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# Patients with atherosclerotic syndrome, negative in anti-cardiolipin assays, make IgA autoantibodies that preferentially target domain 4 of $\beta_2$-GPI

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**Abstract**

Autoantibodies targeting $\beta_2$-glycoprotein I ($\beta_2$-GPI), a component of the atherosclerotic plaque, are commonly found in patients with acute ischemic syndromes. Serum samples from APS (antiphospholipid syndrome) patients and from cardiovascular patients exhibiting acute atherosclerotic syndromes were analyzed for IgG and IgA antibodies in both anti-$\beta_2$-GPI and anticardiolipin (aCL) ELISA assays. All of the APS samples used here were positive in both assays. Serum samples from 382 atherosclerosis patients were also analyzed for IgG and IgA antibodies in the same assays. In sharp contrast to the APS samples, we found that only 1% of the samples from atherosclerosis patients were positive for IgA aCL, and 1.6% positive for IgG aCL, whereas 35.6% were positive for IgA anti-$\beta_2$-GPI and only 1.6% for IgG anti-$\beta_2$-GPI. The antigenic specificity of 29 serum samples from atherosclerosis patients was evaluated. Six different recombinant domain-deleted mutants (DM) of human $\beta_2$-GPI and full-length human $\beta_2$-GPI (wild-type) were used in competitive inhibition assays to inhibit the autoantibodies from binding in the anti-$\beta_2$-GPI ELISA assays. Domain-deleted mutants D—345 and D—45 inhibited the binding in the IgA anti-$\beta_2$-GPI assay, suggesting that these autoantibodies recognize domain 4 of the $\beta_2$-GPI molecule. These results clearly show that IgA anti-$\beta_2$-GPI autoantibodies from atherosclerotic patients are distinct from IgA autoantibodies found in APS samples.
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*Keywords:* $\beta_2$-GPI; Atherosclerosis

## 1. Introduction

IgA class autoantibodies to beta 2-glycoprotein I ($\beta_2$-GPI) have recently been reported in patients with acute myocardial infarction [1] and also in patients with ischemic stroke [2, 3]. A striking observation from two of these studies was that the IgA $\beta_2$-GPI autoantibodies were usually detected in patients that were negative for IgA anti-cardiolipin antibodies (ACA) [1, 3]. This finding is in sharp contrast to that observed in patients with antiphospholipid syndrome (APS), where both anti-$\beta_2$-GPI and anticardiolipin antibodies are usually positive.

$\beta_2$-GPI is a serum protein composed of five homologous domains numbered 1–5 from the N terminus. Domains 1–4 are composed of ~60 amino acids [4] that contain a motif characterized by a framework of four conserved cysteine residues, which form two internal disulfide bridges. The fifth domain differs from domains 1–4 in that it contains 82 amino acid residues with six cysteines. The fifth domain contains the phospholipids-binding site [5].

Conflicting findings have been published concerning the domain specificity of anti-$\beta_2$-GPI. There are reports that IgG anti-$\beta_2$-GPI autoantibodies in patients with APS recognize epitopes on domain 3 [6] and domain 4 [6,7] and domain

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5 [8] of $\beta_2$-GPI. Using recombinant anti-$\beta_2$-GPI and $\beta_2$-GPI domain-deleted mutants (Dms) expressed in insect cells we and others have reported that these autoantibodies recognize, recognize domain 1 of $\beta_2$-GPI [9–11].

In the present study we explored the possibility that the predominant antibody profile exhibited by APS patients (cardiolipin IgG positive/$\beta_2$-GPI IgG/IgA positive) differed from the predominant profile exhibited by atherosclerotic patients with acute ischemic disease (cardiolipin IgG/IgA negative/$\beta_2$-GPI IgA positive) because of differing domain specificity of the APS and atherosclerosis patient's antibodies.

Using a series of full-length $\beta_2$-GPI and $\beta_2$-GPI Dm, we tested a large number of serum samples from patients with APS and various atherosclerotic populations for IgG, IgA, and IgM antibodies to these constructs by using a competitive inhibition ELISA. All specimens were also tested for IgG, IgA, and IgM aCL antibodies.

The investigation presented here found that 29 of 29 IgA anti-$\beta_2$-GPI positive samples from atherosclerosis patients specifically recognized domain 4 of $\beta_2$-GPI.

## 2. Materials and methods

### 2.1. Recombinant $\beta_2$-GPI

The recombinant $\beta_2$-GPI and $\beta_2$-GPI domain-deleted mutants (Dms) used are as previously described [12]. Briefly, TN5 insect cells were infected with high titre viral stock produced in Sf9 insect cells. Each construct contained a six His tail that was used for purifying the protein from culture media. The nomenclature for domain deletion mutants uses numbers to indicate the presence of domains while a dash symbolizes the domain is missing. Thus, D—345 is the name given to the recombinant protein that contains domains 3, 4 and 5 while lacking domains 1 and 2.

### 2.2. Patient sample selection

The diagnosis of each syndrome described was done according to clinical presentation, ultrasound, angiography or magnetic angioresonance studies. Patients were enrolled consecutively in a tertiary center (University Hospital). Patients with infective endocarditis, osteonecrosis, neoplasms, cerebral hemorrhage, infection by HIV or treponema pallidum, presence of known heritable causes of thrombosis such as homocistinuria or factor V (Leiden) mutation, previous diagnosis of APS, or other connective tissue disorder (CTD) were excluded. Detailed inclusion and exclusion criteria for each clinical situation have been described in detail in our previous publications. Control patients were recruited from patients admitted to the Orthopedic clinic for fractures or musculoligamentous disorders and without acute myocardial infarction, stroke, or other cardiac conditions [1, 3].

A total of 511 archived specimens consisting of 382 sera from individuals with various atherosclerosis conditions and 129 sera from individuals with antiphospholipid syndrome were studied. The atherosclerosis group included sera from individuals with peripheral arterial disease (117), acute coronary syndrome (117), and acute myocardial infarction (90). We randomly selected for this study 10 samples from APS patients that were positive for both IgG and IgA in the anti-$\beta_2$-GPI ELISA, and 29 samples from atherosclerosis patients that were positive for IgA in the anti-$\beta_2$-GPI ELISA.

Table 1
Frequency of aCL and anti-$\beta_2$-GPI antibodies in APS and cardiovascular groups by ELISA testing

| Total sera = 511 | APS patients ($n = 129$) | Cardiovascular patients ($n = 382$) |
|---|---|---|
| aCL screen (IgG/IgA/IgM) | 78% | 12% |
| anti-$\beta_2$-GPI Screen (IgG/IgA/IgM) | 79% | 46% |
| aCL IgG | 64% | 1% |
| aCL IgA | 9% | 1% |
| anti-$\beta_2$-GPI IgG | 43% | 1% |
| anti-$\beta_2$-GPI IgA | 48% | 33% |

### 2.3. Anti-$\beta_2$-GPI and Anticardiolipin ELISA

All samples, both from the APS and atherosclerosis groups, were tested for the presence of aCL antibodies and anti-$\beta_2$-GPI antibodies by ELISA. Specimens were first tested for the presence of IgG, IgA, or IgM aCL and $\beta_2$-GPI antibodies using polyvalent aCL and anti-$\beta_2$-GPI screening ELISA tests. All ELISA kits used in this study were manufactured by INOVA

Table 2
Competitive inhibition assay using 10 different APS serum samples with indicated recombinant B2GPI and deletion mutants

| Antibody number | D12345[a] | | D12— | | D—45 | |
|---|---|---|---|---|---|---|
| | Max[b] | 50%[c] | Max | 50% | Max | 50% |
| *IgG* | | | | | | |
| 6612 | 89 | 9.5 | 98 | 24.0 | 17.0 | >125 |
| 6626 | 88 | 31.8 | 90 | 88.0 | 3.5 | >125 |
| 6635 | 95 | 11.8 | 96 | 29.5 | 3.0 | >125 |
| 6647 | 92 | 25.8 | 94 | 66.0 | 5.0 | >125 |
| 6656 | 64 | 38.4 | 67 | 85.0 | 10.0 | >125 |
| 6666 | 79 | 27.2 | 85 | 20.3 | 2.4 | >125 |
| 6674 | 90 | 20.5 | 94 | 52.0 | 12.0 | >125 |
| 7002 | 58 | 53.7 | 53 | 178.0 | 7.0 | >125 |
| 7005 | 77 | 23.5 | 71 | 58.0 | 1.9 | >125 |
| 7010 | 83 | 15.8 | 86 | 41.6 | 14.0 | >125 |
| *IgA* | | | | | | |
| 6612 | 80 | 13.1 | 74 | 36 | 28 | >125 |
| 6626 | 62 | 42.4 | 39 | >125 | 61 | >125 |
| 6635 | 57 | 44.7 | 67 | 29.6 | 0 | >125 |
| 6647 | 68 | 30.7 | 58 | 66.4 | 0.7 | >125 |
| 6656 | 74 | 24.3 | 42 | >125 | 40 | >125 |
| 6666 | 48 | 67.3 | 42 | >125 | 11 | >125 |
| 6674 | 85 | 39.9 | 88 | 39.9 | 4 | >125 |
| 7002 | 29 | 116.3 | 21 | >125 | 0 | >125 |
| 7005 | 35 | 50.6 | 45 | >125 | 40 | >125 |
| 7010 | 63 | 16.7 | 59 | 50 | 12 | >125 |

>, Highest concentration tested.
[a] Domains included in construct.
[b] Maximum inhibition observed at concentrations tested.
[c] Concentration (micromolar) to give 50% inhibition.

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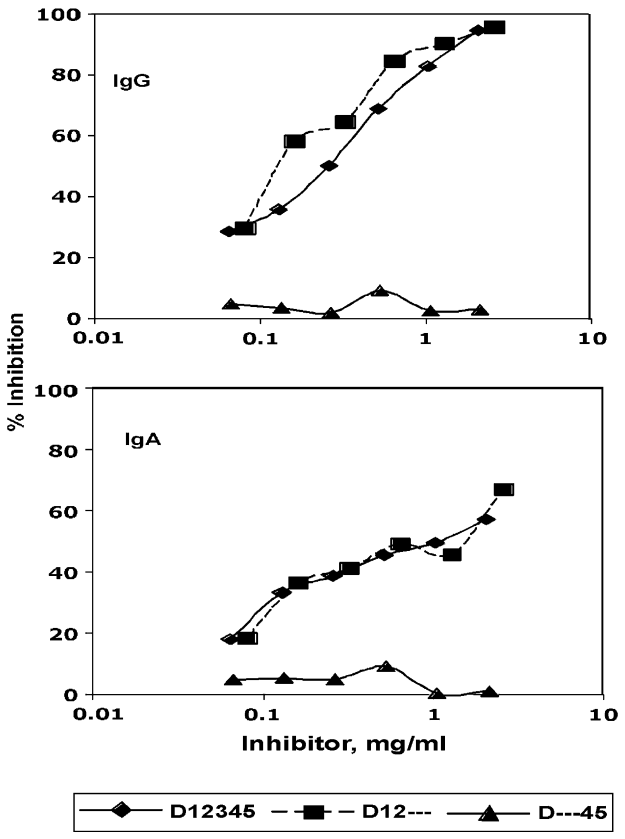


Fig. 1. Competitive inhibition of APS sample 6635 binding to $\beta_2$-GPI by recombinant $\beta_2$-GPI and Dms. A constant amount of antibody was mixed with varying concentrations of inhibitor in wells coated with $\beta_2$-GPI. Recombinant $\beta_2$-GPI and DMs were used as inhibitors. Upper panel measures inhibition of IgG antibodies. Lower panel measures inhibition of IgA antibodies.

Diagnostics (INOVA Diagnostics, San Diego, CA) and run according to the manufacturer's instructions.

### 2.4. Competitive inhibition ELISA

Tests were performed using the appropriate (IgG and/or IgA) anti-$\beta_2$-GPI ELISA kit from INOVA Diagnostics. Each serum was titred to determine the dilution required to give approximately 80% of maximum binding. Test inhibitors were diluted in the sample dilution buffer provided in the kits and 25 μl of each dilution or sample diluent alone was added to the wells. The serum samples were diluted in sample dilution buffer and 25 μl of a constant dilution was added to the wells. The contents of the wells were mixed and plates were incubated at room temperature for 30 min. Wells were washed three times with the wash buffer provided in the kits, 50 μl of the HRP conjugated anti-IgG or IgA was added, incubated for 30 min washed three times with wash buffer and 50 μl of substrate solution was added. Wells were incubated at room temperature for 30 min and 50 μl of stop solution was added. The OD 450 for each well was determined in an Anthos Labtec HT2 microplate reader (Salzburg, Austria). The percent inhibition was determined as follows: [(mean $A_{450}$ obtained from the control wells without inhibitor less $A_{450}$ of background) − ($A_{450}$ obtained in the presence of inhibitor less $A_{450}$ of background)/mean $A_{450}$ obtained from the control wells without inhibitor less $A_{450}$ of background)] × 100.

Table 3
Anti-aCL and anti-$\beta_2$-GPI profile of samples from cardiovascular patients[a] for inhibition study

| Sample | $\beta_2$-GPI IgA units | $\beta_2$-GPI IgG units[b] | $\beta_2$-GPI IgM units | aCL IgA units | aCL IgG units | aCL IgM units |
|---|---|---|---|---|---|---|
| ACS-52 | 58.9 | 0 | 10.1 | n.t. | n.t. | n.t. |
| ACS-53 | 20.7 | 0 | 4.0 | n.t. | n.t. | n.t. |
| ACS-54 | 33.1 | 9.6 | 0 | n.t. | n.t. | n.t. |
| ACS-58 | 86.4 | 0 | 5.3 | n.t. | n.t. | n.t. |
| ACS-65 | 234.4 | 0 | 4.0 | n.t. | n.t. | n.t. |
| ACS-67 | 38.7 | 0 | 0 | n.t. | n.t. | n.t. |
| ACS-71 | 77.6 | 0 | 0 | n.t. | n.t. | n.t. |
| ACS-74 | 154.8 | 0 | 5.7 | n.t. | n.t. | n.t. |
| ACS-104 | 23.7 | 0 | 58.5 | n.t. | n.t. | n.t. |
| ACS-136 | 32.0 | 64.4 | 0.2 | n.t. | n.t. | n.t. |
| ACS-144 | 28.3 | 3.3 | 16.9 | n.t. | n.t. | n.t. |
| CAS-5 | 65.4 | 0 | 2.8 | n.t. | n.t. | n.t. |
| CAS-6 | 55.5 | 1.2 | 0 | n.t. | n.t. | n.t. |
| CAS-8 | 61.2 | 0 | 0 | n.t. | n.t. | n.t. |
| CAS-13 | 39.9 | 1.7 | 11.7 | n.t. | n.t. | n.t. |
| CAS-15 | 130.1 | 0.1 | 0 | n.t. | n.t. | n.t. |
| CAS-18 | 35.5 | 0 | 0 | n.t. | n.t. | n.t. |
| CAS-28 | 60.5 | 0 | 0 | n.t. | n.t. | n.t. |
| CAS-29 | 51.8 | 4.1 | 5.7 | n.t. | n.t. | n.t. |
| MI-5 | 70.0 | 0 | 25.6 | n.t. | n.t. | n.t. |
| MI-7 | 90.4 | 0 | 0 | n.t. | n.t. | n.t. |
| MI-10 | 183.9 | 0 | 25.4 | n.t. | n.t. | n.t. |
| MI-15 | 58.4 | 0 | 77.4 | n.t. | n.t. | n.t. |
| MI-37 | 32.7 | 0 | 41.2 | n.t. | n.t. | n.t. |
| MI-45 | 25.5 | 0 | 11.6 | n.t. | n.t. | n.t. |
| PAD-30 | 27.6 | 0 | 0 | n.t. | n.t. | n.t. |
| PAD-39 | 22.1 | 0 | 0 | n.t. | n.t. | n.t. |
| PAD-42 | 45.5 | 0 | 0 | 27.9 | n.t. | n.t. |
| PAD-101 | 26.4 | 3.0 | 25.4 | n.t. | n.t. | n.t. |

n.t. = specimens testing negative on aCL screening assay weree not tested on isotype-specific assays.

[a] ACS: Acute Coronary Syndrome. MI: Myocardial Infarction. CAS: Carotid Artery Study. PAD: Peripheral Artery Disease.

[b] $\beta_2$-GPI results with negative values (resulting from extrapolation at bottom of the standard curve) were assigned a value of 0.

## 3. Results

### 3.1. Anti-$\beta_2$-GPI and anticardiolipin

Serum samples from APS and atherosclerosis patients were analyzed for IgG and IgA autoantibodies in both the anti-$\beta_2$-GPI and the anti-cardiolipin (aCL) assays. Almost 80% of the APS samples were positive by polyvalent IgG/IgA/IgM aCL and B2GPI screening assays (Table 1). Specific isotype testing of the APS sera revealed that approximately 64% were IgG and 9% were IgA aCL antibody positive, while 43% were IgG and 48% were IgA anti-$\beta_2$-GPI positive. Serum samples from 382 atherosclerosis patients were similarly tested for total (IgG/IgA/IgM) and specific IgG and IgA antibodies in both the aCL and anti-$\beta_2$-GPI assays. In sharp contrast to the APS samples, where IgG aCL and IgG anti-$\beta_2$-GPI

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Table 4
Competitive inhibition assays using 29 different serum samples, from patients with cardiovascular problems,[a] with indicated recombinant B2GP1 and deletion-mutants

| Ab# | 12345[b] | | 12— | | 123– | | 1234- | | —345 | | —45 | | ——5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Max[c] | 50%[d] | Max | 50% | Max | 50% | Max | 50% | Max | 50% | Max | 50% | Max | 50% |
| ACS-104 | 62 | 20 | 0 | >125 | 13 | >83 | 0 | >63 | 79 | 30 | 76 | 24 | 10 | >250 |
| ACS-136 | 74 | 12 | 3 | >125 | 0 | >83 | 0 | >63 | 72 | 6 | 76 | 26 | 3 | >250 |
| ACS-144 | 52 | 12 | 12 | >125 | 12 | >83 | 8 | >63 | 63 | 6 | 68 | 7 | 9 | >250 |
| ACS-52 | 70 | 17 | 0 | >125 | 14 | >83 | 30 | >63 | 86 | 6 | 87 | 2 | 21 | >250 |
| ACS-53 | 56 | 23 | 15 | >125 | 12 | >83 | 24 | >83 | 76 | 10 | 80 | 14 | 10 | >250 |
| ACS-54 | 71 | 5 | 11 | >125 | 0 | >83 | 0 | >63 | 67 | 3 | 85 | 2 | 3 | >250 |
| ACS-58 | 57 | 24 | 0 | >125 | 7 | >83 | 24 | >63 | 84 | 20 | 90 | 16 | 2 | >250 |
| ACS-65 | 82 | 12 | 0 | >125 | 18 | >83 | 88 | 5 | 92 | 4 | 94 | 8 | 0 | >250 |
| ACS-67 | 65 | 21 | 8 | >125 | 2 | >83 | 49 | >63 | 75 | 21 | 83 | 23 | 20 | >250 |
| ACS-71 | 72 | 12 | 18 | >125 | 7 | >83 | 13 | >63 | 90 | 4 | 93 | 4 | 49 | 227 |
| ACS-74 | 69 | 10 | 3 | >125 | 2 | >83 | 15 | >63 | 90 | 5 | 91 | 1 | 29 | >250 |
| CAS-13 | 54 | 34 | 7 | >125 | 0 | >83 | 10 | >63 | 74 | 17 | 72 | 33 | 15 | >250 |
| CAS-15 | 83 | 2 | 0 | >125 | 1 | >83 | 0 | >63 | 92 | 2 | 91 | 17 | 11 | >250 |
| CAS-18 | 72 | 24 | 0 | >125 | 13 | >83 | 0 | >63 | 85 | 31 | 82 | 46 | 0 | >250 |
| CAS-28 | 65 | 18 | 7 | >125 | 22 | >83 | 39 | >63 | 84 | 26 | 84 | 29 | 0 | >250 |
| CAS-29 | 63 | 26 | 8 | >125 | 17 | >83 | 33 | >63 | 76 | 36 | 75 | 28 | 0 | >250 |
| CAS-5 | 70 | 29 | 0 | >125 | 19 | >83 | 29 | >63 | 90 | 6 | 84 | 20 | 0 | >250 |
| CAS-6 | 68 | 24 | 14 | >125 | 14 | >83 | 27 | >63 | 78 | 6 | 77 | 17 | 15 | >250 |
| CAS-8 | 75 | 23 | 14 | >125 | 0 | >83 | 6 | >63 | 88 | 6 | 87 | 17 | 0 | >250 |
| MI-10 | 74 | 14 | 8 | >125 | 12 | >83 | 64 | 43 | 90 | 13 | 89 | 4 | 0 | >250 |
| MI-15 | 71 | 16 | 6 | >125 | 19 | >83 | 25 | >63 | 71 | 34 | 80 | 12 | 19 | >250 |
| MI-37 | 37 | 52 | 0 | >125 | 0 | >83 | 0 | >63 | 50 | 7 | 58 | 16 | 2 | >250 |
| MI-45 | 60 | 27 | 5 | >125 | 16 | >83 | 20 | >63 | 67 | 38 | 66 | 8 | 0 | >250 |
| MI-5 | 73 | 18 | 30 | >125 | 0 | >83 | 0 | >63 | 90 | 2 | 58 | 5 | 0 | >250 |
| MI-7 | 85 | 2 | 0 | >125 | 35 | >83 | 48 | 55 | 76 | 21 | 90 | 8 | 56 | 80 |
| PAD-101 | 27 | >50 | 3 | >125 | 0 | >83 | 14 | >63 | 67 | 51 | 72 | 55 | 6 | >250 |
| PAD-30 | 57 | 24 | 0 | >125 | 19 | >83 | 58 | 41 | 78 | 30 | 79 | 24 | 0 | >250 |
| PAD-39 | 59 | 19 | 14 | >125 | 10 | >83 | 54 | 46 | 75 | 30 | 77 | 24 | 0 | >250 |
| PAD-42 | 40 | >50 | 9 | >125 | 6 | >83 | 9 | >63 | 79 | 24 | 83 | 29 | 14 | >250 |

>, Greater than highest concentration tested.
[a] ACS: Acute Coronary Syndrome. MI: Myocardial Infarction. CAS: Carotid Artery Study. PAD: Peripheral Artery Disease.
[b] Domains included in construct.
[c] Maximum inhibition observed at concentrations tested.
[d] Concentration (μM) to give 50% inhibition.

antibodies were found in 64% and 43% of the specimens, respectively, we found that IgG aCL and IgG-$\beta_2$-GPI antibodies were present in only 1% of the samples from atherosclerosis patients. Even more striking was the observation that while the pattern of reactivity for IgA aCL and IgA-$\beta_2$-GPI was similar in the APS and atherosclerosis patients (both had low levels of IgA aCL and moderate levels of IgA anti-$\beta_2$-GPI), IgA anti-$\beta_2$-GPI was the only major antibody present in the atherosclerosis group. In contrast, the APS patients had moderate levels of antibodies to IgG aCL, IgG anti-$\beta_2$-GPI, and IgA anti-$\beta_2$-GPI (Table 1).

### *3.2. Epitope(s) of $\beta_2$-GPI recognized by both IgG and IgA anti-$\beta_2$-GPI from APS patients*

Recombinant $\beta_2$-GPI and two deletion mutants were used to determine the antigenic specificity of both the IgG and IgA autoantibodies from 10 different APS patients. Each recombinant form of $\beta_2$-GPI was tested, in a dose-dependent fashion, for its ability to inhibit these autoantibodies from binding to full-length $\beta_2$-GPI (Table 2, Fig. 1). Only those constructs that contained domain 1 inhibited both the IgG and IgA autoantibodies. As shown in Table 2, both the IgG and IgA anti-$\beta_2$-GPI binding antibodies from all 10 patients were inhibited by both constructs that contain domain 1. None of the samples were effectively inhibited, even at the highest concentration tested, by the construct that lacked domain 1. $ID_{50}$ values for mutants that contain domain 1 ranged from 1 to 50 μM for the IgG antibody and 13 to 100 μM for the IgA antibody. By contrast the mutant that did not contain domain 1 (D—45) did not effectively inhibit either the IgG or the IgA antibody.

### *3.3. Epitope(s) of $\beta_2$-GPI recognized by IgA anti-$\beta_2$-GPI from patients with acute atherosclerotic syndromes*

The differing $\beta_2$-GPI and aCL profiles of the APS and atherosclerosis sera (Table 1) suggested to us that the IgA anti-$\beta_2$-GPI antibodies in atherosclerosis patients may be distinct from those present in APS patients and might target a different domain on the $\beta_2$-GPI protein.

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We therefore selected 29 samples from the atherosclerosis patient cohort which were IgA anti-$\beta_2$-GPI antibody positive and aCL IgG, aCL IgM, and with the exception of one sera, aCL IgA negative. The detailed $\beta_2$-GPI and aCL profiles of these sera are shown in Table 3.

Seven different recombinant $\beta_2$-GPI mutant proteins were used to determine the antigenic specificity of the IgA $\beta_2$-GPI binding antibodies from 29 different samples from patients with various atherosclerosis conditions, including acute coronary syndrome (11), acute myocardial infarction (6), carotid artery disease (8), and peripheral artery disease (4). Each mutant recombinant $\beta_2$-GPI protein was tested, in a dose-dependent fashion, for its ability to inhibit the IgA antibody from binding to full-length $\beta_2$-GPI (Table 4). An example of the results is shown graphically in Fig. 2. With the exception of the full-length construct, only the D—345 and D—45 constructs inhibited these IgA antibodies. Four of the 29 samples were also inhibited, albeit to a much lesser extent, by the D1234- construct. Only one of the samples was also inhibited by the D——5 construct. $ID_{50}$ values for the D—345 and D—45 mutants ranged from 1 to 55 μM. By contrast, the D12— and D123—mutants did not effectively inhibit the binding of any of the 29 samples tested.

## 4. Discussion

It has been previously shown by many investigators that the antigenic specificity of the IgG autoantibodies found in APS patients recognize domain 1 of the $\beta_2$-GPI molecule [11,13]. The antigenic specificity of the IgA autoantibodies from APS patients, however, was never addressed and has remained unknown. The inhibition studies reported here (Fig. 1 and Table 2) clearly show that the antigenic specificity of the battery of 10 APS samples studied in this report are directed toward an epitope that is contained within domain 1 of the $\beta_2$-GPI molecule. Furthermore, while our study confirmed that APS patients produce antibodies of the IgG isotype directed to domain 1 $\beta_2$-GPI, as we and others have previously demonstrated, we now show for the first time, that APS patients also produce IgA autoantibodies which target domain 1 of the $\beta_2$-GPI. Thus, the antigenic specificity of both the IgG and IgA autoantibodies found in APS patients is domain 1.

In contrast, our study clearly shows (Fig. 2 and Table 3) that IgA $\beta_2$-GPI binding antibodies from patients with several types of atherosclerotic syndromes (acute myocardial infarction, acute coronary syndrome and peripheral artery disease) recognize an epitope on domain 4 of the $\beta_2$-GPI molecule. This should not be confused with earlier studies [12,14] that purported to show autoantibodies from APS patients recognize domain 4 of $\beta_2$-GPI. These studies were designed to study the antigenic specificity of IgG, not IgA, autoantibodies. In our study, the Dms that contained domain 4 inhibited in a similar, but not identical pattern, among the various samples tested. For example, only four were inhibited by the D1234- construct. This suggests that these antibodies recognize comparable, but distinguishable, epitopes present on domain 4. A recent molecular simulation derived from $\beta_2$-GPI crystal structure supports this possibility. This study suggested two discontinuous antigenic sequences in domain 4 $\beta_2$-GPI [15]. Domain 4 may have different conformational states when present in constructs containing different domains. For example, a few samples recognized domain 4 when domain 5 was absent, while the majority only recognized domain 4 when domain 5 was present. Thus, these antibodies may recognize an epitope on domain 4 that is affected by the presence of additional domains. This interpretation was also supported by the simulation experiments.

The precise antigenic epitope(s) for APS autoantibodies to $\beta_2$-GPI has been controversial since their discovery. We have previously reviewed the different technologies that have been used and offer our explanations on the different results [16].

Previously, it has been shown that the orientation of $\beta_2$-GPI on the ELISA plate is important for the binding of anti-$\beta_2$-GPI autoantibodies when measured by ELISA [11]. This could explain why these samples recognize $\beta_2$-GPI when adsorbed onto plastic plates, but do not bind $\beta_2$-GPI when adsorbed onto cardiolipin. The binding of $\beta_2$-GPI to cardiolipin via domain 5 may give a different orientation than when bound to plastic. Binding via domain 5 may alter either the availability of domain 4 or the conformation of domain 4, or both. We do not know the orientation of the $\beta_2$-GPI molecule when adsorbed onto plastic. It is conceivable that sufficient numbers of molecules adsorb to the plastic in such an orientation that domain 4 is neither hindered, that is available for antibody, nor has its

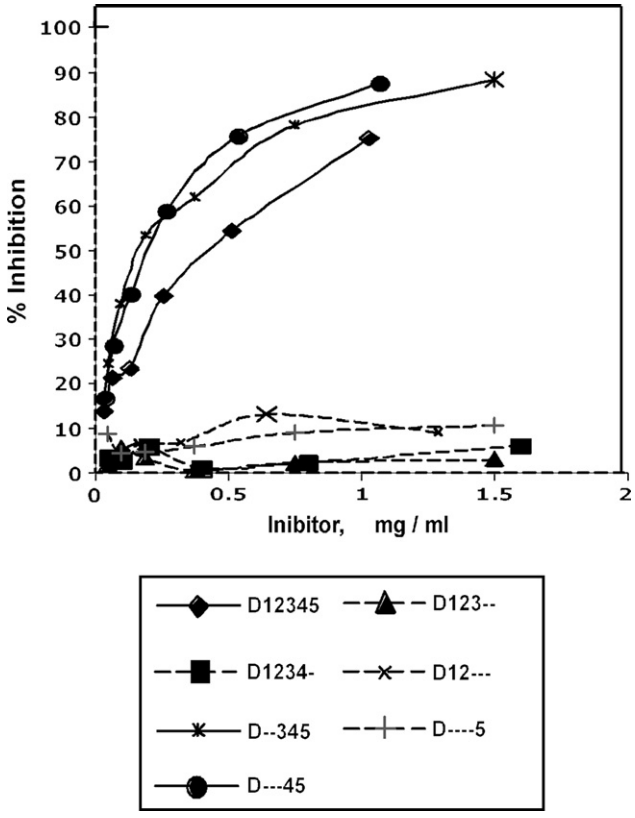


Fig. 2. Competitive inhibition of IgA anti-$\beta_2$-GPI of ACS-71 from binding to $\beta_2$-GPI by recombinant $\beta_2$-GPI and Dms. A constant amount of antibody was mixed with varying concentrations of inhibitors in wells coated with $\beta_2$-GPI. Recombinant $\beta_2$-GPI and Dms were used as inhibitors.

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conformation altered enough to negate the binding of these antibodies.

A pathogenic role for anti-$\beta_2$-GPI antibodies in atherosclerosis has been suspected, but their role remains to be proved. We have recently reviewed possible mechanisms that may be involved in the specific relationship of IgA antibodies to $\beta_2$-glycoprotein of atherosclerosis [17]. Further studies are needed to evaluate the merit of each of these potential mechanisms and to integrate the conclusions of the present study that patients with atherosclerotic conditions preferentially make IgA antibodies that target B2-gp1 domain 4. Understanding of these observations may guide the development of future diagnostic tests and therapeutic interventions.

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