Human $\beta_2$-Glycoprotein I as an Anticardiolipin Cofactor Determined Using Deleted Mutants Expressed by a Baculovirus System

By Makoto Igarashi, Eiji Matsuura, Yoshiko Igarashi, Hisato Nagae, Kenji Ichikawa, Douglas A. Triplett, and Takao Koike

$\beta_2$-Glycoprotein I ($\beta_2$-GPI) consists of five repeats of a homologous domain. We designed a series of human $\beta_2$-GPI mutants, i.e., three mutant genes lacking the domain(s) present in the NH$_2$-terminal region and two of those present in the COOH-terminal region. These mutant genes were expressed in Spodoptera frugiperda insect cells (SF9) infected with recombinant baculoviruses and the mutant proteins were secreted into the culture medium. The molecular mass of the purified mutant proteins, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was fairly consistent with the size calculated from their nucleotide sequences. Binding of $\beta_2$-GPI to solid-phase cardiolipin (CL) was diminished by the deletion of the fifth domain (domain V) from its complete structure. Thus, the phospholipid binding site of $\beta_2$-GPI is located on its domain V. Monoclonal anti-CL antibodies (aCL) derived either from NZW $\times$ BXSB (WB) F1 mice or from patients with antiphospholipid syndrome bound directly to the domain V-deleted mutant protein (DI-IV) absorbed not only on an oxygenated but also on a plain polystyrene surface. We conclude from this study that the epitope for aCL is exposed on a conformationally changed structure of $\beta_2$-GPI by interacting with negatively charged phospholipid or on the mutant protein, DI-IV.

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Submitted March 27, 1995; accepted November 30, 1995.

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0006-4971/96/8708-0025$3.00/0

Human $\beta_2$-Glycoprotein I ($\beta_2$-GPI) is a plasma glycoprotein first described by Schulze et al in 1961. In human plasma, the major amount of $\beta_2$-GPI after ultracentrifugation is found in the lipoprotein fractions, and it activates lipoprotein lipase in vitro. $\beta_2$-GPI has therefore also been designated as apolipoprotein H. In 1984, the complete amino acid sequence of $\beta_2$-GPI was determined by peptide sequencing and $\beta_2$-GPI proved to be a single polypeptide chain containing 326 amino acid residues with five oligosaccharide attachment sites. The complete nucleotide sequence and the deduced amino acid sequence were established by cDNA cloning and sequencing in 1991. These sequence analyses showed a putative signal sequence of 19 amino acids. Two differences were apparent between cDNA-derived amino acids and those determined by peptide sequencing.

$\beta_2$-GPI is composed of five homologous domains. Four of them are composed of approximately 60 amino acids that contain highly conserved cysteines, prolines, and tryptophans. The motif is characterized by a framework of four conserved half-cystine residues involved in the formation of two internal disulfide bridges. These repeating motifs were designated as short consensus repeats/complement control protein repeats or as sushi structures because of their shape.

The fifth domain (domain V) contains 82 amino acid residues and 6 half-cystines. It is known that $\beta_2$-GPI binds to various kinds of negatively charged substances, such as phospholipids and lipoproteins, and inhibits the intrinsic blood coagulation pathway and ADP-dependent platelet aggregation. However, the function $\beta_2$-GPI is not well understood.

It has been widely considered that antiphospholipid antibodies, such as anticardiolipin antibodies (aCL), present in sera of patients with antiphospholipid syndrome, are closely related IgGs that react with negatively charged phospholipids. However, recent studies have shown that a serum/plasma cofactor is also required for aCL binding to solid-phase CL. Purification of the cofactor from normal human plasma suggests that it is a single 50-kD polypeptide chain and proved to be $\beta_2$-GPI, as determined by the N-terminal amino acid sequence. In light of its novel function, $\beta_2$-GPI is regarded as a cofactor, and it is now considered that aCL in systemic lupus erythematosus (SLE) patients binds to the complex of CL and $\beta_2$-GPI but not to either CL or $\beta_2$-GPI alone. We reported that aCL derived from either SLE patients or NZW $\times$ BXSB (WB) F1 mice, an animal model of APS, bound directly to a solid-phase $\beta_2$-GPI adsorbed on a γ-irradiated polystyrene surface onto which a significant amount of oxygen had been covalently introduced. We concluded that aCL bound to a novel epitope appearing on the $\beta_2$-GPI when the protein interacts with an oxygen-introduced solid-phase surface.

In the present report, we designed five $\beta_2$-GPI mutant genes in which various sushi domain regions were deleted. These were expressed in the baculovirus expression system to investigate (1) which domain contains a binding site for negatively charged phospholipids and (2) on which domain(s) the novel epitope for aCL appears. Our data indicate that a binding site for the negatively charged phospholipid exists in domain V in a physiologic salt condition and that an epitope for aCL is cryptic and domain IV may be dominantly involved in exposure of the epitope.

MATERIALS AND METHODS

Amplification of LDI-III and LDI-IV genes (Fig 1) by PCR and construction of pVLLD12 and pVLLD14. An LDI-III gene that is composed of a leader sequence, namely domains 1 to III of $\beta_2$-GPI cDNA, was constructed by polymerase chain reaction (PCR) using pAcGPI plasmid as a template. The 5'-coding PCR primer used was 5PHN1 (Table 1), the sequence of which corresponded to a just upstream region of the ATG initiation codon of the polyhedrin gene. The 3'-anticoding PCR primer used was 3DHE (Table 1), and this sequence corresponded to a complementary sequence of...
The DII-V fragment was amplified from pUC118:BGPI. The 5'-coding primer used was SDIIII (Table 1) and the sequence corresponded to nucleotides 226 to 252 and was introduced to the EcoRI site just upstream of cystein$^e$ to facilitate construction of the fused protein. The 3'-anticoding primer used was M13 primer (Takara Shuzo, Kyoto, Japan). The DIII-V and DIV-V fragments were amplified by PCR using the same template and 3'-anticoding primer, as described above. The 5'-coding primer used in amplification of the DIII-V fragment was the SDIIIII primer and the sequence corresponded to nucleotides 406 to 432 and was introduced at the EcoRI site. Sequence of the SDIVN primer used in amplification of the DIV-V fragment corresponded to nucleotides 592 to 618 and was also introduced at the EcoRI site.

After digestion of the amplified products DII-V, DIII-V, and DIV-V with EcoRI/BamHI, the fragments were cloned into plasmid pVLLI1 digested with EcoRI/BglII IL and the resulting plasmids were designated as pVLLD25, pVLLD35, and pVLLD45, respectively. The resulting plasmids pVLLD13, 14, 25, 35, and 45 were abbreviated to pVLLDs.

Preparation of recombinant $\beta_2$-GPI or its deleted mutant proteins. Recombinant $\beta_2$-GPI or its mutant proteins were purified from the culture supernatant of $\beta_2$-GPI cDNA (or mutant genes) transfected Sf9 cells by sequential antibody-affinity column chromatography and gel filtration. The antibody-affinity columns contained monoclonal antibody (MoAb) against human $\beta_2$-GPI (Cof-22 or -23). Briefly, the culture supernatant (100 mL) was applied to the antibody-CN sepharose 4B column and the column was washed by 10 mmol/L sodium phosphate buffer and 150 mmol/L NaCl. The bound proteins were eluted with 1 mol/L glycine, pH 2.5. The eluate was then chromatographed on a TSK-G3000SW column (0.75 $\times$ 60 cm) equipped with a high performance liquid chromatography (HPLC) system.

Preparation of monoclonal anti-$\beta_2$-GPI antibodies. Ten-week-old female BALB/c mice were intraperitoneally immunized and then twice administered a booster with 50 $\mu$g of human $\beta_2$-GPI emulsified with the complete Freund’s adjuvant. Finally, 25 $\mu$g of $\beta_2$-GPI without the adjuvant was administered intravenously. Three days after the final immunization, the spleens were excised from the mice and 1.8 $\times$ 10$^7$ splenocytes were fused with 6 $\times$ 10$^4$ mouse myeloma cells, P3-X63-Ag8-U1, using the method of Kohler and Milstein. These MoAbs were sequentially purified by ammonium sulfate precipitation, protein A-Sepharose affinity chromatography, and CL-6B affinity column chromatography.

WB-CAL-1 and EY2C9. A murine MoAb, WB-CAL-1 (IgG2a, $\kappa$), was derived from NZW $\times$ BXSB (WB) F1 male mice, an animal model of human APS. WB-CAL-1 antibody was obtained from ascites fluid and purified sequentially by ammonium sulfate precipitation and protein A-Sepharose affinity chromatography. Six human monoclonal aCLs, including EY2C9 (IgM, $\kappa$), were established, as

### Table 1. PCR Primers Used for Amplification of Deletion Mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Nucleotides†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPHN1</td>
<td>5'-GTAATAAAAAAACCTATATAAAT</td>
<td>226 to 252</td>
</tr>
<tr>
<td>SDIN1</td>
<td>5'-ACCTGGAATCTCTACGGGAGGTGTT</td>
<td>406 to 432</td>
</tr>
<tr>
<td>SDIV1</td>
<td>5'-CCGAGATCGAAGGAGGAGGTGTT</td>
<td>592 to 618</td>
</tr>
<tr>
<td>3DIIIIV</td>
<td>5'-ACAGAAATCTTAACACGGGAGGTGTT</td>
<td>792 to 873</td>
</tr>
<tr>
<td>3DIIIIV</td>
<td>5'-TGTTTATATCATGATGGAGGTGTT</td>
<td>612 to 583</td>
</tr>
<tr>
<td>3LS1</td>
<td>5'-GGGAGAATCTGATGAGGTGTT</td>
<td>72 to 49</td>
</tr>
</tbody>
</table>

* EcoRI site (GAATTC) engineered into primers in bold print. The STOP codon (TTA and TCA) engineered into primers is underlined. † Nucleotide numbering system is based on $\beta_2$-GPI ATG initiation codon representing residues -1, +2, and +3. ‡ Primers represent sequence in the noncoding DNA strand.
peripheral lymphocytes isolated from aCL-positive patients transformed with Epstein-Barr virus were fused with the mouse-human heterohybridoma cells (SHM-D3; #1688-CRL; American Type Culture Collection, Rockville, MD) to establish hybridoma cells secreting aCL.

Evaluation of specificity of anti-β2-GPI MoAbs: competitive inhibition assay using human, rat, and bovine β2-GPI. Wells of 96-well microtiter plates (plain polystyrene plates) were coated with 50 μL of human β2-GPI (10 μg/mL, dissolved in phosphate-buffered saline [PBS]) for 16 to 20 hours at 4°C. After the incubation, the β2-GPI-coated wells were washed three times with 200 μL of PBS (pH 7.4) containing 0.05% Tween 20 (PBS-Tween) and were then incubated with 200 μL of HEPES buffer (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4) containing 3% gelatin (Difco Laboratories, Detroit, MI) for 1 hour at room temperature. After washing in the same manner, the wells were simultaneously incubated with 25 μL of β2-GPI solution (human, bovine, or rat β2-GPI at final concentrations of 0.026, 0.13, 0.64, 3.2, 16, 80, and 400 μg/mL) and with 25 μL of MoAb solutions (Cof-18, -19, -20, -21, -22, or -23 at 1.6, 32, 1.6, 8.0, 8.0, and 1.6 μg/mL, respectively) were added and the preparation incubated for 1 hour at room temperature. The wells were washed again three times and incubated with 100 μL of 10-fold diluted solution of horseradish peroxidase (HRP)-labeled goat antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature. After another washing, the wells were incubated with 100 μL of 0.3 mmol/L tetramethylbenzidine containing 0.01% H2O2. After 10 minutes of incubation, the reaction was terminated by adding 100 μL of 2 N H2SO4 and the OD was measured at 450 nm.

Evaluation of binding property to solid-phase CL: competitive inhibition assay. CL (2.5 μg in 50 μL of ethanol/well) was coated on the surface of wells of 96-well microtiter plates (plain polystyrene) by evaporation. The CL-coated wells were incubated with 50 μL of 10 mmol/L HEPES and 150 mmol/L NaCl, pH 7.4, containing 0.3% bovine serum albumin (BSA: HEPES-BSA) for 1 hour at 37°C, and washed three times with 200 μL of PBS-Tween. The wells were incubated with 50 μL of HEPES-BSA containing β2-GPI for 30 minutes at room temperature and washed in the same manner. After washing with PBS-Tween, the wells were incubated with 25 μL of WB-CAL-1 solution (0.4 μg/mL) and 25 μL of solution containing β2-GPI or the DI-IV mutant protein (0.64, 3.2, 16, 80, and 400 μg/mL) for 1 hour at room temperature. After washing in the same manner, the wells were incubated with 100 μL of HRP-labeled goat antitoxins IgG (1 hour at room temperature. Color was developed and the OD was measured as described previously.

Anti-β2-GPI ELISA: direct binding assay using the DI-IV protein. CL-coated wells of plain polystyrene plates were prepared as previously described. The CL-coated wells were incubated with 50 μL of HEPES-BSA for 1 hour at 37°C and washed three times. The wells were incubated with 50 μL of HEPES-BSA containing β2-GPI (30 μg/mL) for 30 minutes at room temperature and washed in the same manner. After washing with PBS-tween, the wells were incubated with 25 μL of WB-CAL-1 solution (0.4 μg/mL) and 25 μL of solution containing β2-GPI or the DI-IV mutant protein (0.64, 3.2, 16, 80, and 400 μg/mL) for 1 hour at room temperature. After washing in the same manner, the wells were incubated with 100 μL of HRP-labeled goat antitoxins IgG (1 hour at room temperature. Color was developed and OD was measured as previously described.

RESULTS

Construction and expression of β2-GPI mutant genes. We designed five β2-GPI mutant genes by deleting a certain sushi domains(s) from the complete β2-GPI gene. These genes were amplified by PCR from cDNA. The mutant genes shown schematically in Fig 1 were structurally characterized into two types: (1) type 1 mutants, LDII-III and LDI-IV genes, lacked domain(s) positioned in the C-terminal region; and (2) type 2 mutants, LDII-V, LDIII-V and LDIV-V genes, lacked domain(s) positioned in the N-terminal region. Each

![Fig 2. SDS-PAGE of β2-GPI mutant proteins. Coomassie brilliant blue staining of 12% polyacrylamide gels. Lane 1, native β2-GPI purified from human serum; lane 2, recombinant β2-GPI obtained from insect cells (complete form); lane 3, DIV-V mutant protein; lane 4, DIII-V mutant protein; lane 5, DII-V mutant protein; lane 6, LDIII mutant protein. The positions of the 94-, 67-, 43-, 30-, 20-kDa, and 14.4-kD molecular mass markers are indicated. (A) Reduced condition. Samples were heated at 100°C for 3 minutes in sample buffer containing 1 mmol/L 2-ME and loaded on the gel. (B) Nonreduced condition. Samples were heated without 2-ME.](www.bloodjournal.org)
Table 2. Molecular Mass of Deletion Mutants of β₂-GPI

<table>
<thead>
<tr>
<th>β₂-GPI</th>
<th>Estimated* by SDS-PAGE</th>
<th>Calculated From Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native†</td>
<td>50,000</td>
<td>36,254</td>
</tr>
<tr>
<td>Complete‡</td>
<td>43,000</td>
<td>36,254</td>
</tr>
<tr>
<td>DIV-V</td>
<td>18,900</td>
<td>16,750</td>
</tr>
<tr>
<td>DIII-V</td>
<td>32,700</td>
<td>23,472</td>
</tr>
<tr>
<td>DI-III</td>
<td>25,600</td>
<td>19,936</td>
</tr>
<tr>
<td>DI-IV</td>
<td>38,400</td>
<td>26,651</td>
</tr>
</tbody>
</table>

* Molecular mass of the proteins was estimated by migration of a major band on SDS-PAGE under reducing conditions (Fig 2A).
† Native β₂-GPI purified from human serum.
‡ Recombinant β₂-GPI expressed in insect cells.

PCR product encoding the β₂-GPI mutant gene was sub-cloned into the baculovirus transfer vector pVL1393 to generate pVLLDs and sequenced for confirmation of its DNA sequence (data not shown). Cotransfection of Sf9 cells with wild-type AclacZ DNA digested with EcoRI and respective pVLLDs resulted in the production of a recombinant baculovirus. The recombinant viruses were purified from plaques and amplified in Sf9 cells. In the resulting recombinant viruses, each mutant gene was placed under control of the polyhedrin promoter. Sf9 cells infected with the recombinant baculovirus were cultured in a serum-free medium, as described. At 72 hours after infection, the mutant proteins were purified from each culture supernatant.

Analysis of purified β₂-GPI mutant proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified proteins were resolved by 12% SDS-PAGE (Fig 2) and each mutant protein showed single or diffused homogeneous bands. In particular, the distinct two bands were seen in the preparation of DIV-V protein. The mutant proteins reacted with the rabbit polyclonal anti-β₂-GPI antibodies, as seen on Western blot analyses (data not shown). Table 2 indicates the molecular mass of β₂-GPI and its mutant proteins, as estimated by migration on SDS-PAGE.

Determination of the N-terminal amino acids sequence. Human β₂-GPI has a signal sequence for secretion of 19 amino acid residues. The LDII-V, LDIII-V, and LDIV-V genes were artificially combined with the DNA fragment encoding signal peptide. The three mutant genes were subjected to N-terminal amino acid sequence analysis through at least 7 residues to confirm the integrity of the signal peptide cleavage. As shown in Fig 3, the 19 amino acid sequence identified as the signal peptide protein was removed from the three mutant proteins. The N-terminal amino acids sequences, Gly-Arg-Asn-Ser, and the following three amino acids proved to be identical to those deduced from the DNA sequence. The N-terminal amino acids, Gly and Arg, were derived from the N-terminus of β₂-GPI and the following two amino acids, Asn and Ser, were derived from the EcoRI site.

Isolation and epitope mapping of MoAbs against human β₂-GPI. Six MoAbs raised in mice against human β₂-GPI, namely Cof-18, -19, -20, -21, -22, and -23, were prepared

![Fig 3. N-terminal amino acid sequence of β₂-GPI mutant proteins. cDNA, nucleotide sequence of the mutant DNA; 1, amino acid sequence deduced from DNA; 2, N-terminal amino acid sequence of the purified mutant protein determined by peptide sequencing.](from www.bloodjournal.org by guest on October 27, 2017. For personal use only.)
Species-specificity of these Cofs MoAbs was examined by an inhibition assay, using human β₂-GPI-coated plates and human, rat, or bovine β₂-GPI as an inhibitor. As shown in Fig 4, the addition of rat or bovine β₂-GPI had an apparent influence only on binding of MoAb Cof-21 to solid-phase human β₂-GPI coated on the plates. In contrast, the binding of other Cofs to human β₂-GPI was not influenced by the addition of rat or bovine β₂-GPI. Direct binding of these MoAb to the mutant proteins coated on the plain plates was evaluated (Fig 5). Each MoAb showed domain-specific binding, i.e., Cof-18 and -19 only bound to mutant proteins having domain V, Cof-20 and -22 bound to those having domain III and Cof-21 and -23 bound to those having domain IV. In a control study, MoAb G02 against with human IgG bind neither to β₂-GPI nor to all those mutant proteins.

Binding of mutant proteins of β₂-GPI to solid-phase CL. Purified DI-III and DI-IV mutant proteins did not inhibit the binding of β₂-GPI to solid-phase CL (Fig 6). In contrast, the DII-V, DIII-V, and DIV-V mutant proteins significantly inhibited the binding of β₂-GPI to CL as well as to complete protein.

aCL binding to β₂-GPI and its mutant proteins on a plain or an oxygenated polystyrene surface. Figure 7 shows evidence for the binding of monoclonal mouse aCL, WB-CAL-1, to the complete form of β₂-GPI coated on plain polystyrene plates. WB-CAL-1 antibody did not bind to the plain plates coated with DIV-V, DIII-V, and DII-V mutant proteins, but did bind to the plates coated with the DI-IV mutant protein. However, the binding was significantly reduced once domain IV was removed from the DI-IV. Thus, WB-CAL-1 antibody directly recognized not only the β₂-GPI complete form but also the DI-IV and DI-III (but very weakly) absorbed onto the oxygenated polystyrene plates.
Fig 5. Binding of anti-β2-GPI MoAbs to each mutant protein in an anti-β2-GPI ELISA. The MoAbs Cofs were incubated in mutant protein-coated wells. The data represent the mean OD at 450 nm of duplicate determinations.

As shown in Fig 8, it was ascertained whether the soluble DI-IV protein competitively inhibited a binding of WB-CAL-1 to β2-GPI on CL-coated polystyrene surface. The DI-IV protein at 40 μg/mL inhibited 40% of binding activity of WB-CAL-1 to β2-GPI, whereas the complete form had no inhibition activity. We also evaluate specificity of a series of human monoclonal aCL antibodies in the same assay systems. As shown in Fig 9, a binding profile of EY2C9 antibody was similar to that of WB-CAL-1 antibody. Three of five other MoAbs had similar binding properties (data not shown).

**DISCUSSION**

We have shown that the baculovirus expression system is ideally suited for production of an incomplete form of a protein. A prokaryotic fusion protein system such as the glutathione-s-transferase system is usually used for expression of low molecular weight polypeptides, because short polypeptides or incomplete forms of a protein are more susceptible for protease. This baculovirus expression system can provide substantial quantities of mutated protein for detailed immunologic and biochemical analyses. Each mutant protein purified from the culture supernatant was detected as a single polypeptide band on Western blot and SDS-PAGE analysis,
and peptides with a molecular mass lower than that of the mutant proteins that reacted with rabbit anti-β₂-GPI antibodies or mouse MoAbs were not detected with either analysis. Moreover, the signal sequence artificially conferred on mutant proteins were recognized and cleaved correctly by insect cells.

Human β₂-GPI is a highly glycosylated protein with five potential N-glycosylation sites. The molecular masses of recombinant β₂-GPI expressed in insect cells and native protein purified from normal human serum were estimated as 43 kD and 50 kD on SDS-PAGE, respectively, but values are significantly different from the molecular mass of 36,254 calculated from the cDNA nucleotide sequence. However, the molecular mass of these proteins treated with N-glycans was reduced to the almost identical molecular weight (Mr) of 36,254.\textsuperscript{20} The molecular mass of all mutant proteins determined by migration on SDS-PAGE was slightly greater than that calculated from the nucleotide sequences. As with the complete form, the carbohydrate content of the mutant proteins might significantly affect the Mr as estimated by migration on SDS-PAGE. We previously reported that native protein derived from human serum is composed of several bands, as noted under reducing and nonreducing conditions on SDS-PAGE (Fig 2, lane 1).\textsuperscript{16} Such diffused patterns were detected in the complete form (lane 2) and in the mutant proteins (lanes 3 through 7). However, the DIV-V mutant protein was observed as two protein bands (20.6 and 18.9 kD, lane 3). We suspected that upper band (20.6 kD) was
glycosylated form of it because a potential glycosylation site existed in domain IV. To confirm this, the DIV-V protein treated with N-glycanase was resolved by SDS-PAGE. This treatment resulted in both a fainter 20.6-kD band and a corresponding increase in the 18.9-kD band, as expected (data not shown).

β2-GPI is known to bind to negative-charged substances such as phospholipids, and a highly positive-charged region is located in domain V.23 Moreover, Hunt and Krilis23 reported that the amino acid sequence, Cys231-Lys-Asn-Lys-Glu-Lys-Lys-Cys, is located in domain V and is a dominant phospholipid binding site. However, it was not clear whether the phospholipid binding site existed in other domains. We present evidence here that the phospholipid binding site exists only in domain V. The mutant proteins containing domain V only inhibited a binding of β2-GPI to CL (Fig 6).

We attempted to produce an MoAb against human β2-GPI because there are reports challenging our conclusion on the exact nature of the epitope recognized by aCL. Other investigators reported that aCL are identical to anti-β2-GPI antibodies that directly bind to β2-GPI without binding to CL.14,26,27 Thus, we compared WB-CAL-1 and Cofs MoAbs with regard their reactivity with β2-GPI derived from various species. All Cofs MoAbs except Cof-21 showed specific bindings to human β2-GPI coated on polystyrene plates. These observations are of interest because there is high homology in the amino acid sequence of β2-GPI among human, rat, and bovine proteins. For example, bovine β2-GPI is composed of 326 amino acid residues, 84% of which are identical with those of human protein. Homology is present in all parts of the protein molecules. The binding properties of Cofs MoAbs to β2-GPI clearly differed from those of WB-CAL-1 or aCL from SLE patients. aCL reacted with human, rat, and bovine β2-GPI that undergo a conformational change with binding to solid-phase CL.

We determined the domains containing the epitope of Cofs MoAbs using mutant proteins. Each Cof MoAb strongly reacted with the mutant proteins containing the epitope, as well as the complete protein, but did not react with the protein(s) in the absence of the epitope (Fig 5). Thus, the epitopes of Cof-18 and -19 would be present in domain V. Similarly, those of Cof-20 and -22 would be mapped within domain III and those of Cof-21 and -23 within domain IV, respectively. Therefore, mutant proteins are useful to determine domains containing an epitope.

Advanced studies performed with these mutant proteins provided evidence regarding the epitope recognized by WB-CAL-1 antibody, aCL, as well as the epitopes of Cofs MoAbs. We measured the binding activity of aCL to the mutant protein absorbed on oxygenated polystyrene plates instead of on CL-coated plates. In β2-GPI–dependent aCL ELISA using CL-coated plates, it was impossible to evaluate the binding activity of aCL to the DI-III or DI-IV mutant proteins because a phospholipid binding site was missing.

As previously reported, oxygen atoms are introduced onto a polystyrene surface by irradiation with γ-ray radiation, and aCL are able to recognize directly β2-GPI interacting with an oxygen-substituted solid-phase surface.28 In anti-β2-GPI (or its mutant proteins) ELISA, WB-CAL-1 antibody binds to the DI-IV mutant protein that is deficient in domain V and not to the DI-II, DI-III, and DIV-V mutant proteins, which contain this domain. Our findings are at variance with the hypothesis that the neoepitope of aCL is exposed on domain V after binding to phospholipid.23,28 In our view, the most probable explanation is that the binding of phospholipid to domain V is required for exposure of the cryptic epitope, but the epitope is absent on domain V.

Because the degree of reactivity to the DI-III mutant protein was significantly lower than that to the DI-IV mutant protein, domain IV may play a critical role in exposure of the cryptic epitope. There is also the possibility that domain I is involved in exposure of the cryptic epitope. Interestingly, WB-CAL-1 antibody bound to the DI-IV mutant protein coated on plain polystyrene plates as well as to oxygenated polystyrene plates. The degree of binding to the DI-IV was about twice that to the complete form. These data suggest that the DI-IV mutant peptide undergoes a conformational change by the removal of domain V, which thereby exposes the epitope. However, the conformational change may be insufficient for exposure of the epitope because the degree of binding to the DI-IV coated on plain plates was relatively lower than that detected in experiments using oxygenated plates. Moreover, the soluble phase of the deletion mutant, the DI-IV protein, competitively inhibited the WB-CAL-1 binding to the complex of the complete structure of β2-GPI and solid-phase CL (Fig 8). In contrast, a slight enhancement rather than inhibition was observed when the complete structure of β2-GPI was added as a control, because additional β2-GPI might further bind to solid-phase CL to expose the epitope for aCL.

All six MoAbs established from lymphocytes of patients with APS recognize the altered structure of β2-GPI interacting with an oxygen-introduced polystyrene surface.24 We measured binding properties of the six antibodies to a series of mutant proteins. Four of these six human monoclonal aCL bound specifically to the DI-IV protein either on a plain and on an oxygenated surface, which is similar to WB-CAL-1 (manuscript in preparation). Therefore, typical binding of EY2C9 antibody was indicated in Fig 9.

Finally, we showed that the present study using a series of mutant proteins of human β2-GPI is available for definition of epitopes of anti-β2-GPI MoAbs and aCL derived from an animal model of APS or an APS patient. Indeed, a distinct domain was not determined, but the fact that a removal of domain V from the intact protein induced exposure of epitope should be an important clue in the investigation as to whether that epitope is cryptic. We are now attempting to express sushi domains individually and to design another type of mutant genes that lack domains present in the middle of the protein molecule. Furthermore, we should evaluate specificities of a large number of aCL found in APS patients. The system contributes to a better understanding of some pathogenic features of aCL in APS.

ACKNOWLEDGMENT

We thank Dr Dennis R. Voelker (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) for helpful suggestions and comments and Dr Yoshisharu Matsuura (National Institute of Health, Tokyo, Japan) for pertinent advice on technology.
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Human beta2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system

M Igarashi, E Matsuura, Y Igarashi, H Nagae, K Ichikawa, DA Triplett and T Koike