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Patient-derived anti- β 2GP1 antibodies recognize a peptide motif pattern and not a specific sequence of residues

Philippe de Moerloose,¹ Céline Fickentscher,¹ Françoise Boehlen,¹ Jean-Marie Tiercy,² Egbert K.O. Kruithof¹ and Karim J. Brandt^{1*}

¹Division of Angiology and Hemostasis and ²National Reference Laboratory for Histocompatibility, Transplantation Immunology Unit, Department of Genetic and Laboratory Medicine, University Hospital of Geneva and Faculty of Medicine, Switzerland

*K.J.B is currently affiliated with Division of Cardiology, Department of Internal Medicine, Faculty of Medicine, University of Geneva, Switzerland

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ABSTRACT

Antiphospholipid antibody syndrome is an autoimmune disease characterized by the presence of so-called antiphospholipid antibodies and clinical manifestations such as recurrent thromboembolic or pregnancy complications. Although the main antigenic determinant for antiphospholipid antibodies has been identified as the β -2-glycoprotein 1 (β 2GP1), the precise epitope recognized by antiphospholipid antibodies still remains largely unknown. In the study herein, we wanted to identify a sequence in domain I of β 2GP1 able to induce the proliferation of CD4⁺ T cells isolated from antiphospholipid antibody syndrome patients, but not from healthy donors, and to interact with antiphospholipid antibodies. We have characterized a sequence in domain I of β 2GP1 that triggers CD4⁺ T-cell proliferation. A comparison of this sequence with the previously reported binding of antiphospholipid antibodies to discontinuous epitope R39-R43 reveals the presence of an indeterminate motif in β 2GP1, in which the polarity determines the characteristics and specificity of antiphospholipid antibodies-interacting motifs. Using point mutations, we characterized the main antiphospholipid antibodies-interacting motif as $\phi\phi\phi\zeta\zeta\text{FxC}$, but also established $\phi\phi\phi\zeta\zeta\text{F}\phi$ -related motifs as potential antiphospholipid antibodies epitopes, in which ϕ represents nonpolar residues and ζ polar residues, with charges of the residues not being involved. Of specific importance, these different motifs are present at least once in all antiphospholipid antibodies-related receptors described so far. We have further demonstrated, *in vitro*, that peptides and domains of β 2GP1 containing these motifs were able to interact with antiphospholipid antibodies and inhibit their monocyte activating activity. These results established that the antiphospholipid antibodies-interacting motifs are determined by the polarity, but not by the sequence or charge, of amino acids. These data could also contribute to the future development of more sensitive and specific diagnostic tools for antiphospholipid antibody syndrome determination and potential peptide- or β 2GP1 domain-based clinical therapies.

Correspondence:

karim.brandt@hcuge.ch

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Introduction

Antiphospholipid syndrome (APS) is described as a common risk factor for recurrent thromboembolic events and/or pregnancy complications resulting from circulating antiphospholipid antibodies (aPLA).¹ It is now widely accepted that the plasma phospholipid binding protein β -2-Glycoprotein 1 (β 2GP1) is the main antigenic target for aPLA.² β 2GP1 is a protein of 43 kDa composed of 5 short consensus repeat domains called “sushi” domains. Two different conformations exist for β 2GP1: a circular plasma conformation and a fishhook conformation.³ Epitopes within domains I and V are involved in maintaining a circular conformation,

Table 1. Clinical and laboratory profiles of the 9 patients providing the aPLA.

	LA	aCL IgG	a β 2GP1 IgG	Clinical manifestations
aPLA 1	+	> 100	POSITIVE	Arterial thrombosis
aPLA 2	+	> 60	POSITIVE	Fetal loss
aPLA 3	ND	> 100	POSITIVE	Venous thrombosis
aPLA 4	+	30.2	POSITIVE	Recurrent fetal loss
aPLA 5	ND	> 60	POSITIVE	Arterial thrombosis
aPLA 6	+	46	POSITIVE	Arterial thrombosis
aPLA 7	+	> 60	POSITIVE	Fetal loss
aPLA 8	+	> 60	POSITIVE	Fetal loss
aPLA 9	+	> 57	POSITIVE	Thromboembolism

LA: lupus anticoagulant; aCL IgG: anticardiolipin IgG (normal value < 5GPL); a β 2GP1 IgG: anti- β -2-glycoprotein 1 IgG; ND: not done (patient under vitamin K treatment); aPLA: antiphospholipid antibodies; Recurrent fetal loss: >2 fetal losses.

whereas binding of domain V to anionic surfaces induces a fishhook conformation and exposure of a cryptic epitope in domain I.^{3,4} This cryptic epitope is described as being located around residues 39 and 43; however, Iverson *et al.* have identified additional residues involved in the recognition of residues by pathogenic anti- β 2GP1 antibodies in domain I.^{5,6} Ioannou *et al.* have also studied mutations including residues R39 to R43 describing complex, and probably discontinuous, epitopes.⁷ Their data suggest that the epitope(s) are not “classical” and that several epitopes are present in domain I and could potentially be present elsewhere in β 2GP1.

Humoral immunophysiology studies of APS and the treatment of APS patients with an anti-CD20 monoclonal antibody (rituximab) have aroused interest in B cells as therapeutic targets. Anti-CD20-treated APS patients have a normal distribution of anti- β 2GP1, anti-cardiolipin (aCL) and Lupus anticoagulant (LAC) antibody titers and improved clinical manifestations.⁸ The isotype of anti- β 2GP1 antibody is mainly immunoglobulin G (IgG), suggesting that the production of these antibodies requires antigen-specific CD4⁺ T helper cells.⁹ Hattori *et al.* have shown that β 2GP1 induce an *in vitro* proliferative response of T cells from APS patients. These β 2GP1-specific CD4⁺ T cells are able to induce the production of anti- β 2GP1 antibodies by autologous peripheral blood B cells through human leukocyte antigen-D related (HLA-DR) interactions.^{10,11} The identity of the principal T-cell epitopes on β 2GP1 has not been established as of yet. It seems that all 5 β 2GP1 domains are able to induce a T-cell proliferative response depending on the APS patient.¹¹ Moreover, analysis of T-cell responses to a β 2GP1-derived peptide library have shown that CD4⁺ T cells are reactive to different peptides independently of HLA.¹² In the study herein, we have investigated and identified an immunodominant β 2GP1-specific CD4⁺ T-cell epitope using a peptide-associated major histocompatibility complex (pMHC) II tetramer-based assay. We have shown that the immunodominant β 2GP1-specific CD4⁺ T-cell epitope shares a common peptide motif, which is present in the β 2GP1 peptide sequence R39-R43. We have further determined that the characteristic $\phi\phi\phi\zeta\zeta\text{F}\phi\phi$ motif, in which ϕ represents nonpolar residues (AVILMFWCPCG) and ζ polar residues (YTSHKREDQN), as well as motifs closely related to $\phi\phi\phi\zeta\zeta\text{F}\phi\phi$ are not only present several times in β 2GP1 but also in every receptor described for aPLA.¹³

Methods

Ethics statement

Buffy coats of blood from healthy donors were provided by the Geneva Hospital Blood Transfusion Center. In accordance with the ethical committee of the Geneva Hospital and with the Declaration of Helsinki, the blood bank obtained informed consent from the donors, who were informed that part of their blood would be used for research purposes.

Patient characteristics

All patients had an APS, as defined by the revised Sapporo criteria.² Control antibodies and peripheral blood mononuclear cells (PBMC) were isolated from the blood plasma of healthy volunteers. The characteristics of the patients used in this study are listed in Table 1.

Recombinant β 2GP1 fusion proteins and peptide libraries

Recombinant fusion proteins, corresponding to sushi domains I and II, and domains III, IV and V, respectively, of β 2GP1 were generated. Briefly, a series of β 2GP1 complementary DNA (cDNA) constructs, encoding these domains, were inserted into the vector pcDNA3.1_mycHis_A_A130 between cloning sites BamHI / XhoI. The plasmids were prepared by Life Technology (Carlsbad, CA, USA) and transfected into HEK293 cells. The fusion proteins were purified using nickel resin affinity chromatography (GE Healthcare) and dialysed with Amicon® Ultra (Millipore, Billerica, MA, USA). The concentration was then adjusted to 5mg/ml in peripheral blood smear (PBS). The peptides libraries were generated by Mimotopes (Clayton, VIC, Australia). Lyophilized non water-soluble peptides were reconstituted in 50% dimethyl sulfoxide (DMSO) and 7.5% acetic acid before dilution in PBS. All peptides had 95% purity as assessed by analytical reverse phase high performance liquid chromatography (RP-HPLC). Native β 2GP1 was purified from human plasma with 96% purity (Prospecbio, NJ, USA).

T-cell proliferation assays

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Lymphoprep™ (Axis-Shield PoC) according to the manufacturer's instructions. T-cell proliferation was evaluated by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assays (eBioscience). PBMC were stained with 0.1 μ M CFSE (eBioscience), according to the manufacturer's instructions. Cells were cultured in the presence of anti-

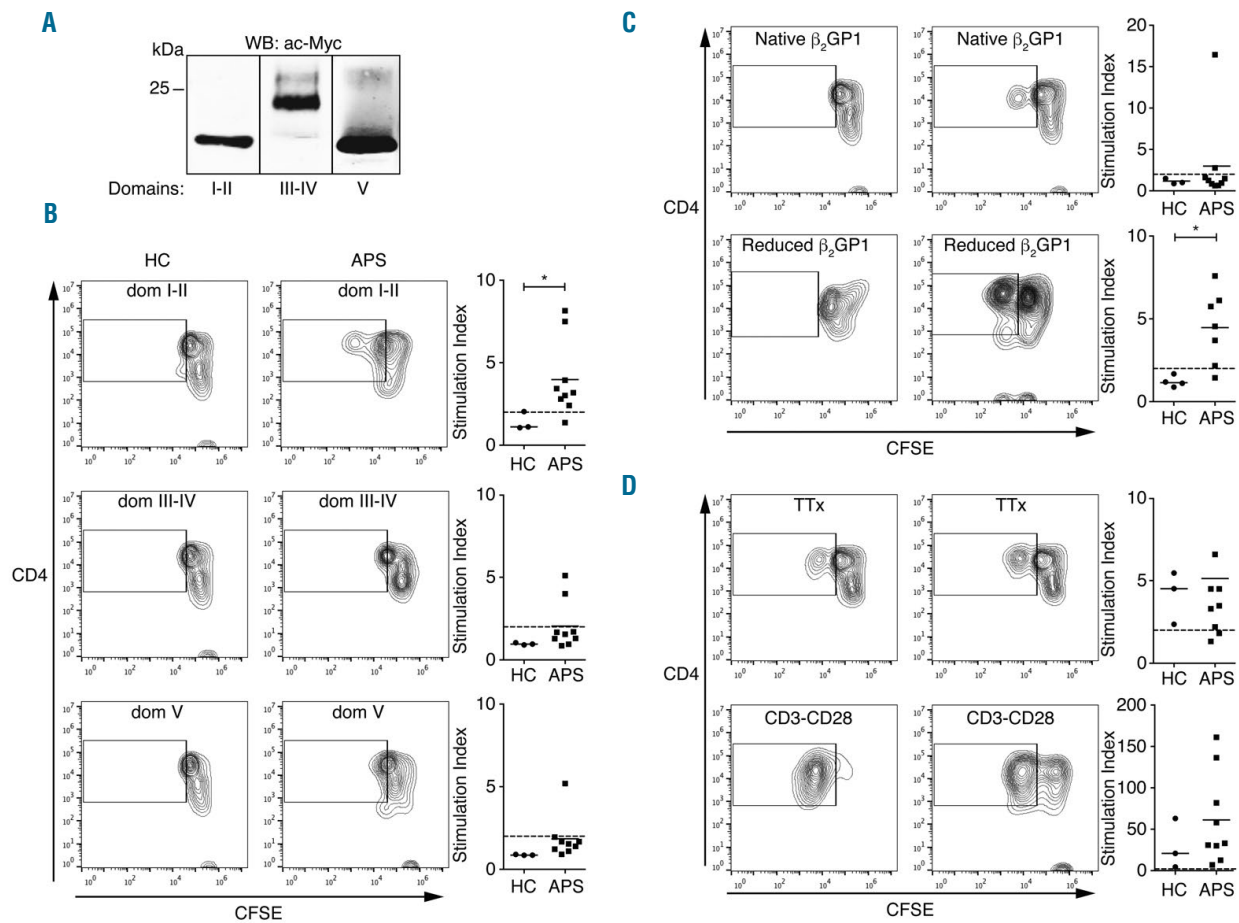


Figure 1. Domain I-II and reduced β 2GP1 induce proliferation of T cells from APS patients. (A) β 2GP1 recombinant domains are purified and analyzed by Western blot. Data are representative of 3 independent experiments. (B-D) Flow cytometry analysis of the proliferation assay performed on PBMC of APS patients in the presence of domains I-II, II-IV, V β 2GP1, reduced β 2GP1 and tetanus toxin (TTx). CD3-CD28 is positive proliferation control. CD4⁺CFSE⁺ population is gated on CD3⁺ cells. Data are mean \pm SEM of 9 different donors. Statistical significance was determined by Mann-Whitney U analysis. HC: healthy controls; APS: Antiphospholipid syndrome; WB: western blot; ac-MYC: anti-c-Myc; CFSE: carboxyfluorescein diacetate succinimidyl ester.

gens (10 μ g/ml) for 10 days. T-cell proliferation was assessed by flow cytometry evaluation of CFSE dilution. Proliferation was expressed as the cell division index (defined as the number of CFSE^{low} T cells cultured with antigen/number of CFSE^{low} T cells without antigen). In all cases, the culture medium consisted of X-VIVO 15 (Lonza, Walkersville, MD, USA).

Flow cytometry

Single-cell suspensions stained with CFSE (eBioscience) were incubated with anti-CD32 (Biolegend) to prevent nonspecific antibody binding, then stained with antibodies against AF488-CD3, AF647-CD4, (BD Biosciences) and major histocompatibility complex (MHC) class II tetramer (Benaroya Research Institute). Staining was assessed with ACCURI C6 flow cytometer (BD Biosciences).

ELISA epitope mapping assay

MaxiSorp™ 96 well plates (Nunc) were coated with 10 μ g/ml recombinant domains or peptides of β 2GP1 prior to incubation with aPLA or control IgG. Secondary anti-human antibodies conjugated to IR800CW (Rockland) were used. Protein- or peptide-bound antibodies were detected and quantified by the Odyssey system (Li-Cor Biosciences).

Statistical analysis

When required, the significance of differences between groups was assessed using the nonparametric Mann-Whitney U test. * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$. All data are represented as mean \pm SEM of at least 3 independent experiments.

Results

Identification of β 2GP1 domains recognized by T cells

It was previously demonstrated that β 2GP1-specific T cells are present in APS patients, although the specific domain(s) and peptide(s) inducing T-cell proliferation were not well defined.^{10,12} To identify β 2GP1 domain-specific T cells in APS patients, PBMCs were isolated from 9 APS patients (Table 1), and 3 healthy controls (HC) were stimulated with native β 2GP1, reduced β 2GP1 and recombinant β 2GP1 domain I-II, domain III-IV, and domain V (Figure 1A). We tested the proliferation of PBMC by the CFSE dilution assay. In contrast with HC, we detected CD4⁺ T-cell proliferative responses in primary culture to β 2GP1 domain I-II and reduced β 2GP1 from APS patients (Figure 1B,C). While the cryptic epitope exposed by reduced β 2GP1 was recognized by β 2GP1 reactive T cells

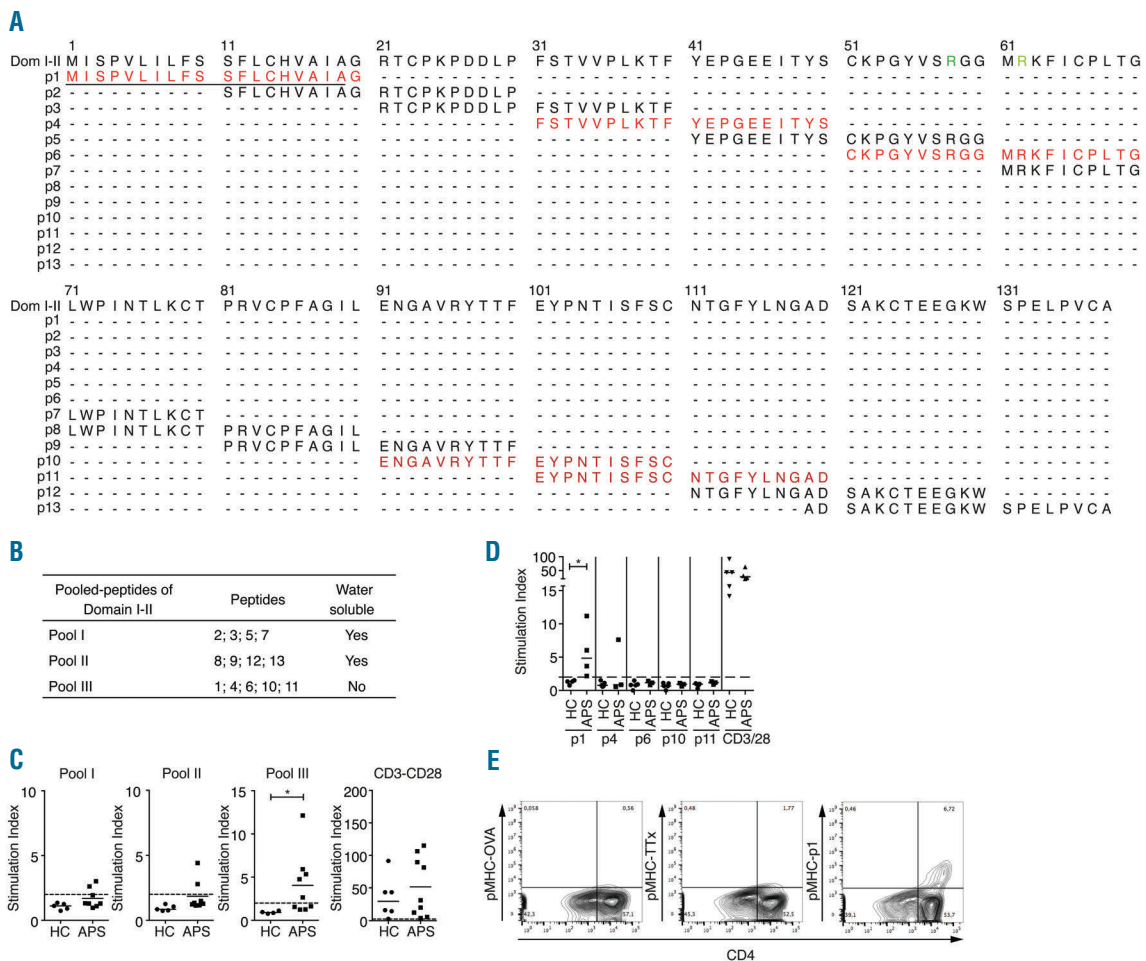


Figure 2. Peptide 1 of β 2GP1 carries antigenic determinant for CD4⁺ T-cell proliferation. (A) Representation of the peptide library of domain I-II. Peptides in red are peptides contained in Pool III. Residues in green represent R39-R43 epitopes. Underlining represents a signal peptide. (B) Table of peptide distribution for proliferation assay. (C) Flow cytometry analysis of proliferation assay performed on PBMC of APS patients in the presence of pool I, II or III. CD3-CD28 shows positive proliferation control. CD4⁺CFSE^{low} population is gated on CD3⁺ cells. Data are mean ± SEM of 9 different donors. Statistical significance was determined by Mann-Whitney U analysis. (D) Flow cytometry analysis of proliferation assay performed on the PBMC of APS patients in the presence of p1, p4, p6, p10 and p11. CD3-CD28 is positive proliferation control. CD4⁺CFSE^{low} population is gated on CD3⁺ cells. Data are mean ± SEM of 9 different donors. Statistical significance was determined by Mann-Whitney U analysis. (E) Flow cytometry analysis of pMHC class II tetramer staining loaded with p1 on PBMCs of APS patients pulsed with recombinant domain I-II. pMHC-OVA (ovalbumin) and pMHC-TTx (Tetanus toxin) are negative controls. Populations are gated on CD3⁺ cells. HC: healthy controls; APS: antiphospholipid syndrome.

in the normal T-cell repertoire of APS patients, native β 2GP1 was unable to significantly stimulate T-cell proliferation (Figure 1C). The proliferation controls performed with tetanus toxoid (TTx) and CD3-CD28-beads, i.e., specific and unspecific T-cell proliferation stimuli, showed that the proliferative ability of T cells from HC and APS were unaltered (Figure 1D). These results suggest that the core sequence(s) of the β 2GP1 peptide(s) inducing T-cell proliferation were present in β 2GP1 domain I-II.

Identification of a core peptide fragment recognized by T cells

Having identified candidate regions of β 2GP1 containing T-cell determinants, we then tested proliferative responses of APS patients against a pool of β 2GP1 peptides. For this purpose, we tested the proliferation of PBMC from APS patients and HC to the pools of peptides containing 4 to 5 β 2GP1 domain I-II peptides from a library of 13 synthetic overlapping 20-mer peptides pres-

ent in the 138 amino-acid sequence of β 2GP1 domain I-II (Figure 2A,B). By CFSE dilution assay, we identified proliferative responses in primary cultures to β 2GP1 domain I-II peptide pool III (Figure 2C). T-cell proliferations of HC and APC triggered by CD3-CD28 have shown similar responses. β 2GP1 domain I-II peptide pool III was composed of 5 peptides consistently insoluble in water with the cryptic character of epitope recognized by β 2GP1 reactive T cells. Interestingly, this pool of peptides contained the region targeted by aPLA, i.e., peptide n°6 (p6).¹⁴ We then examined the responses to individual β 2GP1 peptides contained in pool III. T-cell proliferation response against leader peptide n°1 (p1) was significantly increased (but not against peptides n°4, 6, 10 or 11), while T-cell proliferations of HC showed a similar response to CD3-CD28 (Figure 2D). To confirm the presence of circulating T cells specific for p1, we defined the HLA class II haplotype profiles of APS patients used previously (Table 2). We then used pMHCII-p1 tetramer DRB3* 02:02 staining to detect

Table 2. Haplotype profiles of 6 patients from Table 1.

	DRB1*	DRB3*	Haplotype			Clinical manifestations	
			DRB4*	DQB1*	DPB1*	Pregnancy loss	Thrombosis
Patient 1	04:03/-			03:02/03:05	04:01/-	+	-
Patient 2	09:01/13:01	02:02	01:03	03:02/06:03	05:01/14:01	+	-
Patient 3	03:01/11:04	02:02		02:01/03:01	03:01/04:02	+	+
Patient 4	04:03/-			03:02/03:05	04:01/09:01	-	+
Patient 5	01:01/13:01	01:01		05:01/06:03	NT	+	+
Patient 6	04:01/13:01	02:02	01:03	03:02/06:03	04:01/04:02	-	+

Table 3. Different motifs present in antiphospholipid antibodies (aPLA)-related receptors and proteins.

Motif		$\phi\phi\phi\zeta\zeta\text{FxC}$		
aPLA-related receptors	Names and References	Localization	Number	Orientation
	β 2GPI ²⁷	Domain I	2	
	TLR ²⁸	Extracellular	1	NH ₂ => COOH
	ApoER ²⁹	Extracellular	2	
Motif		$\phi\phi\phi\zeta\zeta\text{Fx}\phi$		
aPLA-related receptors	Names and References	Localization	Number	Orientation
	β 2GPI ²⁷	Domain I	2	
	TLR ³⁰	Extracellular	1	COOH => NH ₂
	ANXA ²¹	Annexin repeats	1	
Motif		$\phi\phi\phi\zeta\text{Fx}\phi$		
aPLA-related receptors	Names and References	Localization	Number	Orientation
	β 2GPI ²⁷	Domain III	1	
	TLR ¹³²	Extracellular	1	
	TLR ⁴³³	Extracellular	3	COOH => NH ₂
	TLR ⁶³²	Extracellular	1	
	TLR ⁷³⁴	Extracellular	2	
	TLR ⁹³⁵	Extracellular	2	
Motif		$\phi\phi\phi\zeta\zeta\phi\text{Fx}\phi$		
aPLA-related receptors	Names and References	Localization	Number	Orientation
	β GPIb α ³⁶	Extracellular	1	COOH => NH ₂

specific circulating T cells in β 2GPI domain I-II pulsed T-cell population from APS patients. Although, as expected, no population was observed with negative control pMHC-OVA (tetramer with a peptide of ovalbumin), a pMHC-p1 positive population was strongly present in β 2GPI domain I-II specific pulsed T-cell population (Figure 2E). pMHC-TTx staining was used as a positive control (Figure 2E). These results demonstrated that the immunodominant β 2GPI-specific CD4⁺ T-cells' epitope in patients with APS is present in a sequence of amino acids corresponding to MISPVLLIFSFLCHVAIAG, which correlates with the signal peptide of β 2GPI.

Characterization of common motif shared by p1 and p6 of domain I-II

To identify a potential consensus motif between p1 and p6, which was previously reported as containing the aPLA binding discontinuous epitope R39-R43, we aligned their sequences and observed a common motif present around a phenylalanine (Figure 3A). This motif seemed to be determined by intrinsic physical properties (polar and

nonpolar) and not by amino acid sequences. To investigate the ability of p1 to interact with patient-derived anti- β 2GPI antibodies and to identify critical residues in the motif, we generated an alanine scanning library of p1 and p6 including the common motif (Figure 3B) to perform an ELISA epitope mapping assay. As shown in Figure 3B, aPLA are able to bind p1 as potently as p6. The mutations I7A, L8A F9A, S11A, F12A and C14A of p1 significantly decreased the binding of aPLA. Similarly, mutations R58A (R39), G59A (G40), G60A (G41), M61A (M42), R62A (R43), F64A and C66A were involved in the binding of aPLA to p6 (Figure 3B) (numbering corresponds to the numbering in the mature β 2GPI protein, i.e., without the signal peptide). aPLA interacted similarly with both peptides, although the sequences of amino acids present in the motif were not the same. It seems that the aPLA interacting motif, $\phi\phi\phi\zeta\zeta\text{FxC}$, is dependent on polar or nonpolar properties more than hydrophathy classes or charge of residues. Alanine is nonpolar, but it decreased the ability of aPLA to bind I7A, L8A, F9A, and C14A p1-mutated peptides as well as R58A, G59A, G60A, M61A, and C66A

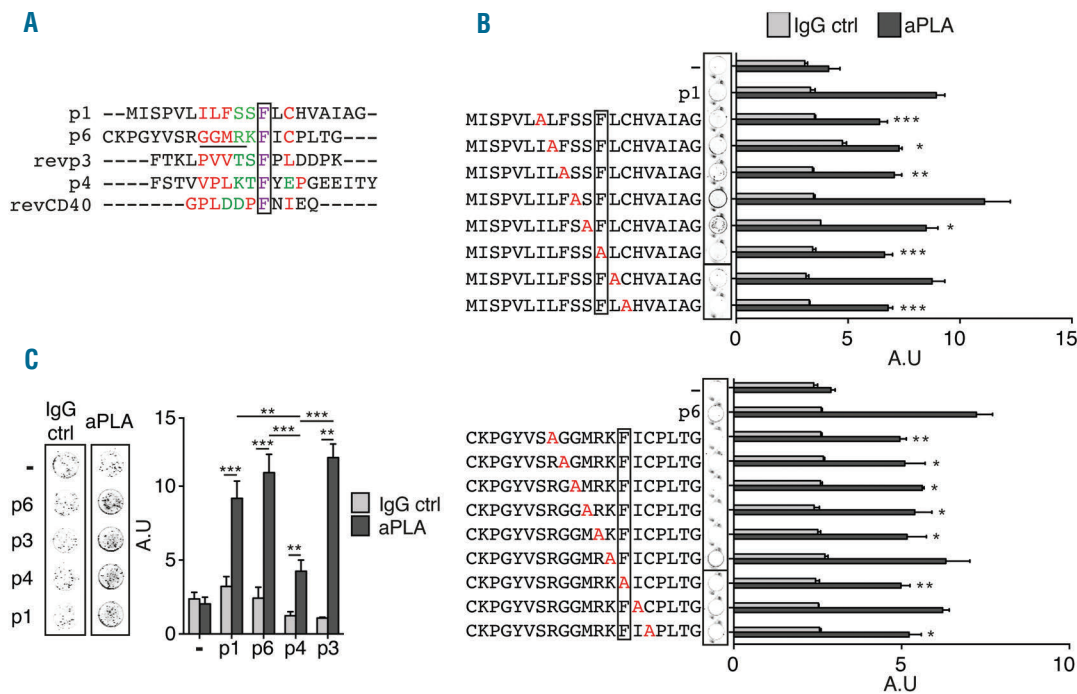


Figure 3. aPLA epitope contains $\phi\phi\phi\zeta\zeta Fx\phi$ motifs. (A) Alignment of p1, p6, p4, anti-sense p3 and CD40 consensus sequence. Red color shows nonpolar residues while green color shows polar residues in the motif. Underlining represents R39-43 epitopes and a frame represents conserved residue. (B) Binding of aPLA to microplate wells coated with p1 and p6 with consecutive point mutations. Substituted residues are in red. Frame represents conserved residue. Data are mean \pm SEM of 9 different donors. (C) Representative picture of IgG ctrl (control) or aPLA binding to peptide-coated well. Quantification of aPLA binding to p1-, p6-, p4- and p3-coated wells. Data are mean \pm SEM of 9 different donors. Statistical significance was determined by Mann-Whitney U analysis. aPLA: antiphospholipid antibodies; IgG: immunoglobulin G; A.U.: arbitrary unit.

p6-mutated peptides. These data suggest that the volume of amino acids and the ability to form electrostatic or hydrogen bonds may also contribute to interactions between aPLA and the motif.

We then performed bioinformatical analysis using Prosite resource (SIB Swiss Institute of Bioinformatics) to find not only the $\phi\phi\phi\zeta\zeta Fx\phi$ motif (N- to C-term) but also the $\phi x F\zeta\zeta\phi\phi\phi$ motif which is the reverse motif (C- to N-term) in the human proteome. Four sequences were present in domain I of $\beta 2GP1$; 2 $\phi\phi\phi\zeta\zeta Fx\phi$ motifs and 2 $\phi x F\zeta\zeta\phi\phi\phi$ motifs (Table 3). $\phi x F\zeta\zeta\phi\phi\phi$ motifs were present in p1 and p3 (Figure 3A). We thus assessed the ability of aPLA to interact with p3. We also included a slightly different motif that was present in p4 to investigate whether a degenerated motif was able to interact with aPLA. As expected, aPLA bound to p3 with a similar efficiency compared to p1 and p6 (Figure 3C). We further observed that p4, although less potent in interactions with aPLA, bound significantly to the aPLA (Figure 3C). Altogether, these data demonstrated that aPLA interact with a $\phi\phi\phi\zeta\zeta Fx\phi$ motif, and more generally with a $\phi\phi\phi\zeta\zeta Fx\phi$ motif, in which the polarity of the residues was more important than the hydrophobicity classes. This motif could also interact with aPLA even if the motif was slightly modified by insertion or deletion of an amino acid before or after the key phenylalanine residue. Finally, aPLA were able to bind motifs in sense or anti-senses.

aPLA-related receptors described possess closely related motifs

Vlachoyiannopoulos *et al.*¹⁵ identified a consensus motif between CD40 and p3 capable of interacting with aPLA. Although this sequence had one additional nonpolar amino acid between the polar and phenylalanine ($\phi x F\phi\zeta\zeta\phi\phi\phi$) amino acids, it was strongly related to the initial motif (Figure 3A). In addition, it seemed that both polar amino acids present in the $\phi\phi\phi\zeta\zeta Fx\phi$ motif were not fully required (Figure 3B). Thus we performed additional bioinformatical analysis relative to $\phi x F\zeta\zeta\phi\phi\phi$ and $\phi x F\phi\zeta\zeta\phi\phi\phi$ motifs. Among the proteins containing these motifs, we identified $\beta 2GP1$ domain I, toll-like receptor (TLR)1, TLR4, TLR6, TLR7, TLR8, TLR9 and GPIIb α (Table 3). In addition to identified proteins containing the motifs $\phi\phi\phi\zeta\zeta Fx\phi$ and $\phi x F\zeta\zeta\phi\phi\phi$, all potential aPLA receptors described in the literature possess at least 1 of these 2 motifs. These results suggest that $\phi\phi\phi\zeta\zeta Fx\phi$ and other closely related motifs constitute the potential links between controversial data obtained by different research groups working on aPLA.

Functional characterization of recombinant domains of $\beta 2GP1$, peptides 1 and 6

To examine the functional ability of recombinant domains of $\beta 2GP1$, p1, p4 and p6 to inhibit the activity of aPLA isolated from APS patients studied in our previous

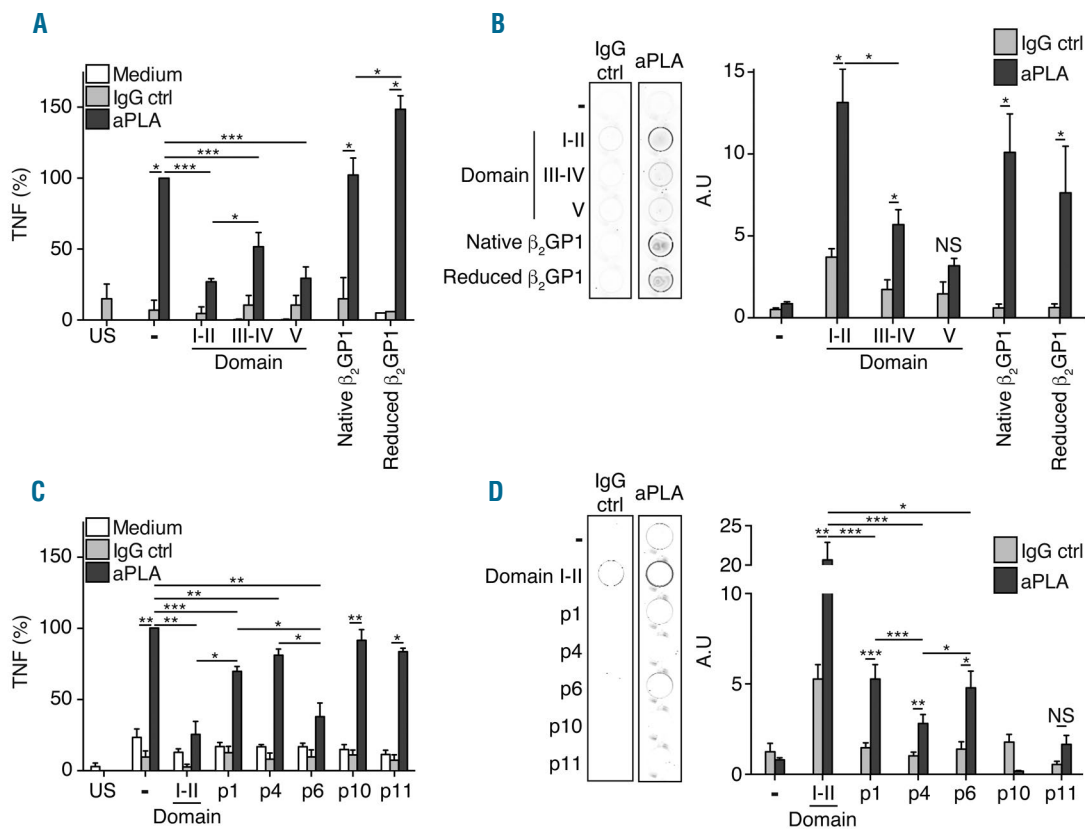


Figure 4. Motif containing domains and peptides inhibits aPLA activity. (A) Inhibition of tumor necrosis factor (TNF) production induced by aPLA by domains I-II, III-IV, V, β_2 GP1 of reduced β_2 GP1. (B) Representative picture of IgG ctrl (control) or aPLA binding to domains I-II, III-IV, V and β_2 GP1-coated well. Quantification of aPLA binding to domains I-II, III-IV, V or β_2 GP1-coated well. aPLA to well coated with domains I-II, III-IV, V, β_2 GP1 of reduced β_2 GP1. (C) Inhibition by domain I-II, p1, p4, p6, p10 and p11 of TNF production induced by aPLA. (D) Representative picture of IgG ctrl or aPLA binding to domains I-II, p1, p4, p6, p10- and p11-coated well. Quantification of aPLA binding to domains I-II, p1, p4, p6, p10 and p11-coated well. aPLA to well coated with domains I-II, III-IV, V, β_2 GP1 of reduced β_2 GP1. Data are mean \pm SEM of 9 different donors. Statistical significance was determined by Mann-Whitney U analysis. aPLA: antiphospholipid antibodies; IgG: immunoglobulin G; US: unstimulated; NS: non significant.

experiments (Table 1), we evaluated tumor necrosis factor (TNF) production in human monocytes activated with aPLA which had previously been treated with peptides or recombinant domains. While incubation of human monocytes with aPLA pretreated with β_2 GP1 and reduced β_2 GP1 have no effect on aPLA activity, incubation of the aPLA with domain I-II, domain III-IV and domain V significantly decreased the production of TNF induced by aPLA (Figure 4A). This inhibition of TNF production involved the binding of aPLA to β_2 GP1, and reduced β_2 GP1, domain I-II and domain III, but not domain V (Figure 4B). Similarly, pre-incubation with peptides n^o1, 4 and 6 (p1, p4 and p6) diminished the production of TNF induced by aPLA, whereas p10 and p11 had no effect (Figure 4C). This inhibition showed that p1, p4 and p6, but not p10 or p11, interact with aPLA (Figure 4D). We observed that p1 and p6 had similar aPLA binding activity, while p4 was significantly less potent in its interaction with aPLA (Figure 4D). These results were consistent with the presence of 4 aPLA interaction motifs in domain I-II, while p1 and p6 had only 1 motif (considering that p1 and revp1 were not simultaneously available) (Figure 5). As shown in Figure 3C, the interaction of p4 with aPLA was less effective due to a slightly degenerated motif (Figure 4D and 3A). Taken together, our results clearly revealed that the motifs recog-

nized by aPLA, which were present in p1, p4 and p6 (Figure 3A), but not in p10 or p11 (Figure 2A), were able to functionally decrease the activity of aPLA. They further demonstrated that the aPLA interacting motif (ϕ xF ζ ϕ ϕ) present in domain III-IV (Table 3) efficiently interacted with aPLA and inhibited its activity, although to a lesser extent than domain I-II (Figure 4B), confirming the potential targets exposed in Table 3.

Discussion

The present study was undertaken to investigate the T-cell response associated with the production of autoantibodies against β_2 GP1 and their related epitopes in APS patients. We established that the β_2 GP1 signal peptide (p1) induced the proliferation of CD4⁺ T cells from APS patients but not from healthy donors. Furthermore, autoreactive CD4⁺ T cells were detected in APS patient blood samples and bound HLA-DRB3* 02:02 tetramer loaded with p1, thus endorsing the fact that specific HLA-DR alleles are associated with susceptibility to APS.^{10,16,17} We further observed that the signal peptide contained a motif that was similar to that of the R39-R43 epitope. We thus identified the aPLA interacting motif (ϕ ϕ ζ ζ F ϕ) to be

such as proteins involved in coagulation processes. Thus, thrombin, proteinase-activated receptor 2 (PAR-2 and 3) and the complements C5, C4a and C4b are some of the aPLA-targeted proteins carrying $\phi\phi\phi\zeta\zeta\text{F}\phi$ motifs (*data not shown*).

Although additional developments are necessary in order to find the exact association of residues needed to obtain the highest aPLA affinity, the study herein offers the opportunity to provide an accurate tool for the detection of β 2GP1 antibodies for diagnostic purposes. Furthermore, aPLA interacting motifs present in peptides

have the ability to inhibit aPLA activity and represent a prevention strategy for APS as an alternative to the use of anticoagulants. Finally, p1-tetramers associated with inducers of cell death could be used to specifically disrupt autoreactive T cells in APS patients, thus representing a potential therapeutic approach.

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