Use of Single Point Mutations in Domain I of β_2-Glycoprotein I to Determine Fine Antigenic Specificity of Antiphospholipid Autoantibodies[1]

G. Michael Iverson,[2,3]* Stephen Reddel,[2†] Edward J. Victoria,* Keith A. Cockerill,* Ying-Xia Wang,[†] Marc A. Marti-Renom,[‡] Andrej Sali,[‡] David M. Marquis,* Steven A. Krilis,[†] and Matthew D. Linnik*

Autoantibodies against β_2-glycoprotein I (β_2GPI) appear to be a critical feature of the antiphospholipid syndrome (APS). As determined using domain deletion mutants, human autoantibodies bind to the first of five domains present in β_2GPI. In this study the fine detail of the domain I epitope has been examined using 10 selected mutants of whole β_2GPI containing single point mutations in the first domain. The binding to β_2GPI was significantly affected by a number of single point mutations in domain I, particularly by mutations in the region of aa 40–43. Molecular modeling predicted these mutations to affect the surface shape and electrostatic charge of a facet of domain I. Mutation K19E also had an effect, albeit one less severe and involving fewer patients. Similar results were obtained in two different laboratories using affinity-purified anti-β_2GPI in a competitive inhibition ELISA and with whole serum in a direct binding ELISA. This study confirms that anti-β_2GPI autoantibodies bind to domain I, and that the charged surface patch defined by residues 40–43 contributes to a dominant target epitope. ***The Journal of Immunology,*** **2002, 169: 7097–7103.**

Autoantibodies that bind β_2-glycoprotein I (β_2GPI)[4] are recognized to be strongly associated with the antiphospholipid syndrome (APS) (1–3). Abs detected by the anticardiolipin (aCL) assay from patients with clinical features of APS generally bind to β_2GPI, but samples from patients with other causes of a positive aCL assay, such as a variety of infections, do not (4). Likewise, lupus anticoagulant activity in APS patient plasma is often due to anti-β_2GPI Abs (1). Knowing the location of an epitope for pathogenic Abs may permit the design of therapeutic agents able to act as toleragens for the specific B cells producing the autoantibodies (5). This would provide a far more specific approach to treatment than the anticoagulation or general immunosuppression currently used in APS.

β_2GPI is a member of the short consensus repeat protein family, which is characterized by repeating domains of ~60 aa with two disulfide bridges. β_2GPI has five domains; the amino-terminal first through fourth domains are typical, and the fifth domain contains an extra ~20-aa C-terminal loop ending in an extra terminal disulfide bond and a lysine-rich region that functions as the major anionic phospholipid binding site (6, 7). The recent structure of β_2GPI, as determined by x-ray crystallography, shows that it has a J shape (8, 9). This suggests that the base of the J shape containing domain 5 provides the membrane binding interface. The remaining domains thus extend progressively away from the phospholipid surface. In this situation domain I would have the greatest exposure and accessibility (8, 9). No specific function has been ascribed to domains I–IV.

The identification of which region(s) of β_2GPI is bound by autoantibodies has been the subject of some debate (10–14). Wang et al. (10) tested several human mAbs generated from patients with APS that bound to cardiolipin in the presence of β_2GPI. Several short peptides containing sequences from domain V of β_2GPI enabled these mAbs to bind to cardiolipin or inhibited binding of mAb to β_2GPI. However, polyclonal autoantibodies from APS patients did not bind these peptides. It was suggested that these mAbs bound epitopes on domain V. Arvieux et al. (15) identified patients who had anti-β_2GPI Abs that were aCL assay negative and inhibited the binding of β_2GPI to cardiolipin, suggesting binding to the cardiolipin binding site on domain V of β_2GPI. Yang et al. (13) have recently described direct binding of autoantibodies from patients with systemic lupus erythematosus to a recombinant domain V-GST fusion protein. However, contrary to the studies of Arvieux et al. (15), these patient samples were also aCL assay positive.

Several studies have used recombinant mutants of β_2GPI with one or more domains deleted (11, 12, 16). Igarashi et al. (16) found that several mouse and human monoclonal β_2GPI autoantibodies bound to wild-type β_2GPI, domains I–IV (i.e., the deletion mutant containing only domains I–IV) and, to a lesser degree, to domains I–III, but not mutants without domain I. This was interpreted to suggest that domain IV may play a critical role in exposure of the cryptic epitope. Using these mutants and purified anti-β_2GPI from four APS patients, George et al. (12) found binding to domains I–IV, but not domains I–III or domains II–V. Three mouse mAbs, two of which were reported to bind to domain IV, were

*La Jolla Pharmaceutical Co., San Diego, CA 92121; †Departments of Medicine and Immunology and Infectious Disease, St. George Hospital, University of New South Wales, Sydney, Australia; and ‡Laboratory of Molecular Biophysics, Rockefeller University, New York, NY 10012

Received for publication March 22, 2002. Accepted for publication October 8, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by the National Health and Medical Research Council of Australia. This work was partly supported by National Institutes of Health Grant GM54762. A.S. is an Irma T. Hirschl Trust Career Scientist.

[2] G.M.I. and S.R. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. G. Michael Iverson, La Jolla Pharmaceutical Co., 6455 Nancy Ridge Drive, San Diego, CA 92121. E-mail address: mike.iverson@ljpc.com

[4] Abbreviations used in this paper: β_2GPI, β_2-glycoprotein 1; aCL, anticardiolipin; APS, antiphospholipid syndrome; AU, absorption units; NFDM, nonfat dried milk.

Copyright © 2002 by The American Association of Immunologists, Inc. 0022-1767/02/$02.00

used in competitive inhibition assays. Binding of one of the two anti-domain IV mAbs was inhibited by three of the four patient anti-β_2GPI preparations. These findings were interpreted as demonstrating that domain IV contains the epitope site for human anti-β_2GPI (12). Using a single point and a triple point domain IV mutant, Koike et al. (17) found significantly decreased binding of anti-β_2GPI to both mutants in an ELISA, again suggesting domain IV as the site of the epitope(s).

Other studies using domain-deleted mutants of β_2GPI reached different conclusions. Using anti-β_2GPI purified from 11 patients with APS, significant competitive inhibition of binding to the wild type could be demonstrated using all mutants containing domain I, most importantly including domain I alone, but there was little if any inhibition using mutants without domain I (11). In a direct binding ELISA study in which the wild-type and domain-deleted mutants were coated onto irradiated polystyrene plates, sera from 21 patients with anti-β_2GPI Abs were also shown to bind to full-length β_2GPI and to the domain I–IV mutant at the same level. In only three was there any degree of binding to domains II–V (14). These studies suggested that domain I, not domain IV or V, is the dominant epitope site on β_2GPI for anti-β_2GPI autoantibodies from the majority of APS patients.

This present study used recombinant wild-type β_2GPI plus variants with single point mutations in domain I in both direct binding and competitive inhibition ELISAs. This was done firstly to attempt to better localize the epitope, and secondly to address the possibility that the deletion of entire domains in the previous studies resulted in conformational changes elsewhere in the protein, which could potentially affect autoantibody binding and/or the coating characteristics on ELISA plates (11, 14). Using these domain I point mutants we have examined the binding of anti-β_2GPI autoantibodies in a wide range of ELISA conditions, with two separate groups of anti-β_2GPI patient samples. In one laboratory (La Jolla Pharmaceutical Co.) the domain I point mutants were examined for their ability to competitively inhibit the binding of affinity-purified anti-β_2GPI autoantibodies from binding to wild-type β_2GPI. The competition assays were performed using both irradiated and Maxisorp microwell plates (Nalge Nunc International, Roskilde, Denmark). In the other laboratory (St. George Hospital) a separate panel of serum samples with anti-β_2GPI autoantibodies was examined for binding to wild-type and domain I single point mutant β_2GPI coated directly onto irradiated plates.

Materials and Methods

Affinity purification of anti-β_2GPI autoantibodies

Autoantibodies binding β_2GPI were purified from 15 patients with APS. No patient selection criteria were applied other than the availability of sufficient volume and titer to yield an adequate amount of affinity-purified Ab for the inhibition studies. Anti-β_2GPI was affinity-purified by sequential fractionation with cardiolipin-containing liposomes, then protein A-agarose beads, as previously described (11).

Patient selection for the direct binding anti-β_2GPI ELISA

Serum samples were screened with an in-house and a commercial anti-β_2GPI ELISA (INOVA Diagnostics, San Diego, CA). Samples scoring above normal with the in-house assay and >40 U in the commercial assay (normal is <20 U) were selected for further study.

Anti-β_2GPI competitive inhibition ELISA

MaxiSorp microplates (Nunc) and irradiated microplates (10 kGy; ICN, Costa Mesa, CA) were coated overnight at 4°C with 50 μl of full-length recombinant β_2GPI at 10 μg/ml in 0.1 M carbonate, pH 9.5, then washed three times with 0.15 M PBS, pH 7.2, and blocked for 1 h at room temperature with 75 μl of 2% nonfat dried milk (NFDM) and PBS. Each Ab preparation was titrated to determine the concentration required to produce ~80% maximum binding, typically between 2.5 and 10 μg/ml. Test inhibitors (wild-type or domain I point mutants) were diluted to the required concentration in 2% NFDM, or 2% NFDM alone was added to coated wells. Affinity-purified anti-β_2GPI Ab was diluted in 2% NFDM, and 25 μl of a constant concentration was added to the wells. The contents of the wells were mixed, and the plates were incubated at 37°C for 1 h. The plates were washed three times with PBS, then 50 μl of alkaline phosphatase-conjugated anti-human IgG or anti-mouse IgG (γ-chain specific; Zymed, South San Francisco, CA) diluted in 2% NFDM was added and incubated at 37°C for 1 h. The plates were washed three times with PBS, 50 μl of alkaline phosphatase chromogenic substrate was added, and the plates were incubated for 30 min at room temperature. The A_{550} was measured in a microplate autoreader (Bio-Tek Instruments, Winooski, VT). The percent inhibition was determined as follows: [(mean A_{550} obtained from the control wells without inhibitor less A_{550} of background) $-$(A_{550} obtained in the presence of inhibitor less A_{550} of background)/(the mean A_{550} obtained from the control wells without inhibitor less A_{550} of background)] $\times$ 100. Each inhibitor was tested at seven different concentrations, allowing an inhibition curve to be constructed. This was then used to calculate the amount of mutant or wild-type β_2GPI required to give 50% inhibition (IC_{50}). The IC_{50} of each mutant was expressed as a ratio with the IC_{50} of wild-type β_2GPI for each anti-β_2GPI tested.

Anti-β_2GPI direct binding ELISA

Flat-bottom microtiter polystyrene plates (ICN Biomedical) were used after gamma irradiation to 10 kGy (7). Preparations of β_2GPI were wild-type β_2GPI and 10 mutants with a single point mutation in domain I, as described above. The washing buffer was 1 M NaCl, 20 mM sodium phosphate buffer adjusted to pH 7.2 plus 0.1% (v/v) Tween 20, and the blocking buffer comprised wash buffer with the addition of fatty acid-free 1% (w/v) BSA (Sigma-Aldrich, Sydney, Australia). The final wash was with 0.15 M NaCl and 20 mm sodium phosphate, pH 7.2, without Tween 20. Second Abs were alkaline phosphatase-conjugated goat anti-mouse IgG, anti-human IgG, or anti-human IgM (Sigma-Aldrich). The colorimetric substrate was *p*-nitrophenyl phosphate (Sigma-Aldrich; 1 mg/ml) in 1 M diethanolamine and 0.5 mM $MgCl_2$, pH 9.8, and reading was performed in a TiterTek Mulitskan MCC instrument (Eflab, Espoo, Finland) at A_{405}. The wild-type and point mutant β_2GPI preparations were coated at 10 μg/ml in 50 μl/well of 50 mM sodium carbonate/bicarbonate buffer, pH 9.6, at 4°C overnight, a concentration that ensured adequate Ag coating to enable patient autoantibodies to bind as previously determined (14). The wells were washed three times with 200 μl of wash buffer, blocked with 200 μl of blocking buffer for 1 h at 4°C, and washed three times. Patient serum, diluted 1/100 in blocking buffer, was added and incubated for 2 h at room temperature, then wells were washed three times. Where mAbs were used these were at the stated concentration diluted in blocking buffer. The appropriate alkaline phosphatase-conjugated anti-Ig was diluted 1/1000 in blocking buffer, and 50 μl/well was added and incubated for 2 h at room temperature. The plates were washed three times with wash buffer, then once with final wash buffer, before 50 μl/well of prewarmed substrate was added and incubated in the dark at 37°C.

Patient samples were read 30 min after the addition of substrate. Assays were accepted if the positive standard control was within 10% of 1.5 absorption units (AU), and the normal serum control within the normal range. All samples were tested in parallel in duplicate wells. All samples tested against point mutants were also tested against the wild type at the same time. Assays with mAbs were read when the positive control OD was in the range 1.5–1.9 AU.

The direct binding ELISA was reproducible with an intraassay coefficient of variation of 5.3, and an interassay coefficient of variation of 2.6% for the positive standard and of 8.4% for all samples. Forty normal blood donors had a mean of 0.081 AU and an SD of 0.054 AU. The mean $\pm$ 5 SD (0.353 AU) was used as the cut-off for abnormality.

Anti-β_2GPI mAbs

mAbs 1, 10, 11, and 16 were obtained from immunized mice and were used at 0.1, 0.2, 0.1, and 0.2 μg/ml, respectively. mAbs 1, 11, and 16 bind domain I, while mAb 10 binds domain IV (18). mAb FC1, used at 1 μg/ml, is an IgG that binds domain I and was obtained from an autoimmune NZW $\times$ BXSB F_1 mouse (19). FC3 is an isotype-matched non-β_2GPI-binding control from the same mouse. IgM mAbs EY2C9 (anti-β_2GPI) and TH1B9 (negative control; a gift from Dr. T. Koike) were used at 2 μg/ml and were derived from an autoimmune patient as previously described (20). mAb 4E7H10 was obtained from mice immunized with a domain I-keyhole limpet hemocyanin conjugate.

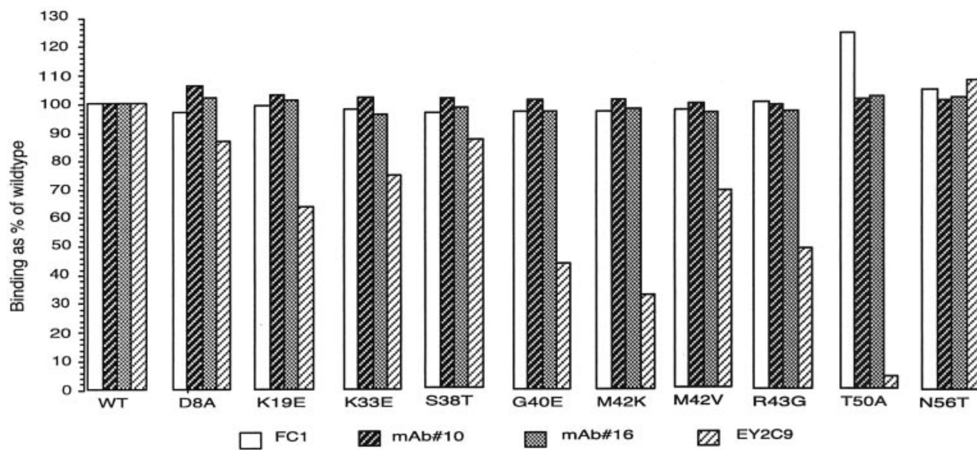

FIGURE 1. Binding of anti-β_2GPI mAb to wells coated with wild-type β_2GPI and domain I point mutants. Binding is displayed as a percentage of binding to the wild type in parallel. Abs FC1, 10, and 16 are mouse mAbs, and EY2C9 is a human Ab.

β_2GPI point mutation models

Three-dimensional models of the first domain of β_2GPI were calculated using a method of comparative modeling by satisfaction of spatial restraints implemented in the MODELLER program (21). Ten different models were produced for each point mutation based on the x-ray structure of the native β_2GPI (Protein Data Bank code 1c1z (8)). The final models of each point mutant were selected based on the value of the MODELLER objective function (21). To study changes in antigenicity caused by point mutations in the β_2GPI sequence, the surface electrostatic potentials of the native structure and the mutant models were calculated with the GRASP program (22) using the atomic charges from the CHARMM22 force field (23).

Results

Construction, expression, and purification of domain I single point mutants

To further examine the epitope(s) recognized by these autoantibodies we made full-length recombinant β_2GPI proteins with single point mutants in domain I. First we determined which, if any, of the amino acid positions in domain I of β_2GPI are important for autoantibody binding by screening filamentous phage-displayed peptide libraries in which mutants of domain I were expressed as an amino-terminal fusion with the pIII protein. The mutations were created by error-prone PCR, such that an average of one mutation in domain I was generated in each phage clone. A phage micropan assay was used to screen individual phage for the ability to bind affinity-purified Abs from patients with antiphospholipid syndrome. Using this approach we rapidly identified phage with missense mutations covering approximately half the 63 domain I residues. This allowed us to identify a number of mutants that adversely affected Ab binding. Ten mutants, some of which affected Ab binding and some of which did not, were chosen for further study as follows: D8A, K19E, K33E, S38T, G40E, M42K, M42V, R43G, T50A, and N56T, where the nomenclature of D8A signifies a change from D to A at position 8. These were incorporated into full-length β_2GPI mutant proteins produced in insect cell cultures as previously described (11).

Direct binding anti-β_2GPI ELISA

mAbs were used to assess the ability of the point mutants to coat the wells of gamma-irradiated microplate wells. Both mouse anti-domain IV mAb 10 and mouse anti-domain I mAb 16 showed no reduction in

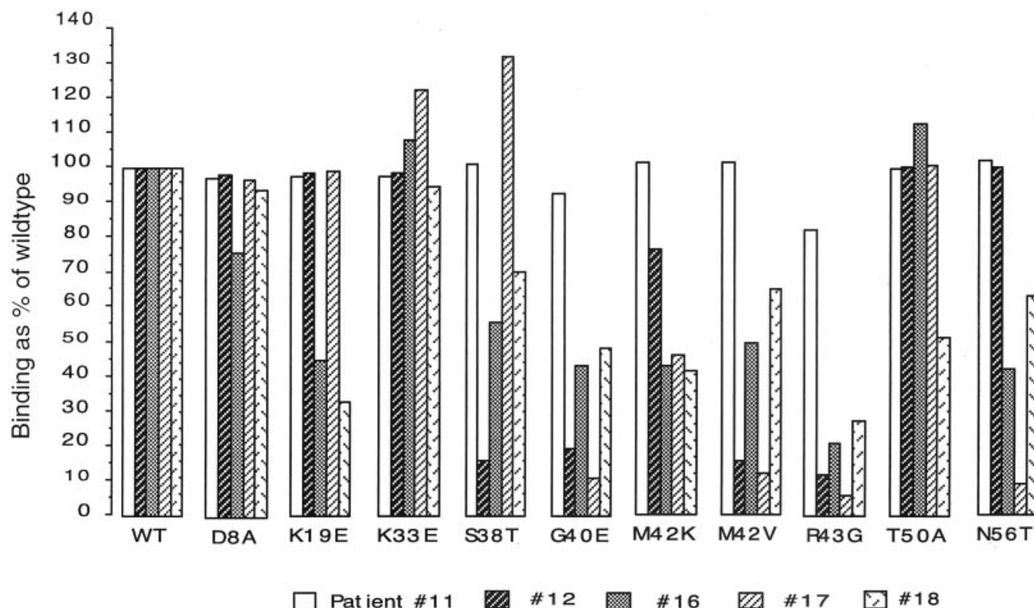

FIGURE 2. Antisera from five selected patients were tested for their ability to bind to wells coated with recombinant β_2GPI, each with a single amino acid substitution in domain I. Binding is displayed as a percentage relative to wild-type binding.

Table I. *Binding of all patients' sera in the direct binding ELISA*[a]

Patient No.	D8A	K19E	K33E	S38T	G40E	M42K	M42V	R43G	T50A	N56T
1	88	64	94	102	102	70	101	34	106	82
2	98	82	98	101	102	94	105	77	100	98
3	98	63	104	103	93	76	102	22	45	57
4	88	84	93	95	78	68	84	62	106	95
5	74	45	74	82	47	64	84	42	109	102
6	69	42	181	87	72	59	73	47	153	75
7	76	39	160	85	98	55	61	68	51	77
8	102	84	101	96	92	89	92	53	70	90
9	99	86	99	106	89	96	103	76	101	100
10	79	29	90	50	77	30	24	18	86	48
11	98	98	98	101	93	101	101	82	99	101
12	98	98	98	16	19	76	16	12	100	100
13	91	56	87	84	76	73	72	56	108	95
14	80	69	94	75	93	52	85	27	166	32
15	97	89	110	97	95	87	87	51	77	94
16	76	45	108	56	43	43	49	21	112	42
17	97	99	122	132	11	46	12	6	101	9
18	94	33	95	70	48	41	64	28	51	63
19	101	108	107	74	74	67	98	31	111	109
20	86	64	43	77	121	21	103	30	92	105
21	86	20	76	81	76	45	75	28	32	68
22	98	98	98	101	101	101	101	96	100	101
23	117	52	130	76	61	53	70	39	109	41
24	96	64	88	105	81	86	108	45	80	98
25	93	45	71	69	62	84	72	33	132	63
26	142	71	95	128	75	74	97	89	110	91
27	95	36	86	97	74	75	98	61	57	85

[a] Direct binding of autoantibodies from APS patients to wells coated with recombinant β_2GPI, each with a different single amino acid substitution in domain I. Values are the percent binding relative to binding to wild-type β_2GPI.

binding to the point mutants compared with wild type, suggesting similar microplate coating for each mutant (Fig. 1). The autoimmune mouse mAb FC1 showed a 20% increase in binding to T50A, but otherwise similar binding compared with the wild type. The binding of the human mAb EY2C9 to mutant T50A was negligible and was reduced by >50% to G40E, M42K, and R43G. The results suggest that the antigenic epitope recognized by EY2C9 is located on domain I.

Serum samples from 27 APS patients were similarly tested for their ability to bind each of the point mutants and wild-type β_2GPI adsorbed onto gamma-irradiated microplate wells. The patient samples showed a decreased binding to several point mutants, in particular to those with mutations between G40E and R43G. The results from five patients are shown graphically in Fig. 2, and the results for all 27 samples are tabulated in Table I. A majority of the samples (60%) had a >50% decrease in binding to R43G compared with the wild type. Nine sera (33%) showed a >50% decrease in binding to K19E. Two sera (no. 12 and 17) showed little to no binding to the G40E, M42V, and R43G mutants while displaying very good binding to the remaining seven single point mutants and the wild type.

Competitive inhibition ELISA

Wild-type β_2GPI and 10 different mutants were tested for their ability to inhibit mouse mAbs 4E7H10 and 16 from binding to wild-type β_2GPI immobilized on ELISA plates (both Nunc MaxiSorp and irradiated polystyrene). These mAbs were chosen because they recognize conformational epitopes on domain I and because they bind to different epitopes than the human autoantibodies (data not shown). The results (Fig. 3) show that on both types of ELISA plates all 10 mutants inhibited the binding of both anti-β_2GPI mAbs to wild-type β_2GPI. The IC_{50} for each of the single point β_2GPI mutants was within a 10-fold range of the wild-type value. This suggests that the single amino acid substitutions in domain I did not alter the overall conformation of the domain, inasmuch as that part of the domain recognized by these two mAbs remained unaffected.

The same mutants were also tested, in a dose-dependent fashion, for their ability to inhibit affinity-purified APS patient autoantibodies from binding to wild-type β_2GPI immobilized on both Nunc and gamma-irradiated ELISA plates. The amount of each of the mutants required to give 50% inhibition was divided by the amount of wild type required to give 50% inhibition. This value, a

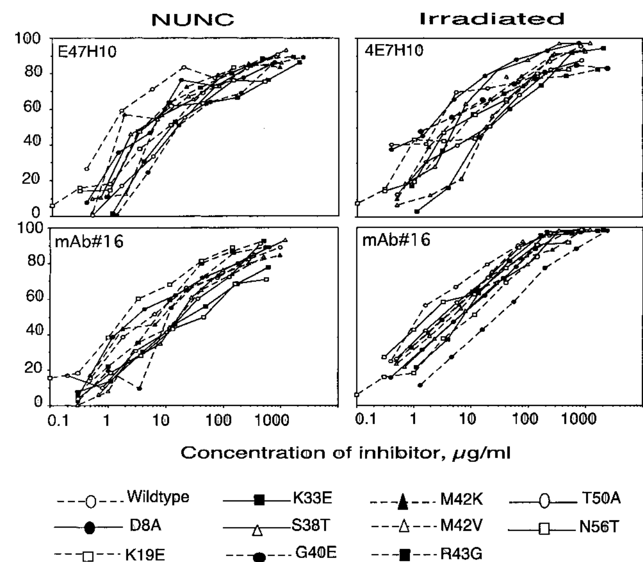

FIGURE 3. Competitive inhibition of anti-β_2GPI mAbs with domain I point mutants. The percent inhibition was determined and plotted as a function of that concentration. Each graph is labeled, in the *upper left* corner, with the mAb used.

fold increase, was used as a quantitative measurement, so that thereactivity of each mutant could be compared from one Ab sample to another. The complete inhibition results from two of the Ab preparations are shown graphically in Fig. 4, and the tabulated results of all 15 of the autoantibody preparations tested are shown in Table II. The data show that most of the mutants were generally within 10-fold of the wild-type β_2GPI concentration in their ability to achieve 50% inhibition. By contrast, the G40E and R43G mutant β_2GPI proteins were much less effective in inhibiting the autoantibodies than was wild-type β_2GPI, and in most cases were unable to achieve 50% inhibition at concentrations up to 1000-fold greater than that required for the wild type. The K19E mutation generally required concentrations 10- to 500-fold greater than wild-type β_2GPI to achieve 50% inhibition.

Eight of the affinity Ab preparations were also analyzed by direct binding. The results, tabulated in Table III, show results similar to those obtained in the competitive inhibition assays.

Modeling

The amino acids R43 and G40 are exposed to solvent and located close together on one face of β_2GPI domain I. Comparative modeling and electrostatic calculations of the G40E and R43G mutants predict an alteration of the surface shape and electrostatic charge of domain I. The disrupted region includes both a protruding mobile loop and the adjacent concave surface (Fig. 5).

Discussion

This study was designed to test the conclusions of our earlier report, which showed that autoantibodies from APS patients recognize antigenic epitopes on domain I of β_2GPI (11). In that report we used a competitive inhibition ELISA employing a panel of whole domain deletions in recombinant β_2GPI. It is possible that the deletion of whole domains might have altered the conformation of the remaining domains in such a way as to prevent the binding of autoantibodies, thereby giving a false impression of their domain specificity. In this study we employed only full-length β_2GPI

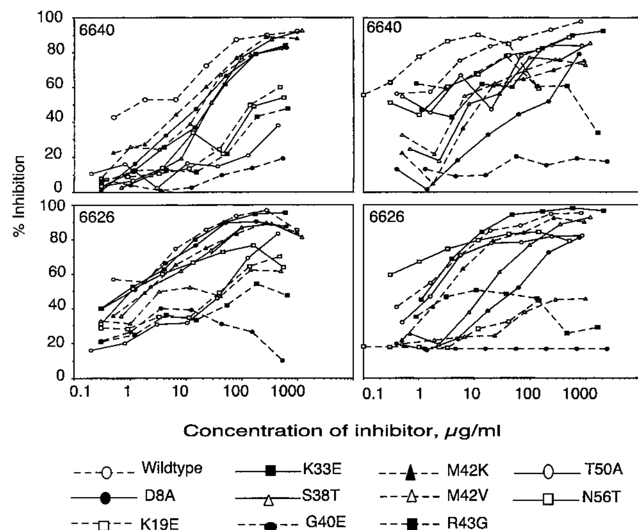

FIGURE 4. Same as in Fig. 3, except human Abs were used for these graphs.

Table II. *Binding of all patients' antibodies in the competitive inhibition ELISA*[a]

Sample	β2GPI		D8A		K19E		K33E		S38T		G40E		M42K		M42V		R43G		T50A		N56T	
	I	N	I	N	I	N	I	N	I	N	I	N	I	N	I	N	I	N	I	N	I	N
4E7H10	1	1	0.2	0.8	35	25	0.5	21	0.1	4	0.1	0.7	5	4	89	14	1	7	17	9	34	5.7
mAb-16	1	1	3	1	18	0.4	15	14	0.1	3	0.5	9	0.1	1.2	0.5	5	0.2	4	0.4	4	0.4	4.6
6501	1	1	0.4	2	16	109	0.5	8	13	13	>1000	>1000	9	8	0.9	14	>1000	>1000	4	119	3	56
6701	1	1	0.6	0.7	12	5	0.2	0.4	0.1	1	>1000	>1000	0.3	1.1	0.1	0.5	>1000	>1000	0.2	1	>1000	>1000
6632	1	1	2	0.6	11	16	4	0.3	3	0.8	1	0.9	16	1	4.9	1	0.3	4	24	0.1	33	167
7019	1	1	1	2	529	352	4	38	2	7	143	110	8	8	3.3	5	>1000	817	3	101	7	22
7209	1	1	3	0.7	36	21	1	5	9	4	>500	>1000	23	1.3	25	3	>1000	>1000	15	1.6	ND	9
7101	1	1	3	0.8	0.3	3	1	0.5	2	0.3	33	2	1	ND	2	ND	17	10	0.3	ND	0.2	ND
7211	1	1	1	3	37	37	0.4	4	1	3	>1000	>1000	0.5	7	1	3	>1000	>1000	2	8	3	18
6625	1	1	7	0.2	25	13	10	3	6	0.8	>1000	>1000	8	2	7	2	>1000	>1000	5	4	51	4
6666	1	1	2	46	26	199	3	5	8	15	>1000	>1000	2.2	1	1	0.4	>1000	>1000	1	5	3	13
6660	1	1	2	9	45	3	3	141	3	44	>1000	>1000	3.9	55	3	44	>1000	>1000	4	45	4	29
6664	1	1	5	8	14	81	3	141	12	16	>1000	>1000	3.3	55	3	44	>1000	>1000	4	40	60	152
7208	1	1	2	57	15	37	4	20	2	15	>1000	>1000	4.8	0.3	2	0.4	>1000	>1000	12	3	2	17
6626	1	1	47	6	66	68	0.5	6	16	16	>1000	>1000	4.4	13	41	169	>1000	>1000	3	213	4	13
6640	1	1	71	14	0.8	21	25	18	104	22	>1000	>1000	180	7	67	11.7	>1000	>1000	6	4	4	283
7217	1	1	37	1	33	41	26	5	10	1	9	25	42.3	31	44	3.2	177	137	2	4	2	5

[a] Concentration of soluble inhibitor, relative to wild-type β_2GPI (fold increase), required to give 50% inhibition of binding of patient Ab to wells coated with wild-type β_2GPI. I, irradiated (10 kGy) plate; N, Nunc plate; ND, not done.

Table III. *Binding of affinity-purified autoantibodies in direct binding ELISA*[a]

Patient No.	D8A	K19E	K33E	S38T	G40E	R43G	M42K	M42V	N56T	T50A
6632	101	80	100	81	87	103	69	73	89	65
6625	127	96	130	126	31	29	147	143	127	123
6701	135	112	139	142	25	9	179	189	5	136
6666	173	75	113	89	54	47	190	198	112	98
7101	117	94	119	120	99	64	118	119	131	99
7209	119	115	128	102	40	25	124	120	83	113
7217	116	88	120	113	109	87	119	115	95	92
7211	109	99	109	96	25	30	122	114	99	99

[a] Direct binding of autoantibodies from APS patients to Nunc wells coated with recombinant β_2GPI and using 2% NFDM as diluent. Values are the percent binding relative to wild-type β_2GPI.

(all five domains), each having a single amino acid substitution in domain I. These single point mutants were compared with full-length wild-type β_2GPI for their ability to bind anti-β_2GPI autoantibodies from APS patients.

mAbs were used in both direct binding and competitive inhibition assays to assess whether the selected mutations in domain I altered the conformation of the molecule. With one exception (APS patient-derived mAb EY2C9), binding of mAbs was unaffected by single amino acid substitutions. These results confirm that the overall conformation of β_2GPI was not changed. The data further show that the substituted amino acids are not critical components of the epitopes recognized by the mouse mAbs. Importantly, the data show that all the mutants were coated equally on the microplate wells in the direct binding assays, as mouse mAbs FC1, 10, and 16 bound equivalently to wild-type β_2GPI and to each of the mutants.

Contrary to this finding, binding of human mAb EY2C9 was affected by a number of mutations in domain I. There was essentially no binding to T50A and markedly decreased binding to the mutations at aa 40–43. Paradoxically, EY2C9 was previously reported to bind domain IV (17). The current study strongly supports localization of an epitope for EY2C9 on domain I.

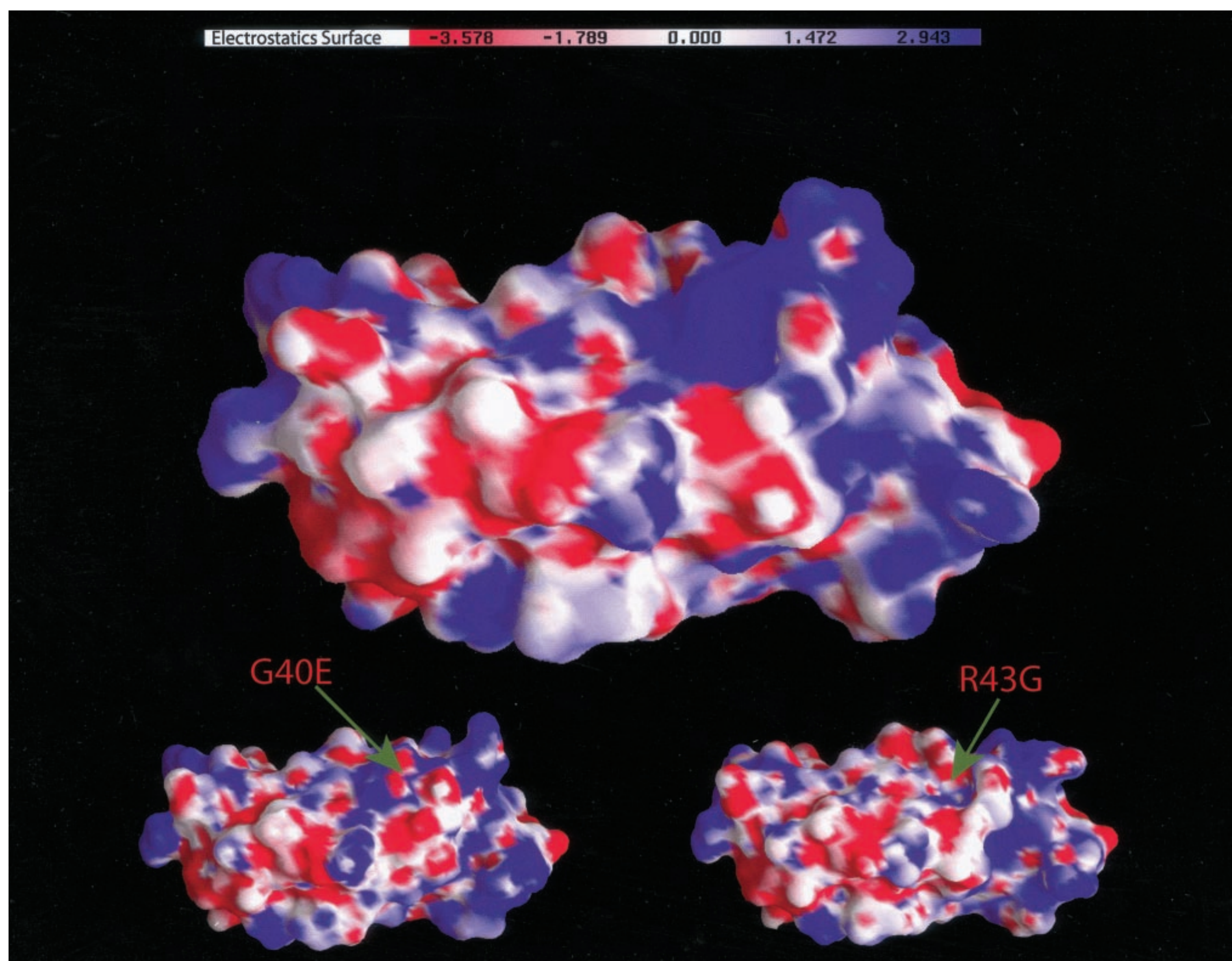

FIGURE 5. The surface electrostatic potential is displayed for B2GPI domain I wild type (*top*), G40E mutation (*bottom left), and R43G (*bottom right), as shown by the color bar (in units of kT; 1 kT unit = 0.58 kCal/electron mol). Arrows point the location of the amino acid substitutions.

Autoantibodies from patient sera were also affected by mutations in domain I, particularly in the region of aa 40–43 (G40E, M42V, and R43G). With some patient sera there was a reduction in direct binding by 80–90% relative to wild-type β_2GPI (Table I). In the competitive inhibition studies the same mutants were generally unable to achieve 50% inhibition at concentrations 1000-fold higher than required with the wild type (Table II). These data clearly show that domain I contains the dominant epitope(s) for anti-β_2GPI associated with APS.

While there are some differences in the results from the two different assay methods, the overall pictures from both are similar. The main difference is that mutation K19E had a greater effect in the direct binding assays than in the competitive inhibition assays. However, autoantibody binding to K19E was diminished in both assays. It is unclear whether K19 is part of the same epitope as that affected by G40E and R43G for some Abs, if K19E affects other amino acids that are part of that epitope, or if K19E affects a second, distinct epitope.

The conformation of a region in a protein can be altered by a point mutation to a degree that depends on the specific substitution and the residues adjacent to that substitution (24). Modeling of domain I predicts that the G40E and R43G mutations are surface exposed, and they are likely to induce conformation and electrostatic changes over a surface region of the domain, as shown in Fig. 5. The altered area of the domain is therefore likely to contribute significantly to the Ab-Ag binding either because G40 and R43 are themselves critical residues of the epitope or because the substitutions at these points affect the conformation of the nearby critical residues. The autoantibodies and the mAbs do not bind to reduced and alkylated domain 1 (data not shown), further suggesting that the epitope(s) is conformational and not linear.

A similar restriction of autoantibodies to a single immunodominant region has been shown for other autoantigens, such as thyroid peroxidase (25). Given that potential epitopes would be expected to be found over much of the protein (26), this restriction of autoantibody repertoire found in both our study and another suggests that generation of autoantibody-producing B cell clones is influenced by other factors in addition to the availability of epitopes.

In conclusion, this study has shown that single amino acid substitutions in domain I of full-length β_2GPI disrupt autoantibody binding to β_2GPI. This was demonstrated with competitive inhibition and direct binding assays. The competitive inhibition method used mutants in fluid phase to circumvent difficulties with ensuring adequate Ag binding to the solid phase of the microplate well. This study provides strong support for the hypothesis that the epitopes for anti-β_2GPI autoantibodies from patients with APS are located on domain I of β_2GPI. Amino acids 40–43 form a surface-exposed region that contributes to the immunodominant epitope.

Acknowledgments

We thank Jackie Crisologo, Merle Hayag, and Eric Smith for expert technical assistance, and Prof. T. Koike for providing mAbs EY2C9 and Th1B9.

References

1. Kandiah, D. A., A. Sali, Y. Sheng, E. J. Victoria, D. M. Marquis, S. M. Coutts, and S. A. Krilis. 1998. Current insights into the "antiphospholipid" syndrome: clinical, immunological, and molecular aspects. *Adv. Immunol. 70:507.*
2. Reddel, S. W., and S. A. Krilis. 1999. Testing for and clinical significance of anticardiolipin antibodies. *Clin. Diagn. Lab. Immunol. 6:775.*
3. Guerin, J., R. Sim, B. B. Yu, J. Ferluga, C. Feighery, and J. Jackson. 2000. Heterogeneous recognition of β_2-glycoprotein I by antibodies from antiphospholipid syndrome patients. *Thromb. Haemost. 84:374.*
4. Hunt, J. E., H. P. McNeil, G. J. Morgan, R. M. Crameri, and S. A. Krilis. 1992. A phospholipids β_2-glycoprotein I complex is an antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection. *Lupus 1:75.*
5. Iverson, G. M., D. S. Jones, D. Marquis, M. D. Linnik, and E. J. Victoria. 1998. A chemically defined, toleragen-based approach for targeting anti-β_2-glycoprotein I antibodies. *Lupus 7(Suppl. 2):S166.*
6. Steinkasserer, A., C. Estaller, E. H. Weiss, R. B. Sim, and A. J. Day. 1991. Complete nucleotide and deduced amino acid sequence of human β_2-glycoprotein I. *Biochem. J. 277:387.*
7. Sheng, Y., A. Sali, H. Herzog, J. Lahnstein, and S. A. Krilis. 1996. 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. *J. Immunol. 157:3744.*
8. Schwarzenbacher, R., K. Zeth, K. Diederichs, A. Gries, G. M. Kostner, P. Laggner, and R. Prassl. 1999. Crystal structure of human β_2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J. 18:6228.*
9. Bouma, B., P. G. de Groot, J. M. van den Elsen, R. B. Ravelli, A. Schouten, M. J. Simmelink, R. H. M. W. Derksen, J. Kroon, and P. Gros. 1999:Adhesion mechanism of human β_2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J. 18:5166.*
10. Wang, M. X., D. A. Kandiah, K. Ichikawa, M. Khamashta, G. Hughes, T. Koike, R. Roubey, and S. A. Krilis. 1995. Epitope specificity of monoclonal anti-β_2-glycoprotein I antibodies derived from patients with the antiphospholipid syndrome. *J. Immunol. 155:1629.*
11. Iverson, G. M., E. J. Victoria, and D. M. Marquis. 1998. Anti-β_2-glycoprotein I (β_2GPI) autoantibodies recognize an epitope on the first domain of β_2GPI. *Proc. Nat. Acad. Sci. USA 95:15542.*
12. George, J., B. Gilburd, M. Hojnik, Y. Levy, P. Langevitz, E. Matsuura, T. Koike, and Y. Shoenfeld. 1998. Target recognition of β_2-glycoprotein I (β_2GPI)-dependent anticardiolipin antibodies: evidence for involvement of the fourth domain of β_2GPI in antibody binding. *J. Immunol. 160:3917.*
13. Yang, C. D., S. L. Chen, N. Shen, M. Qi, and F. Xue. 1998. Detection of anti-recombinant β_2-glycoprotein I and anti-recombinant β_2-glycoprotein I fifth domain antibodies in sera from patients with systemic lupus erythematosus. *Rheumatol. Int. 18:5.*
14. Reddel, S. W., Y. X. Wang, Y. H. Sheng, and S. A. Krilis. 2000. Epitope studies with anti-β_2-glycoprotein I antibodies from autoantibody and immunized sources. *J. Autoimmun. 15:91.*
15. Arvieux, J., V. Regnault, E. Hachulla, L. Darnige, B. Roussel, and J. C. Bensa. 1998. Heterogeneity and immunochemical properties of anti-β_2-glycoprotein I autoantibodies. *Thromb. Haemost. 80:393.*
16. Igarashi, M., E. Matsuura, Y. Igarashi, H. Nagae, K. Ichikawa, D. A. Triplett, and T. Koike. 1996. Human β_2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. *Blood 87:3262.*
17. Koike, T., K. Ichikawa, T. Atsumi, H. Kasahara, and E. Matsuura. 2000. β_2-Glycoprotein I-antiβ_2-glycoprotein I interaction. *J. Autoimmun. 15:97.*
18. Sheng, Y., J. G. Hanly, S. W. Reddel, S. Kouts, J. Guerin, T. Koike, K. Ichikawa, A. Sturgess, and S. A. Krilis. 2001. Detection of 'antiphospholipid' antibodies: a single chromogenic assay of thrombin generation sensitively detects lupus anticoagulants, anticardiolipin antibodies, plus antibodies binding β_2-glycoprotein I and prothrombin. *Clin. Exp. Immunol. 124:502.*
19. Monestier, M., D. A. Kandiah, S. Kouts, K. E. Novick, G. L. Ong, M. Z. Radic, and S. A. Krilis. 1996. Monoclonal antibodies from NZW x BXSB F1 mice to β_2-glycoprotein I and cardiolipin: species specificity and charge-dependent binding. *J. Immunol. 156:2631.*
20. Ichikawa, K., M. A. Khamashta, T. Koike, E. Matsuura, and G. R. V. Hughes. 1994. β_2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum. 37:1453.*
21. Sali, A., and T. L. Blundell. 1993. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol. 234:779.*
22. Nicholls, A., K. Sharp, and B. Honig. 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbon. *Proteins: Structure, Function, and Genetics. 11:281.*
23. MacKerrel, A. D., D. Bashford, M. Bellott, R. L. Dunbrack, Jr., J. D. Evanseck, M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, et al. 1998. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B 102:3586.*
24. Wacey, A. I., D. N. Cooper, D. Liney, E. Hovig, and M. Krawczak. 1999. Disentangling the perturbational effects of amino acid substitutions in the DNA-binding domains of p53. *Hum. Genet. 104:15.*
25. Guo, J., Y. Wang, J. C. Jaume, B. Rapoport, and S. M. McLachlan. 1999. Rarity of autoantibodies to a major autoantigen, thyroid peroxidase, that interact with denatured antigen or with epitopes outside the immunodominant region. *Clin. Exp. Immunol. 117:19.*
26. Getzoff, E. D., J. A. Tainer, R. A. Lerner, and H. M. Geysen. 1988. The chemistry and mechanism of antibody binding to protein antigens. *Adv. Immunol. 43:1.*