SIGNIFICANCE OF K(L/V)WX(I/L/V)P EPITOPE OF THE B2GPI IN ITS (PATHO)PHYSIOLOGIC FUNCTION

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ABSTRACT
β2-glycoprotein I (B2GPI) is a major autoantigen of autoimmune thrombophilia, known as the antiphospholipid syndrome. The exact mechanism underlying the B2GPI’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 B2GPI. Obtained K(L/V)WX(I/L/V)P motif, primarily designated as target unrelated, was confirmed as the selective binder of B2GPI. Based on this motif we confirmed the previously suggested role of polar residues in B2GPI interactions, and identified some already known and some new putative B2GPI binding proteins. The latter can help to further elucidate B2GPI’s (patho)physiological role.

INTRODUCTION
B2GPI 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). B2GPI 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 (C281KNKEKKC288) located on the B2GPI’s fifth domain enables it’s binding to negatively charged surfaces such as anionic phospholipids. Upon binding B2GPI opens up and thus converts from circular to “fish-hook” like conformation (2, 3).

The physiological role of B2GPI 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, B2GPI 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 B2GPI interacts with many different proteins (macromolecules). Determination of amino acid sequences recognized by B2GPI, 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 B2GPI by ELISA and surface plasmon resonance. Based on the selected motif we present putative binding partners of B2GPI.
METHODS

BIOPANNING PROCEDURE

To select β2GPI 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 (2×1011 pfu in 0.1% Tween 20 in phosphate buffered saline (PBST)) was added to β2GPI-coated (30 μg/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-β2GPI (HCAL, Inova Diagnostics Inc., San Diego, CA, USA), purified high or low avidity anti-β2GPI (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 β2GPI, were synthesized (GenScript USA Inc., NJ, USA).

IMMUNOASSAYS

Evaluation of phages’ affinity towards β2GPI

The binding affinity of selected phage clones was confirmed by standard phage ELISA. Briefly, microtitre plate (High binding, Costar) was coated with β2GPI (100 μl/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 β2GPI immobilization, as negative controls. Each recovered phage clone (5×109 pfu/well in 0.1% PBST) was added to the β2GPI coated and to the BSA coated wells and incubated for 1h at room temperature (RT). Wells were rinsed (0.05% PBST) and 200 μl/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 μl/well of substrate solution (0.22 mg/ml diammonium 2,2’-azino-di-(3-ethylbenzthiazoline sulfonate in 50 mM citric acid and 1.7 μL of 30% H2O2 /ml, pH 4.0) was added and the absorbance at 405 nm was measured.

Evaluation of phages’ affinity towards β2GPI and lipid A mixtures

To evaluate putative affinity of lipid A to lipid A, the standard phage ELISA (described above) was used. Two mixtures of β2GPI and lipid A, containing 20 μg/ml of β2GPI and 10 or 60 μg/ml of lipid A, were prepared. β2GPI at concentrations 10 and 20 mg/l, and the two mixtures were coated on separate sets of wells and incubated for 2h at RT. After rinsing (0.1% PBST) 3×109 pfu/well of two phage clones corresponding to the common motif K-(L/V)-W-X-(I/L/V)-P-X were added and incubated for 1h 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 μl/well of 2M H2SO4) was added and the absorbance was measured at 450nm.

Competition ELISA

A competitive ELISA was used to evaluate phages’ or peptides’ ability to inhibit binding of anti-β2GPI to the β2GPI. 6.25-25 ng/ml of HCAL or 50-100 ng/ml of anti-β2GPI were incubated with various amounts of each phage clone (0, 1.25×109- 6×1011) or synthetic peptide (250-200 nM) for 2h at RT. The mixtures were subsequently applied to β2GPI coated and BSA blocked microtitre plates (High Binding, Costar). The detection of anti-β2GPI binding proceeded as in anti-β2GPI ELISA (14).

SURFACE PLASMON RESONANCE ANALYSIS OF SELECTED PEPTIDES

The binding affinity of synthetic peptides towards β2GPI 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. β2GPI was amine coupled to the CMS 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 μl/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 β2GPI by Ph.D.-7 library, we used a screening procedure that involved specific elution of bound phages with various subgroups of anti-β2GPI antibodies (monoclonal anti-β2GPI, polyclonal low and high avidity anti-β2GPI and oxidized native IgG with acquired anti-β2GPI 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-β2GPI USED FOR ELUTION | SEQUENCES |
|-----------------------------|-----------|
| **MONOCLONAL ATI-β2GPI ANTIBODY (ECAL)** | KLWVIPQ (4) |
| | KVWTPR (2) |
| | KVVLVQS |
| | KLWKIDT |
| | HNQEPKL |
| | GTLMHTIK |
| | YATTLEH |
| | VTSolphM |
| | LPLYTTI |
| **HIGH AVIDITY ANTI-β2GPI ANTIBODY** | KLWVIPQ (4) |
| | KVWTPR (3) |
| | KVWIVST |
| | KVWLPI |
| | KVLWTPQ |
| | KVWDLK |
| | KVKHIST |
| | KCCFINA |
| | GTLMIPP |
| | HAIPRHM |
| | ISIPIRM |
| **LOW AVIDITY ANTI-β2GPI ANTIBODY** | KLWTPQ (2) |
| | KVWLTPR (2) |
| | KLWLPV |
| | KVWLP |
| | KVWLQG |
| | KLWSIPR |
| | KVWLP |
| | TVSQRTV |
| | AMSVYT |
| | LPLTPLP |
| **OXIDIZED NATIVE IgG WITH ACQUIRED ANTI-β2GPI ACTIVITY** | GTCPLSH (5) |
| | KVWQIPP |
| | KLWTPPM |
| | KVQIQY |
| | KVWQIPQ |
Phage clones' selective affinity towards β2GPI was examined by ELISA in which each of the phage clones was applied to the immobilized β2GPI and BSA. Selected clones exhibited selective binding to β2GPI while no significant binding to BSA was observed (Figure 1).

![ELISA graph](image)

However, the competitive ELISA revealed that despite their significant binding to the β2GPI, the clones were unable to interfere with anti-β2GPI 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 β2GPI (Figure 1). Their interaction with β2GPI was further evaluated by SPR. The binding of peptide KVWTIPRGGGS to β2GPI 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 β2GPI. 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 β2GPI-anti-β2GPI 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 β2GPI-anti-β2GPI 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 β2GPI was suspected as a possible decoy of the selection. Therefore, we evaluated the binding of two phage clones (KLWVIPQ and KVWTIPR) to mixtures of β2GPI and increasing amounts of lipid A. The binding of phage clones to pure β2GPI was the same or even slightly higher than the binding to β2GPI- lipid A mixtures, which averted our suspicions (Figure 3).
The results indicated that phagotopes corresponding to K(L/V)W-X(I/L/V)-P-X motif were moderate but true β2GPI 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 β2GPI. The most intriguing of human proteins was T-cell immunoglobulin- and mucin-domain-containing molecule-4 (Tim-4) with 256 KVWDLP 261 segment. Tim4 is the macrophage phosphatidylinerse receptor for engulfment of apoptotic cells, recently reported to be involved in pathogenesis of SLE (16, 17). Recently suggested Tim-4–β2GPI interaction is appealing, since binding of β2GPI to phosphatidylinerse-expressing procoagulant platelet microvesicles was reported to promote their clearance by phagocytosis. Furthermore, autoantibodies to β2GPI 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 KVWPL 114) of human rotavirus A and protein S (67 KVWGL 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 β2GPI 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 pneumonia.

The selected motif also suggests that the interaction with β2GPI is mainly mediated by polar residues. Namely, the negative charge of β2GPI under pH of 7.4 conditions (23) and the presence of at least two positively charged residues (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 hydrophobic, 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-β2GPI paratopes demonstrated that polar residues were crucial for β2GPI’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 β2GPI binding sequence. It exhibits significant resemblance to the segments of some previously identified β2GPI binding macromolecules, and in the same time points to some new potential β2GPI binding partners. The predominately hydrophilic nature of selected peptides further confirms previously suggested role of polar residues in β2GPI interactions.

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