity-purified rabbit polyclonal anti-β_2GPI Ab (31) indicated that there were two immunoreactive bands, for both the native and recombinant preparations of β_2GPI: a major band of 40 kDa and a minor band at ~80 kDa for the recombinant preparations, and a major band of 50 kDa and a minor band at ~100 kDa for the native preparation (Fig. 4*B*). The same result was obtained using a mouse anti-β_2GPI mAb (data not shown). The higher m.w. immunoreactive band is thought to be dimers of β_2GPI, and has previously been reported for a phenylisothiocyanate-modified preparation of native β_2GPI (35).

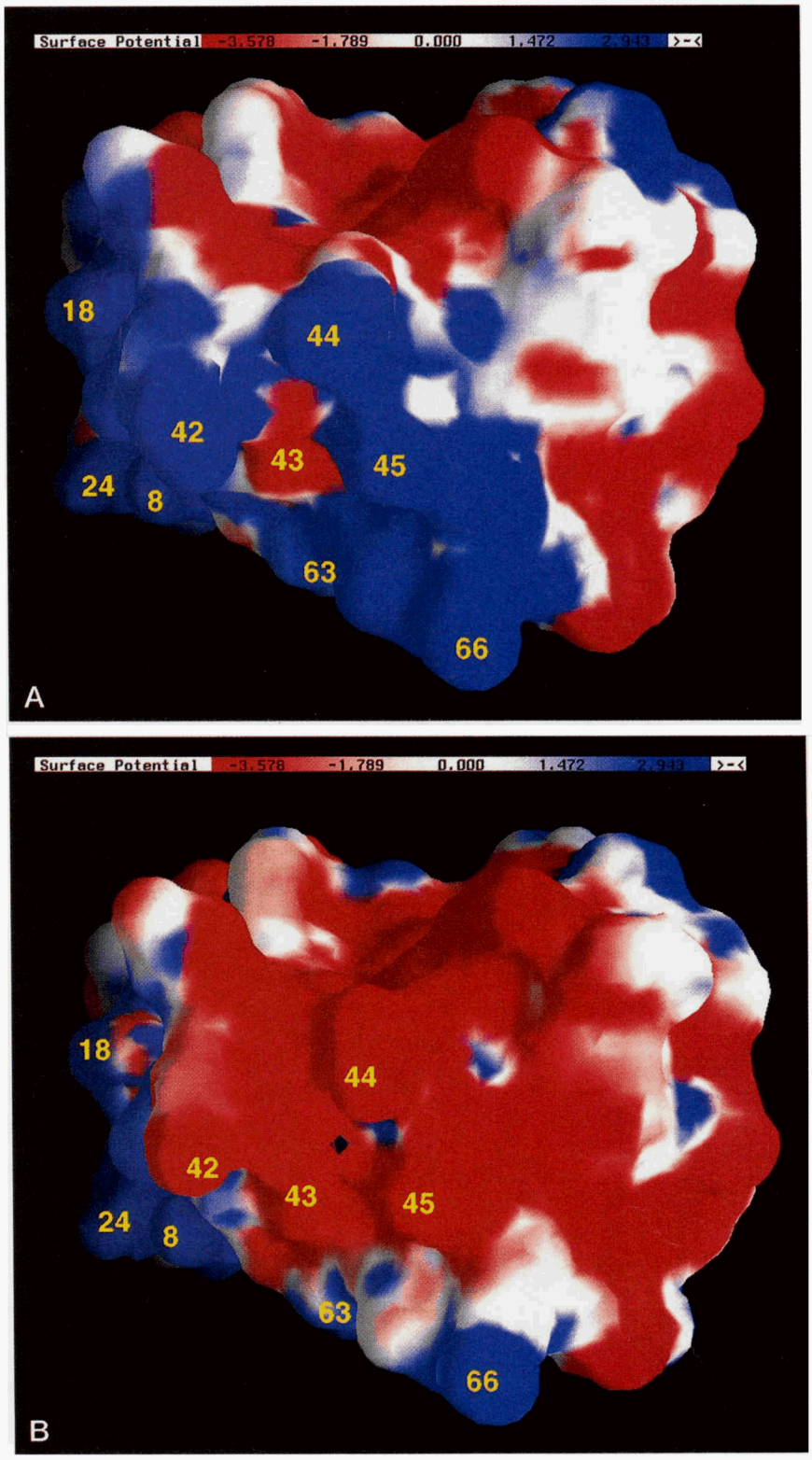

FIGURE 3. Electrostatic potential at the phospholipid binding region of native and mutant β_2GPI-5. *A*, Native β_2GPI-5 at neutral pH. *B*, $Lys^{42/44/45} \rightarrow$ Glu triple mutant at neutral pH. The molecular surfaces of the models are colored by the electrostatic potential, as shown by the color bar on each panel (in units of kT; 1 kT unit = 0.58 kcal/electron mol). The figures were prepared by program GRASP (30), using the relative dielectric constants of 2 and 78 for protein and solvent, respectively, and the salt concentration of 150 mM. The positively and negatively charged residues are numbered in yellow. Relative to Figure 2, β_2GPI-5 is viewed from the *top*.

Determination of N-terminal amino acid sequence

Amino-terminal sequencing confirmed that the first 18 amino acids of each of the affinity-purified proteins from wild-type and mutants 1k, 2k, 2ka, and 3k corresponded to the N terminus of native β_2GPI.

Binding of native ^{125}I-β_2GPI, wild-type ^{125}I-β_2GPI, and ^{125}I mutants of β_2GPI to CL and PC

^{125}I-β_2GPI was able to bind to wells coated with the negatively charged phospholipid CL, but not neutral phospholipid PC (Fig. 5). There is no significant difference in binding to CL of wild-type and

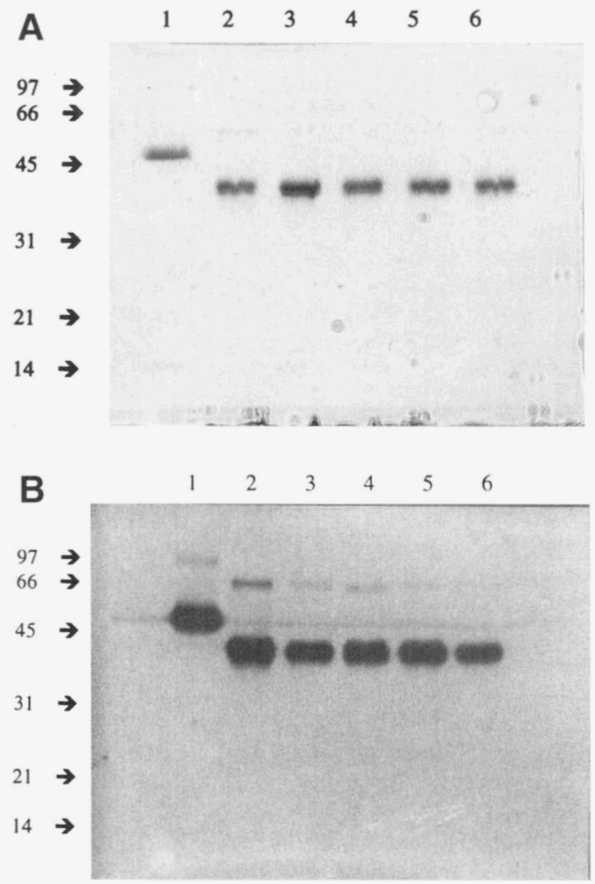

FIGURE 4. SDS-PAGE (*A*) and Western blotting (*B*) analysis of purified β_2GPI. The relative mobilities are shown on the *left*. *Lane 1*, native β_2GPI purified from human serum. *Lane 2*, recombinant wild-type β_2GPI. *Lane 3*, mutant 1k. *Lane 4*, mutant 2k. *Lane 5*, mutant 2ka. *Lane 6*, mutant 3k.

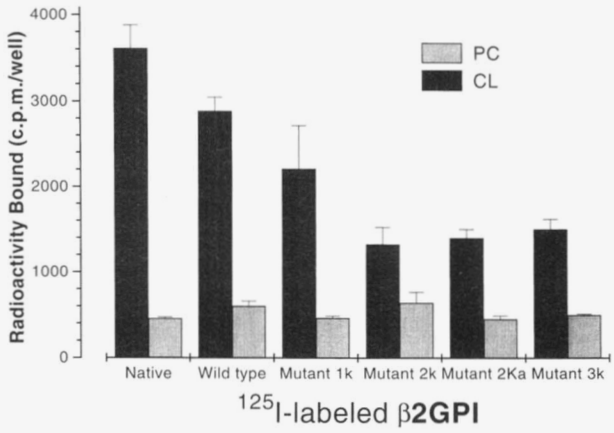

FIGURE 5. Binding of native ^{125}I-β_2GPI, wild-type ^{125}I-β_2GPI, and ^{125}I mutants of β_2GPI, to microtiter wells coated with CL or PC, and bound radioactivity was measured as described in *Materials and Methods*. Results are expressed as total cpm, and represent the mean (± SE) of four samples.

native ^{125}I-β_2GPI (p = 0.1312); however, there was a significant difference in binding to CL between native ^{125}I-β_2GPI and ^{125}I-β_2GPI mutant 1k (p = 0.041), 2k (p = 0.002), 2ka (p = 0.002), and 3k (p = 0.0018).

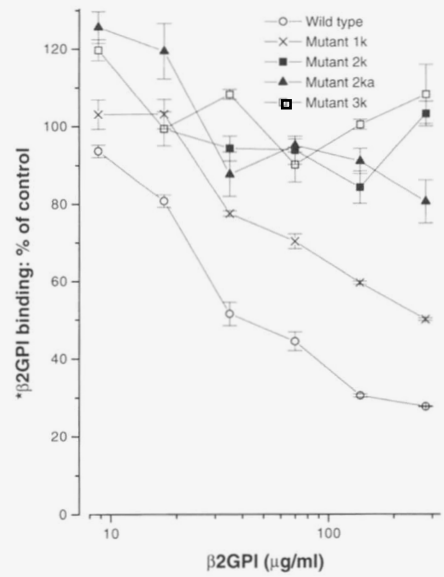

FIGURE 6. The effect of wild-type and mutants (1k, 2k, 2ka, and 3k) of β_2GPI on the binding of native ^{125}I-β_2GPI to CL-coated microtiter wells. The data are expressed as a percentage of the uninhibited control (no added β_2GPI), and represent the mean (± SE) of three samples.

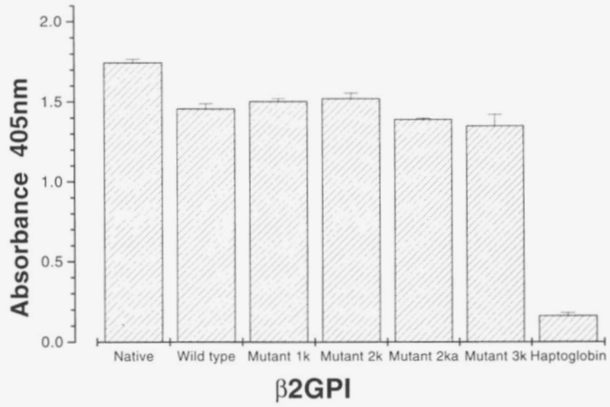

FIGURE 7. Binding of affinity-purified anti-β_2GPI Ab at 10 μg/ml from one autoimmune patient to different preparations of β_2GPI (40 μg/ml) coated on an irradiated microtiter plate.

Inhibition of native ^{125}I-β_2GPI binding to CL by recombinant wild-type β_2GPI and mutants of β_2GPI

Native ^{125}I-β_2GPI bound CL in a manner similar to that previously reported (27). The wild-type β_2GPI was able to inhibit native ^{125}I-β_2GPI binding to CL in a dose-dependent manner similar to that previously reported by our group for native β_2GPI (27). The inhibitory concentration of wild-type β_2GPI for 50% inhibition (IC_{50}) was 35 μg/ml, while IC_{50} for mutant 1k was 280 μg/ml. Mutants 2k, 2ka, and 3k of β_2GPI possessed no inhibitory activity up to 280 μg/ml (Fig. 6).

Binding of anti-β_2GPI Abs to β_2GPI and mutants of β_2GPI in a phospholipid-free system

Purified anti-β_2GPI Abs at 10 μg/ml bound equally well to native, wild-type, and mutants of β_2GPI in an anti-β_2GPI ELISA (Fig. 7). In contrast, no binding was detected to haptoglobin (control).

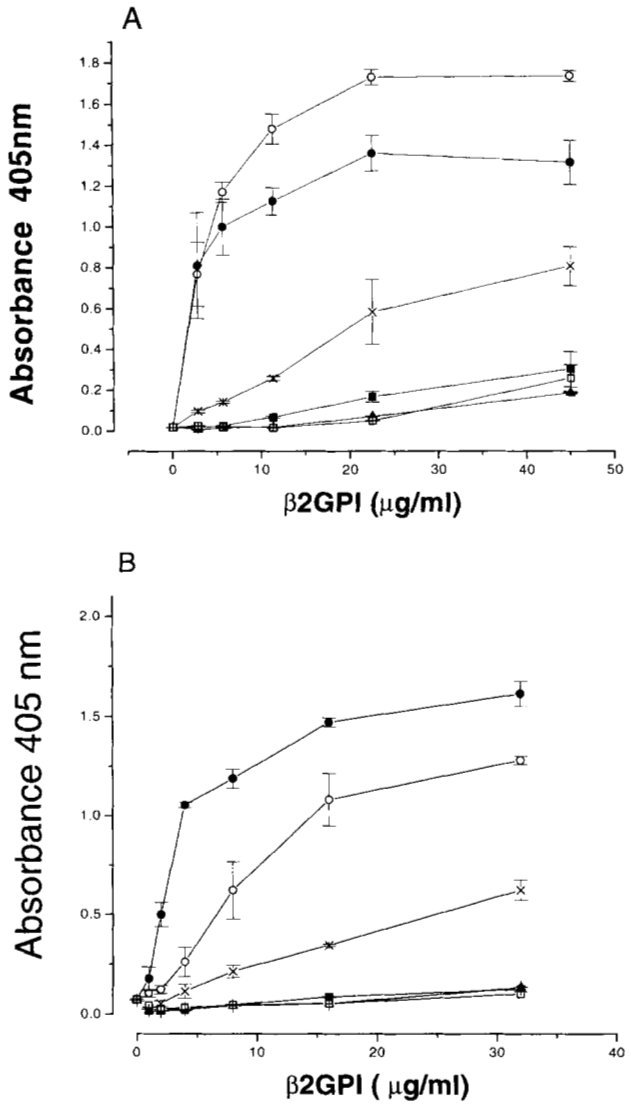

FIGURE 8. Dose response of binding activity of affinity-purified anti-β_2GPI Abs at 2 μg/ml from autoimmune patients (*A, B*) to CL in a modified CL ELISA in the presence of different preparations of β_2GPI. Native β_2GPI (●), recombinant wild-type β_2GPI (○), mutant 1k (×), mutant 2k (■), mutant 2ka (▲), and mutant 3k (□). Results are expressed as the mean ± SE of duplicates.

Comparison of binding activity of native, wild-type, and mutants of β_2GPI in a modified CL ELISA

In the presence of 2 μg/ml of purified anti-β_2GPI Ab (from patients with SLE), binding in a modified CL ELISA increased, with increasing concentration of recombinant wild-type β_2GPI in a dose-dependent manner similar to that obtained with native human β_2GPI. In contrast, no binding activity was obtained with mutants 2k, 2ka, and 3k up to a concentration of 45 μg/ml. However, mutant 1k of β_2GPI exhibited a dose-dependent increase in binding activity that was ~50% of that obtained with wild-type β_2GPI (Fig. 8, *A* and *B*). Experiments performed with affinity-purified anti-β_2GPI Abs from two additional autoimmune patients exhibited similar binding curves (results not shown).

Discussion

β_2GPI is composed of five highly conserved subunits called sushi domains (36) or complement control protein repeats (17). In β_2GPI, the fifth domain is the most variable domain and contains a region critical for its interaction with CL and autoantibodies found in patients with the aPL syndrome. Our group reported that a proteolytic cleavage between Lys317 and Thr318 in the fifth domain of β_2GPI removes the ability of β_2GPI for binding by autoimmune-type aCL Abs when assayed in a CL ELISA (37). In addition, we utilized synthetic peptides spanning the fifth domain of β_2GPI for binding and inhibition experiments, and showed that peptide sequence ^{281}Cys-Lys-Asn-Lys-Glu-Lys-Lys-Cys^{288} binds anionic phospholipids (27). The conformation of this segment is likely to be critical for phospholipid binding, since deletion of the flanking Cys residues or their replacement by Ser residues abolished phospholipid binding. It appears that the flanking Cys residues form a disulfide bond that favors the peptide conformation in the peptide-phospholipid complex, thus increasing the free energy of binding via reducing its entropy. Even though the two flanking Cys residues are not disulfide bonded to each other in the native molecule, their relative position in the model is consistent with such a bond (Fig. 2). As a consequence, a non-native disulfide bond between Cys^{281} and Cys^{288} is expected to favor the native conformation for the intervening peptide segment.

Although homology modeling has been used previously to predict a similar phospholipid binding region (17, 26), electrostatic properties of β_2GPI, which are likely to contribute to phospholipid binding, have not been calculated. To understand or to predict the nature of a complex between two large molecules, it is often useful to look at the global electrostatic potential of the two ligands involved. If the structure of only one ligand is known, it is particularly helpful to examine its electrostatic potential for possible binding sites of the other ligand. This is true in the present case, in which the interaction between a positively (the protein) and a negatively (CL) charged ligand is considered, and the detailed structure of the former is not available.

To investigate more closely which particular amino acid residues are critical for phospholipid binding by the intact fifth domain of β_2GPI, a three-dimensional model of the fifth domain of β_2GPI was constructed with a comparative protein modeling technique based on satisfaction of spatial restraints (28) (Fig. 2). The region with a pronounced positive electrostatic potential containing three central positive charges (Lys residues 284, 286, and 287) was predicted to be the binding site for the negatively charged CL ligand (Figs. 2 and 3*A*). Thus, the mutation of these three Lys residues to Glu residues was also expected to prevent the interaction between CL and β_2GPI-5 (Fig. 3*B*). To evaluate these hypotheses in the context of an intact β_2GPI molecule, β_2GPI and its mutants were expressed and tested for phospholipid and anti-β_2GPI Ab binding.

The baculovirus/insect cell system is a suitable system for expression of large amounts of human β_2GPI, because it overexpresses eukaryotic genes and carries out post-translational modifications. For example, Sf insect cells recognize the putative 19-amino acid sequence of human β_2GPI, and correctly cleave it, so that the expressed protein is secreted into the culture medium in the native conformation. According to SDS-PAGE, the recombinant β_2GPI molecules expressed in the insect cells Sf had m.w. different from human plasma β_2GPI.

The molecular mass of the β_2GPI secreted from insect cells is ~40 kDa, as opposed to 50 kDa for β_2GPI purified from human serum. The most likely explanation is a difference in the glycosylation. Fortunately, the difference in glycosylation between recombinant and native β_2GPI does not appear to influence binding of Abs to β_2GPI in a CL ELISA (Fig. 8). In addition, it has been reported that removal of the *N*-linked sugars of β_2GPI does not affect its anti-β_2GPI activity in a CL ELISA (32, 38), nor its interaction with anionic phospholipids (39).

Although ^{125}I-β_2GPI mutants 2k, 2ka, and 3k can bind to CL at ~38% of the amount bound by native ^{125}I-β_2GPI in a sensitive direct binding assay, the affinity of these mutant proteins for CL was too low to inhibit native ^{125}I-β_2GPI binding to CL in a competitive inhibition assay. Even the single mutant 1k had an approximately eightfold increase in the concentration, resulting in 50% inhibition of native ^{125}I-β_2GPI binding to CL. Mutants 2k, 2ka, and 3k of β_2GPI possessed no inhibitory activity up to 280 μg/ml (Fig. 6). On the other hand, anti-β_2GPI Abs can bind directly to native, wild-type, and mutants of β_2GPI equally well in the absence of CL (Fig. 7), indicating that the Lys residues in the loop Cys^{281}-Cys^{288} of β_2GPI are critical for phospholipid binding, but do not influence binding by anti-β_2GPI Abs.

The results in this study suggest that the major phospholipid binding site on β_2GPI is located in the C-terminal domain. In contrast, Hagihara and colleagues, using bovine β_2GPI and phospholipid liposomes containing CL, concluded that both N-terminal and C-terminal domains have important roles in the interaction of β_2GPI with CL (39). However, they also demonstrated that the affinity of the C-terminal domain for CL was 100-fold higher than that of the N-terminal domain. Binding at physiologic salt concentration significantly reduced the binding of the N-terminal domain to ~25% of that obtained at low ionic strength. In our study to assay for β_2GPI binding, we utilized a negatively charged phospholipid CL coated on microtiter plates. It is possible that the interaction of β_2GPI with bilayer phospholipid membranes is quantitatively and qualitatively different when compared with binding in CL ELISA. This may explain the failure to detect low affinity phospholipid binding sites on β_2GPI in the current study. Although other workers have reported that Lys-rich segments in the fifth domain of β_2GPI are involved in its binding to CL, they did not identify the specific Lys residues that were critical for this interaction (40).

β_2GPI circulates in plasma and has a high affinity for negatively charged phospholipids. These procoagulant phospholipids play a crucial role in promoting the blood coagulation cascade by initiating the contact activation system. Anionic phospholipids are only exposed after endothelial cell damage or platelet activation. After exposure, it is possible that circulating β_2GPI could bind to the endothelial or platelet surface via the exposed anionic phospholipids. The interaction of β_2GPI with phospholipids in in vitro systems is thought to be important for β_2GPI to act as an inhibitor of blood coagulation (41).

The anti-β_2GPI activity in a CL ELISA of β_2GPI can be explained by a ternary complex of the anti-β_2GPI autoantibodies interacting with a previously formed phospholipid-β_2GPI complex. The mechanism of the ternary complex formation is not known, but two explanations have been suggested. It has been proposed that the binding of phospholipids to β_2GPI induces a conformational change in β_2GPI, exposing a cryptic epitope for Abs to bind (1, 13). Alternatively, the phospholipid-β_2GPI complex formation could increase the local concentration of β_2GPI, promoting anti-β_2GPI Ab binding (14). The formation of the ternary complex could lead to the inhibition of the natural anticoagulant effect of β_2GPI in vivo, and thus predispose patients to thrombosis. However, the anticoagulant nature of β_2GPI, as indicated above, is based on in vitro experiments only. There does not appear to be an increased risk of thrombosis in families with β_2GPI deficiency (42), although additional factors predisposing to thrombosis may be involved in situations of low or deficient plasma levels of β_2GPI.

The ability of β_2GPI to bind negatively charged phospholipids is a prerequisite for autoantibodies occurring in patients with aPL syndrome to bind β_2GPI when assayed in a CL ELISA (1). Mutants 2k, 2ka, and 3k possess no binding activity (Fig. 8). Similar results were also obtained for Abs from two other autoimmune patients. The lack of autoantibody binding to the mutant β_2GPI, which also binds phospholipids with significantly lower affinity than native β_2GPI, indicates that the binding of autoantibodies to β_2GPI in vivo may require that β_2GPI be immobilized on an anionic phospholipid membrane surface. The native and mutant β_2GPI described in this study provide reagents for further investigations of the role of β_2GPI in the aPL syndrome.

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