



SIGNIFICANCE OF K(L/V)WX(I/L/V)P EPITOPE OF THE β 2GPI IN ITS (PATHO)PHYSIOLOGIC FUNCTION

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ABSTRACT

β 2-glycoprotein I (β 2GPI) is a major autoantigen of autoimmune thrombophilia, known as the antiphospholipid syndrome. The exact mechanism underlying the β 2GPI's involvement in the disease is not fully elucidated, as it is not its physiological role. We used random phage peptide library to identify sequences binding to β 2GPI. Obtained K(L/V)WX(I/L/V)P motif, primarily designated as target unrelated, was confirmed as the selective binder of β 2GPI. Based on this motif we confirmed the previously suggested role of polar residues in β 2GPI interactions, and identified some already known and some new putative β 2GPI binding proteins. The latter can help to further elucidate β 2GPI's (patho)physiological role.

INTRODUCTION

β 2GPI is the most common and best-characterized antigenic target for antiphospholipid autoantibodies (aPL). aPL are associated with clinical features of different autoimmune disorders especially the antiphospholipid syndrome, which is characterized by vascular thrombosis and pregnancy morbidity, and also systemic lupus erythematosus (SLE) (1).

β 2GPI is a 45kDa plasma glycoprotein with plasma concentration of 50–300 mg/L (2). This 326 amino acids long polypeptide is comprised of five domains existing in at least two different conformations: a circulating plasma conformation and a “fish-hook” like open conformation. The positively charged patch (C281KNKEKCC288) located on the β 2GPI's fifth domain enables its binding to negatively charged surfaces such as anionic phospholipids. Upon binding β 2GPI opens up and thus converts from circular to “fish-hook” like conformation (2, 3).

The physiological role of β 2GPI is not entirely elucidated; in vitro studies have shown its involvement in the coagulation cascade, the removal of apoptotic bodies, triglyceride metabolism and depletion of free radicals (4-7). Recently, β 2GPI was suggested to be involved in the clearance of lipopolysaccharide (LPS) and it was identified as a component of innate immunity (8, 9). Numerous putative functions imply that β 2GPI interacts with many different proteins (macromolecules). Determination of amino acid sequences recognized by β 2GPI, using phage display library, resulted in the selection of a common motif that has been determined also by several other groups studying different targets. We suggested Lipid A of LPS as a possible selection decoy and thus the true binding partner of the selected motif (10). In the present study we tested and confirmed the discriminating and moderate affinity of selected peptides to β 2GPI by ELISA and surface plasmon resonance. Based on the selected motif we present putative binding partners of β 2GPI.

METHODS

BIOPANNING PROCEDURE

To select β 2GPI binding phage peptides a random linear heptamer peptide library (Ph.D.-7, New England Biolabs, Beverly, MA, USA) was used. The selection was carried out according to the manufacturer's instructions (11). Briefly, phage library (2×10^{11} pfu in 0.1% Tween 20 in phosphate buffered saline (PBST)) was added to β 2GPI-coated (30 μ g/ml) and blocked (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)) microtitre plate (High binding, Costar, Cambridge, MA, USA). Unbound phage clones were removed by intensive washing with 0.1% PBST and 0.5% PBST. The bound phage clones were eluted in a specific manner by monoclonal anti- β 2GPI (HCAL, Inova Diagnostics Inc., San Diego, CA, USA), purified high or low avidity anti- β 2GPI (12), or oxidized native IgGs (13). After four rounds of selection the affinity of recovered phage clones was determined, and a single-stranded DNA from selected phage clones was isolated and sequenced (MWG Biotech, Munich, Germany). Two peptides, one presenting most commonly observed motif and other exhibiting the highest affinity towards β 2GPI, were synthesized (GenScript USA Inc., NJ, USA).

IMMUNOASSAYS

Evaluation of phages' affinity towards β 2GPI

The binding affinity of selected phage clones was confirmed by standard phage ELISA. Briefly, microtitre plate (High binding, Costar) was coated with β 2GPI (100 μ l/well; 10 mg/l) and blocked with 1% BSA in PBS pH 7.4. A separate set of wells was coated with 1% BSA in PBS pH 7.4 without previous β 2GPI immobilization, as negative controls. Each recovered phage clone (5×10^9 pfu/well in 0.1% PBST) was added to the β 2GPI coated and to the BSA coated wells and incubated for 1 h at room temperature (RT). Wells were rinsed (0.05% PBST) and 200 μ l/well of horseradish peroxidase-labelled mouse anti-M13 monoclonal antibody (Amersham Biosciences, Little Chalfont, UK) in 1% BSA in 0.1% PBST (dilution 1:5000), was added and incubated for 1 h at RT. Finally, 100 μ l/well of substrate solution (0.22 mg/mL diammonium 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate in 50 mM citric acid and 1.7 μ l of 30% H₂O₂ /mL, pH 4.0) was added and the absorbance at 405 nm was measured.

Evaluation of phages' affinity towards β 2GPI and lipid A mixtures

To evaluate putative affinity of selected phage clones towards lipid A, the standard phage ELISA (described above) was used. Two mixtures of β 2GPI and lipid A, containing 20 μ g/ml of β 2GPI and 10 or 60 μ g/ml of lipid A, were prepared. β 2GPI at concentrations 10 and 20 mg/l, and the two mixtures were coated on separate sets of wells and incubated for 2 h at RT. After rinsing (0.1% PBST) 3×10^9 pfu/well of two phage clones corresponding to the common motif K-(L/V)-W-X-(I/L)-P-X were added and incubated for 1 h at RT. For detection a horseradish peroxidase-labelled mouse anti-M13 monoclonal antibody (Amersham Biosciences) and substrate 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich, MO, USA) were used. After colour development, stop solution (50 μ l/well of 2M H₂SO₄) was added and the absorbance was measured at 450 nm.

Competition ELISA

A competitive ELISA was used to evaluate phages' or peptides' ability to inhibit binding of anti- β 2GPI to the β 2GPI. 6.25-25 ng/ml of HCAL or 50-100 ng/ml of anti- β 2GPI were incubated with various amounts of each phage clone (0, 1.25×10^9 - 6×10^{11}) or synthetic peptide (250-200 nM) for 2 h at RT. The mixtures were subsequently applied to β 2GPI coated and BSA blocked microtitre plates (High Binding, Costar). The detection of anti- β 2GPI binding proceeded as in anti- β 2GPI ELISA (14).

SURFACE PLASMON RESONANCE ANALYSIS OF SELECTED PEPTIDES

The binding affinity of synthetic peptides towards β 2GPI was evaluated by surface plasmon resonance (SPR), using Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). Two peptides selected over unrelated target were used as negative controls. β 2GPI was amine coupled to the CM5 chip (13) reaching the immobilization level of ~ 2500 RU.

Reference cell was prepared by the same immobilization procedure without the addition of the ligand. The running buffer was 0.005% PBST, pH 7.4, and the analysis was performed at 25°C at a flow rate of 15 μ l/ml. Four different concentrations of each peptide (250-2000 nM) were injected over the sensor surface for a period of 1 min. Obtained sensorgrams were corrected by double subtracting the signal obtained on a reference surface and the signal of the running buffer.

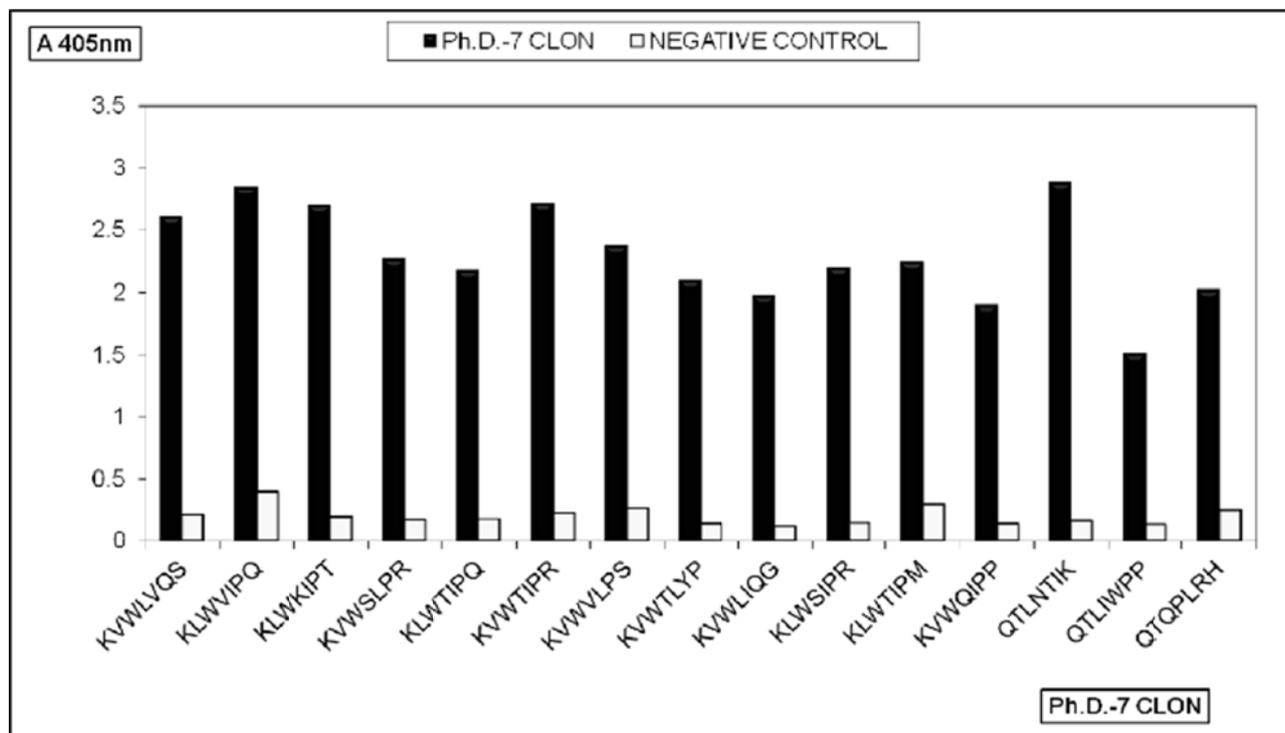
RESULTS AND DISCUSSION

In an attempt to characterize sequences binding to β 2GPI by Ph.D.-7 library, we used a screening procedure that involved specific elution of bound phages with various subgroups of anti- β 2GPI antibodies (monoclonal anti- β 2GPI, polyclonal low and high avidity anti- β 2GPI and oxidized native IgG with acquired anti- β 2GPI activity). As presented in Table 1, the selection yielded series of similar sequences. 53% of the selected sequences corresponded to the consensus motif K-(L/V)-W-X-(I/V/L)-(P)-X. In addition some sequences starting with Q-T-(L/Q)- and motifs known to possess propagation advantage were selected (marked blue and yellow respectively in Table 1) (10).

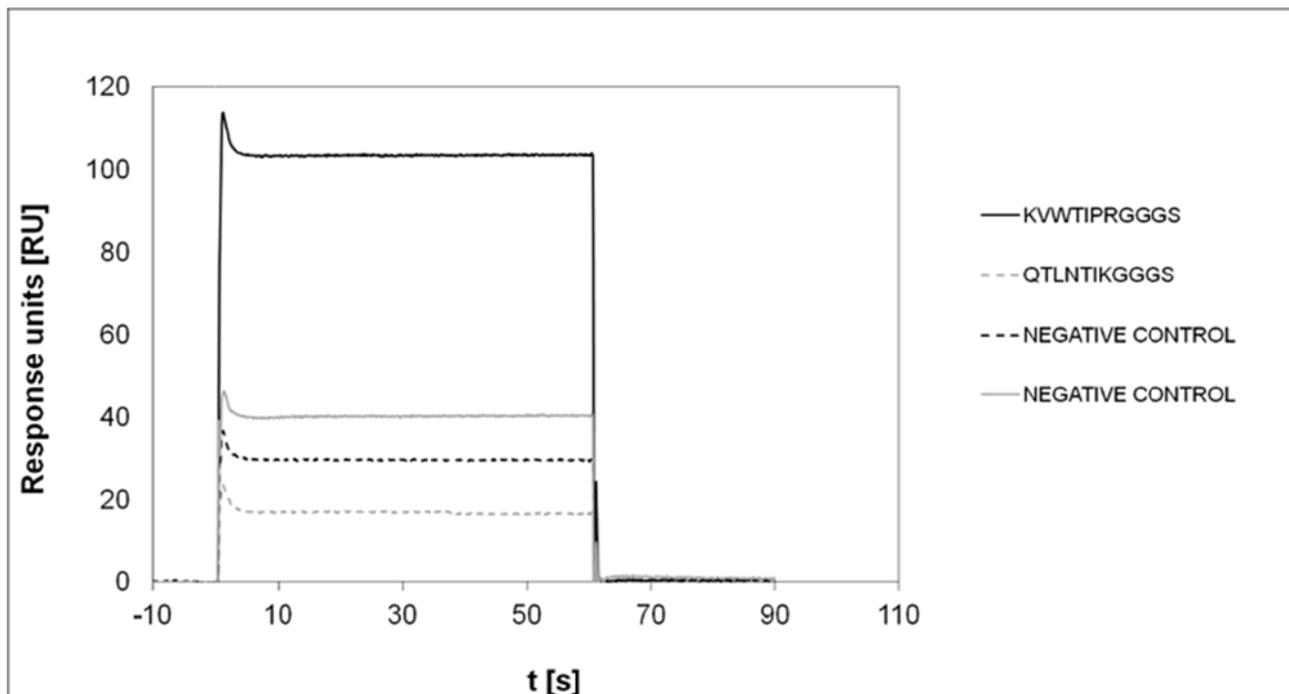
Table 1: Amino acid sequences of selected phage displayed peptides. Residues capable of forming electrostatic or hydrogen bonds are marked red; known target unrelated sequences are marked yellow (10); sequences corresponding to Q-T-(L/Q)- motif are marked blue.

ANTI- β 2GPI USED FOR ELUTION	SEQUENCES
MONOCLONAL ANTI- β 2GPI ANTIBODY (HCAL)	K L W V I P Q (4)
	K V W T I P R (2)
	K V W L V Q S
	K L W K I P T
	H N Q K P K L
	Q T L N T I K
	Y A T T L E H
	V T G S P H M
	L P L Y T T I
HIGH AVIDITY ANTI- β 2GPI ANTIBODY	K L W V I P Q (4)
	K V W T I P R (3)
	K V W I V S T
	K V W V L P I
	K V W S L P R
	K L W T I P Q
	K V W D L K L
	K V W H I S T
	K C C F I N A
	Q T L I W P P
H A I Y P R H	
I S I P R T M	
LOW AVIDITY ANTI- β 2GPI ANTIBODY	K L W T I P Q (2)
	K V W T L P R (2)
	K L W L L P V
	K V W T L Y P
	K V W L I Q G
	K L W S I P R
	K V W V L P S
	T T V Q R T V
	A M Y S V Y T
	L P L T P L P
OXIDIZED NATIVE IgG WITH ACQUIRED ANTI- β 2GPI ACTIVITY	Q T Q P L R H (5)
	K V W Q I P P
	K L W T I P M
	K V W Q I Q Y
	K V W Q I P Q

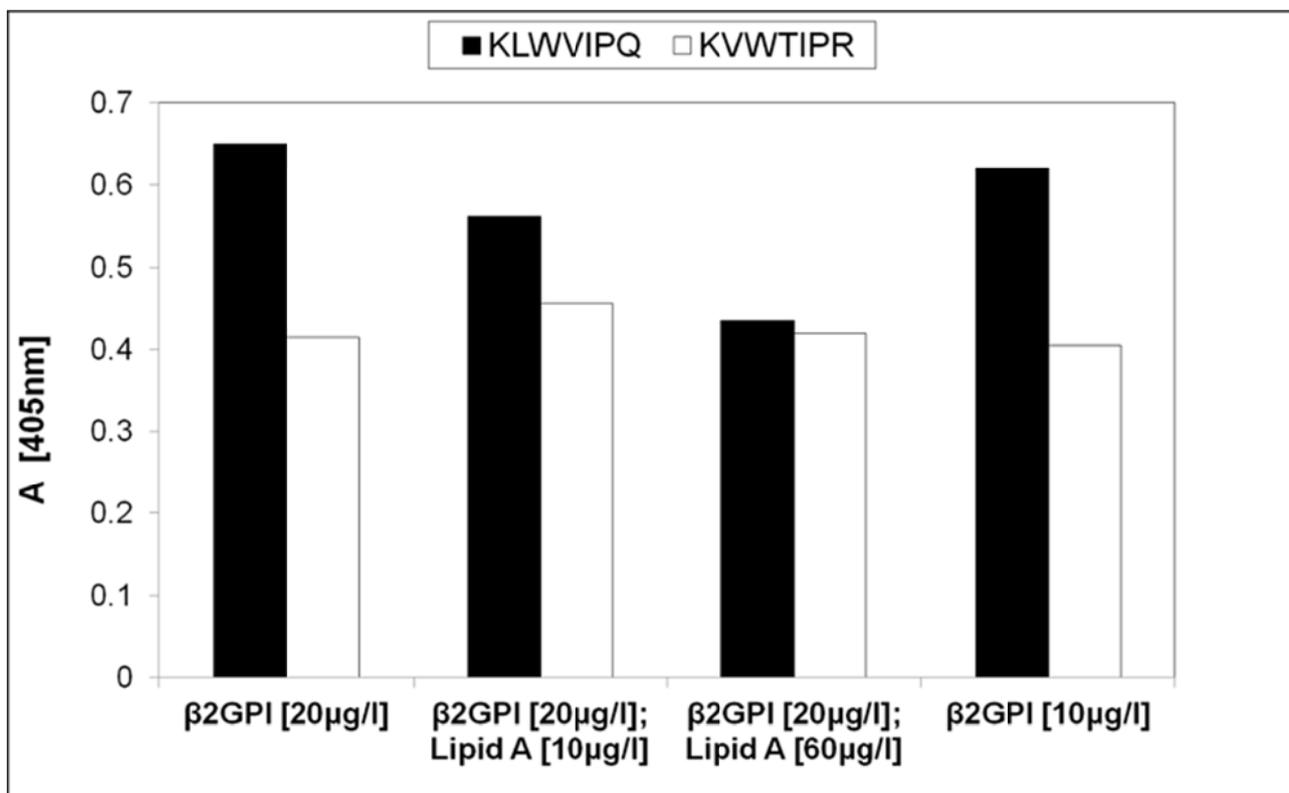
Phage clones' selective affinity towards β 2GPI was examined by ELISA in which each of the phage clones was applied to the immobilized β 2GPI and BSA. Selected clones exhibited selective binding to β 2GPI while no significant binding to BSA was observed (Figure 1).



However, the competitive ELISA revealed that despite their significant binding to the β 2GPI, the clones were unable to interfere with anti- β 2GPI binding to the antigen. The same inability was observed for the two synthesized peptides of the selected phage clones: the KVWTIPRGGGS corresponding to the observed motif and the QTLNTIKGGGS which exhibited the highest affinity toward β 2GPI (Figure 1). Their interaction with β 2GPI was further evaluated by SPR. The binding of peptide KVWTIPRGGGS to β 2GPI in flow conditions was significantly higher than of the QTLNTIKGGGS and the two control peptides (Figure 2). However, the dissociation of KVWTIPRGGGS was extremely fast indicating its low affinity towards β 2GPI. This is not unusual for small molecule such as dodeca-peptide that lacks the effect of avidity. The peptide's fast dissociation also provides the explanation why the respective peptide could not interfere with β 2GPI-anti- β 2GPI interaction in competitive ELISA. Phage clones on the other hand, display five copies of peptide and thus possess higher avidity. Despite selective affinity in phage ELISA, the inability to interfere with β 2GPI-anti- β 2GPI interactions and many studies describing similar sequences lead us to suspicion that selected clones could be target-unrelated. Because slightly diverse peptides as a part of common motif were usually selected, and because peptides with the same motif also emerged from the Ph.D.-12TM library (10), a propagation advantage of all corresponding phage clones seemed very unlikely.



Since phage clones did not exhibit any affinity towards BSA and only minor affinity towards polystyrene surface (data not shown) the lipid A (of LPS), present as contaminant in β 2GPI was suspected as a possible decoy of the selection. Therefore, we evaluated the binding of two phage clones (KLWVIPQ and KVTIPR) to mixtures of β 2GPI and increasing amounts of lipid A. The binding of phage clones to pure β 2GPI was the same or even slightly higher than the binding to β 2GPI- lipid A mixtures, which averted our suspicions (Figure 3).



The results indicated that phagotopes corresponding to K(L/V)-W-X-(I/V/L)-(P)-X motif were moderate but true β 2GPI binders. Therefore, sequences corresponding to the K(L/V)WX(I/L/V)P motif could represent the so called "hot spot" common to various unrelated proteins, especially considering that W, P, Q and R are thought to be among the critical residues at protein-binding sites (15). The consensus motif was therefore used for identification of the best matches on possible protein partners. A search of SWISS-PROT database, using BLASTP resulted in identification of several human, bacterial and viral proteins, exhibiting significant similarity to the motif and thus presenting putative binding partners of β 2GPI. The most intriguing of human proteins was T-cell immunoglobulin- and mucin-domain-containing molecule-4 (Tim-4) with 256 KVDLP 261 segment. Tim4 is the macrophage phosphatidylserine receptor for engulfment of apoptotic cells, recently reported to be involved in pathogenesis of SLE (16, 17). Recently suggested Tim-4- β 2GPI interaction is appealing, since binding of β 2GPI to phosphatidylserine-expressing procoagulant platelet microvesicles was reported to promote their clearance by phagocytosis. Furthermore, autoantibodies to β 2GPI positive in patients with APS may inhibit this process and may causally be related to the procoagulant state (18).

Among viral proteins resembling the consensus motif, the VP7 protein (110 KVVPL 114) of human rotavirus A and protein S (67 KVVGL 71) of hepatitis virus B were the most interesting. The facts that VP7 is a rotavirus outer-layer protein and proteins S are hepatitis B envelope proteins needed for formation of Dane particles are in agreement with reports that β 2GPI is able to bind poly-specifically to viruses, including rotavirus A and Dane particles of hepatitis B virus (19-22).

As for bacterial proteins, the majority of sequences resemble the integrase family proteins of *Salmonella enterica*, *Escherichia coli* and *Klebsiella pneumoniae*.

The selected motif also suggests that the interaction with β 2GPI is mainly mediated by polar residues. Namely, the negative charge of β 2GPI under pH of 7.4 conditions (23) and the presence of at least two positively charged residuals (NH₂-terminal, K, and R or H) in the selected sequences indicate that the interaction is in part mediated by electrostatic bonds. The presence of W, with its indole nitrogen acting as a hydrogen-bond donor, and the fact that the majority of X designated residues are hydrophilic, further suggests that hydrogen bonds are also involved. These results together with the previously reported role of positively charged residues (24) and hydrogen bond forming residues (25) in anti- β 2GPI paratopes demonstrated that polar residues were crucial for β 2GPI's interactions with other proteins.

CONCLUSION

Phage display libraries screening for amino acid sequences of autoantigens' paratopes is a useful tool for elucidating the autoimmune response (26). K(L/V)WX(I/L/V)P is a β 2GPI binding sequence. It exhibits significant resemblance to the segments of some previously identified β 2GPI binding macromolecules, and in the same time points to some new potential β 2GPI binding partners. The predominately hydrophilic nature of selected peptides further confirms previously suggested role of polar residues in β 2GPI interactions.

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