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Recombinant domain V of β_2-glycoprotein I inhibits the formation of a 7-ketocholesteryl-9-carboxynonanoate and β_2-glycoprotein I complex

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Our prior study has been reported the formation of the oxidized low-density lipoprotein (oxLDL)/β_2-glycoproteinI (β_2-GPI)/autoantibody complex facilitated the antiphospholipid syndrome (APS) process. The domain V of β_2-GPI binds to the negatively charged molecules, e.g. 7-ketochoresteryl-9-caboxynonanoate (oxLig-1) derived from the oxLDL and mediates the interaction between oxLDL and β_2-GPI. In the present study, the oxLig-1/β_2-GPI/anti-β_2-GPI Ab (WB-CAL-1) model was established. The recombinant domain V of β_2-GPI (rβ_2-GPI DV) expressed in *Escherichia coli* competitively inhibits the interaction between β_2-GPI and oxLig-1 in the enzyme-linked immunoassay. Moreover, the rβ_2-GPI DV significantly inhibits the formation of the oxLig-1/β_2-GPI/autoantibody complex in an APS patient. The present work suggests a novel possibility that rβ_2-GPI DV could be used to inhibit the formation of oxLDL/β_2-GPI/autoantibody complex, and give us a hint for the development of new therapeutic strategies to prevent the APS process.

Keywords: Antiphospholipid syndrome/β_2-GPI/β_2-GPI domain V/oxLDL/oxLig-1.

Abbreviations: APS, antiphospholipid syndrome; β_2-GPI, β_2-Glycoprotein I; BSA, bovine serum albumin; CL, cardiolipin; DCC, N,N′-dicyclohexy learbodiimide; DMAP, 4-dimethylaminopyridine; ELISA, enzyme-linked immunoassay; HPTLC, high-performance thin-layer chromatography; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria–Bertani; NMR, nuclear magnetic resonance; oxLDL, oxidized low-density lipoprotein; oxLig-1, 7-ketocholesteryl-9-carboxynonanoate; rβ_2-GPI DV, recombinant domain V of β_2-GPI.

The antiphospholipid syndrome (APS) is a multisystem autoimmune disease characterized by vascular lesion, thrombosis and/or recurrent pregnancy loss in patients who test positive for autoantibodies, *e.g.* antiphospholipid and lupus anticoagulant (*1*, *2*). Oxidized low-density lipoprotein (oxLDL) is the proximal source of lipid that accumulates within cells of the atherosclerotic lesions. OxLDL can be taken up by macrophages via endocytosis, resulting in foam cell formation and cell apoptosis. Indeed, macrophage could bind to the circulating oxLDL/β_2-glycoprotein I (β_2-GPI)/autoantibody complexes via Fcγ receptors (*3*). The formation of the oxLDL/β_2-GPI/autoantibody complex is relevant to the development of APS, as evidenced by the autoantibodies facilitate oxLDL uptake and foam cell formation in macrophages (*4*, *5*).

β_2-GPI, a 50-kDa protein present in plasma at ~200 μg/ml is composed of 326 amino acids (*6*) and five sushi domains, namely domain I, II, III, IV and V (*7*). It plays important physiological roles *in vivo* (*8*–*10*). For example, domain I (*10*) and domain IV (*8*) were reported to be an antigen epitope recognized by autoantibodies, WB-CAL-1, EY2C9 and EY1C8, and significantly correlated with arterial thrombosis (*11*–*13*); domain V was reported to bind a negative phospholipid, *e.g.* cardiolipin (CL) and a couple of lipid ligands from oxLDL. The potential binding regions of domain V are reported as Cys281-Cys288 (*14*, *15*) or Cys313-Trp316 (*16*, *17*). It is conceivable that β_2-GPI could bind to the oxidized lipid ligands of oxLDL via its domain V, and recognize the autoantibodies via domain I or/and domain IV, followed by a formation of an oxLDL/β_2GPI/autoantibodies complex. It is noteworthy that autoantibodies against oxLDL/β_2GPI complexes have been demonstrated in patients with systemic lupus erythematosus and/or APS (*2*, *18*).

It has been reported that many components of oxLDL is implicated in atheroselerosis development. Recently, we purified a lipid moiety from oxLDL, a 7-ketocholesteryl-9-carboxynonanoate (oxLig-1), and found that the oxLig-1 is targeted by β_2-GPI/anti-β_2-GPI antibodies, and oxLig-1/β_2-GPI/autoantibodies complex was taken up by the macrophages through scavenger receptors (*19*, *20*), and then results in the foam cell formation and accelerates atheroselerosis process (*3*). The oxLig-1 is a specific ligand of β_2-GPI and contains a carboxyl tail, just like other negative phospholipids, can specially bind to the domain V of β_2-GPI. Since domain V of β_2-GPI plays an important role in the process of forming an oxLDL/β_2-GPI/autoantibody complex, we suspected that domain V could competitively inhibit the formation of an

oxLDL/β_2-GPI/autoantibody complex. To determine our hypothesis, we established an oxLig-1/β_2-GPI/autoantibody complex model, and demonstrated that the recombinant domain V of β_2-GPI (rβ_2-GPI DV) could inhibit the formation of oxLig-1/β_2-GPI/autoantibody complex successfully.

Materials and Methods

Chemicals and specimens

Restriction enzymes *Kpn*I, *Eco*RI, were purchased from TaKaRa Biotechnology Co. (TaKaRa Bio. Japan); IPTG was from BIO BASIC INC. (BBI. Canada); Plasmids pET-32a(+) was from Novagen, Inc (Novagen, Inc. USA); 4-dimethylaminopyridine (DMAP), CL, azelaic acid, 5-cholesten-3β-ol-7-one (7-ketocholesterol) were obtained from Sigma Chemical Co. (St. Louis, MO); N, N′-Dicyclohexy learbodiimide (DCC) was purchased from Sinopharm Chemical Reagent Co. (SCRC, China).

The sera sample from APS patient with episodes of arterial thrombosis and healthy human were obtained from the second affiliated hospital of Dalian Medical University. Informed consent was given for all patients and the study was approved by the Institutional Review Board of Dalian Medical University. The existence of anti β_2-GPI antibody in APS patient and healthy human were detected by anti β_2-GPI IgG enzyme-linked immunoassay (ELISA) kit (EUROIMMUN, Germany).

Monoclonal antibodies

A monoclonal anti-His antibody (mouse IgG) and a goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A monoclonal anti β_2-GPI autoantibody (IgG2a), WB-CAL-1 (*11*), was from Dr Eiji Matsuura (Okayama University, Japan).

Construction of prokaryotic expression plasmid

Total RNA was extracted from human liver tissue sample with TRIzol reagent (Invitrogen, Carlsbad, CA) following the protocol recommended by the manufacturer. Messenger RNA was isolated using a μMACs mRNA Isolation Kit (Miltenyi Biotec., Bergisch Gladbach, Germany). The integrities of total RNA and mRNA were verified by agarose gel electrophoresis and Bioanalyzer with RNA LabChip (Agilent, Palo Alto, CA) following the manufacture's instruction. The mRNA was converted to cDNA using RT-PCR Kit (TAKARA Bio. Inc., Japan).

RT-PCR was performed according to the usual method. The primers were designed depending on the Genebank of NCBI (CCDS ID: CCDS11663.1) with software Primer 5.0. The primer sequences of domain V of β_2-GPI were as follows: the forward primer was 5′-CGGGGTACCAAAGCATCTTGTAAAGTACCTGTG-3′ (the underlined bases indicate a *Kpn*I site) and the reverse primer was 5′-CGGAATTCTTAGCATGGCTTTACATCGGATGC-3′ (the underlined bases indicate an *Eco*RI site). Reaction condition included 94°C for 30 s, 57°C for 45 s and 72°C for 30 s.

PCR products were further purified by Gel extraction (BIO BASIC INC. Canada) and inserted into the pET 32a (+) vector using DNA Ligation kit (TAKARA BIO INC. Japan), and then the vector was transformed into competent JM109. Automated sequencing was carried out by TaKaRa Bio.

Expression and purification of rβ_2-GPI DV

For expression of rβ_2-GPI DV protein, the resultant plasmid, pET 32a(+)-rβ_2-GPI DV, was transformed into *Escherichia coli* Rosetta-gami (DE3) cells. The cells were cultured in the Luria–Bertani (LB) medium containing 50 μg/ml ampicillin, 15 μg/ml kanamycin and 12.5 μg/ml tetracycline. Protein expression was induced by the addition of 0.1 mM Isopropyl-β-D-thiogalactoside (IPTG). And after cells collecting and ultrasonication, the supernatant was remained, the fusion expressed protein was purified by AKATA Explore 100 using Hi Trap affinity column (Amersham Biotech, Sweden).

Western blot analysis

The expressed fusion protein samples of rβ_2-GPI DV were electrophoresed on 12% polyacrylamide gel using Mini Protean II electrophoresis tanks (Bio-Rad, Hercules, CA). After electrophoresis, samples were transferred to PVDF membranes (Immobilon-P, 0.45 μm, Millipore, Bedford, MA). The blots were blocked 5% (w/v) bovine Serum Albumin (BSA) in TBS-T (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 1 h at room temperature. Following incubated with the appropriate anti-His antibody overnight, the slides were washed, and then the blots were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP). Finally, specific proteins were visualized using an ECL system (Amersham Biosciences).

Extraction of lipid components from native LDL and oxLDL

LDL ($d = 1.019$–1.063 g/ml) was isolated by ultracentrifugation, and oxidized using 5 μM $CuSO_4$ for 8 h at 37°C as described (*19*). The lipid components from native and oxLDL were extracted with chloroform–methanol (2 : 1, v/v) and dried by evaporation.

Chemical synthesis of oxLig-1

The oxLig-1 was synthesized according to the method described as previously with a litter modification (*19*). In brief, to a solution of 7-ketocholesterol (5-cholesten-3β-ol-7-one, 49.7 mg, 0.12 mmol) and azelaic acid (106.8 mg, 0.57 mmol) in dichloromethane (5 ml) was added DMAP (17.8 mg, 0.15 mmol) and DCC (31.6 mg, 0.15 mmol). The mixture was stirred at room temperature for 12 h, and then refluxed for other 12 h and filtrated. The extract was successively washed with saturated $NaHCO_3$ and NaCl, dried over sodium sulfate, and evaporated. The residue was subjected to column chromatography on silica gel using ethyl acetate/petroleum ether (4 : 1, v/v) to give oxLig-1.

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectroscopies of oxLig-1 were measured at 500 and 125.8 MHz, respectively, on AVANCE500 spectrometer (Bruker, Switzerland). Infrared spectroscopy of oxLig-1 was recorded on Nicolet 150 (Nicolet Company, USA).

Preparation of β_2-GPI

β_2-GPI was purified from normal human plasma as described (*21*). Briefly, pooled plasma from healthy subjects was chromatographed on a heparin-Sepharose column, on a DEAE-cellulose column and on an anti-β_2GPI affinity column. Finally, the purified protein was reserved by freeze drying.

High-performance thin-layer chromatography immunostaining assay

The lipids were extracted by the method as described previously (*20*). The extracted lipids were spotted on a high-performance thin-layer chromatography (HPTLC) silica gel-60 plate (Silica Gel 60 F-254, Merk, Darmstadt, Germany) and developed with a solvent system of chloroform/methanol (8 : 1, v/v). CL and oxLig-1 (50 μg/plate) were spotted on a HPTLC plate.

HPTLC immunostaining assay for CL and oxLig-1 on HPTLC plate was performed according to the method reported previously (*19*), and with a slight modifications. HPTLC plate was developed with chloroform/methanol (8 : 1, v/v). The dried plate was soaked in a PBS containing 1% gelatin for 1 h and subsequently incubated with rβ_2-GPI DV (pH = 7.4) or β_2GPI for 1 h. Following incubation with the appropriate primary antibodies (anti-His antibody or anti-β_2GPI antibody, WB-CAL-1) overnight, the plates were washed. After washing, the blots were incubated with goat anti-mouse IgG conjugated with HRP for 1 h. The colour was developed with H_2O_2 and 4-methoxy-1-naphtol (Aldrich, Milwaukee, WI).

Enzyme-linked immunoassay

The method of ELISA was performed as described previously (*19*). Briefly, lipids extracted from LDLs and oxLDLs (50 μg/ml, 50 μl/well), CL and oxLig-1 (50 μg/ml, 50 μl/well) were, respectively, absorbed on oxygenated polystyrene plates (carboxylated, Sumilon C-type, Sumitomo Bakelite Co., Ltd.) The plates were blocked with PBS with 1% gelatin for 1 h. The β_2-GPI and/or rβ_2-GPI DV were added. The plates were probed by appropriate first antibodies (1 : 1000) or blood sera (1:100). The plates were incubated with goat anti-mouse IgG or goat anti-human IgG conjugated with HRP (1 : 1000) for 1 h. In each step, the wells were extensively washed with PBS-0.05% Tween-20. Finally the colour was

developed with o-phenylenediamine buffer containing H_2O_2 and terminated by 2 N H_2SO_4. Absorbance was measured at 492 nm.

For binding-inhibition experiments, each well was incubated with β_2-GPI (1.5 μg/50 μl, pH = 7.4) and rβ_2-GPI DV (pH = 7.4) simultaneously at 37°C for 1 h. The amounts of rβ_2-GPI DV were as below: 0 μg/50 μl, 1.5 μg/50 μl, 4.5 μg/50 μl, 9.0 μg/50 μl. To confirm the binding specificity of rβ_2-GPI DV in the experiment, 9.0 μg/50 μl BSA was added instead of rβ_2-GPI DV.

Statistical analysis

Statistical comparisons between data were carried out using the Student's *t*-test with $P < 0.05$ considered as statistically significant and $P < 0.01$ considered as extremely significant.

Results

Expression and purification of rβ_2-GPI DV

To express the rβ_2-GPI DV in *E. coli*, a coding region of the domein V of β_2-GPI cDNA was amplified by PCR from human liver tissue. The PCR product was cloned using a pMD19-T Simple vector and the nucleotide sequence of the insert DNA was determined. The alignment was completely identical to DNA sequence coding (CCDS ID: CCDS11663.1) the domain V of β_2-GPI structural protein (data not shown).

The full-length rβ_2-GPI DV cDNA was amplified by PCR with designed primers (containing enzyme sites). The rβ_2-GPI DV cDNA contains 275 bp (Fig. 1A), and its putative protein consists of 86 amino acid residues. Prokaryotic expression vector pET-32a (+) was used because of containing Trx Tag, which enable to enhance solubility of recombinant target protein. The prokaryotic expression vector containing rβ_2-GPI DV was transformed into *E. coli* Rosetta-gami (DE3) cells. The expressed target protein was shown in Fig. 1B, the synthesis of an abundant fusion protein was found in the supernatant mixture after cells ultrasonication. The molecular weight of recombinant fusion protein is ~30 kDa. Because the prokaryotic expression vector has a hexahistidine (his6), expression product could be purified by HiTrap affinity column (Fig. 1C). Also, expression product can be confirmed by anti-His-tag antibody (Fig. 1D), suggesting that the fusion protein of rβ_2-GPI DV was successfully expressed.

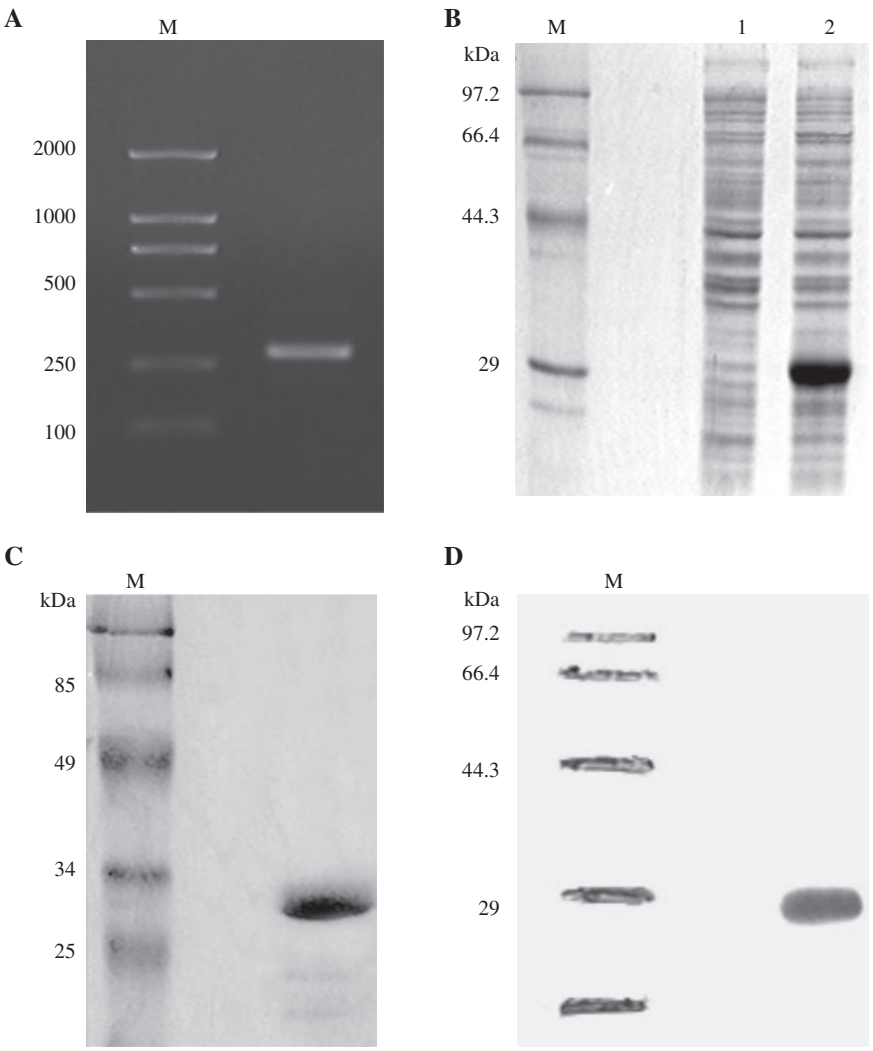

Fig. 1 Expression and purification of rβ_2-GPI DV. (A) Amplification of rβ_2-GPI DV cDNA by PCR. (B) Expression of rβ_2-GPI DV. Lane 1: the soluble fraction without IPTG induction. Lane 2: the soluble fraction after IPTG induction. (C) Purification of rβ_2-GPI DV using Hi Trap affinity column. (D) Western blot of rβ_2-GPI DV by anti-His antibody.

Identification of the rβ$_2$-GPI DV Binding to CL

It has been reported that anti-CL antibody recognized a β$_2$-GPI/CL complex, but not a sole CL (*22*, *23*). Indeed, the β$_2$-GPI binds to CL with domain V and form a β$_2$-GPI/CL complex. In the HPTLC immunostaining assay, we found the rβ$_2$-GPI DV binds to CL as well as β$_2$-GPI does (Fig. 2A). Moreover, in the binding inhibition experiments, we found that the rβ$_2$-GPI DV competitively inhibited β$_2$-GPI binding to CL with ELISA (Fig. 2B). When the amount of rβ$_2$-GPI-DV was up to 9 μg/50 μl, the level of binding inhibition to CL is reached to the level of control. It is conceivable that the rβ$_2$-GPI DV harbored a good binding activity to CL.

Synthesis and analysis of oxLig-1

Lipids were extracted from native LDLs and oxLDL, subjected to binding assays for β$_2$-GPI, by detecting with WB-CAL-1. As shown in Fig. 3A, the lipid extracts from oxLDL, but not from native LDL, showed highly specific binding to β$_2$-GPI. Also, we found that OxLig-1, a lipid component of oxLDL was a specific lipid ligand of β$_2$-GPI (*19*, *20*) (data not shown).

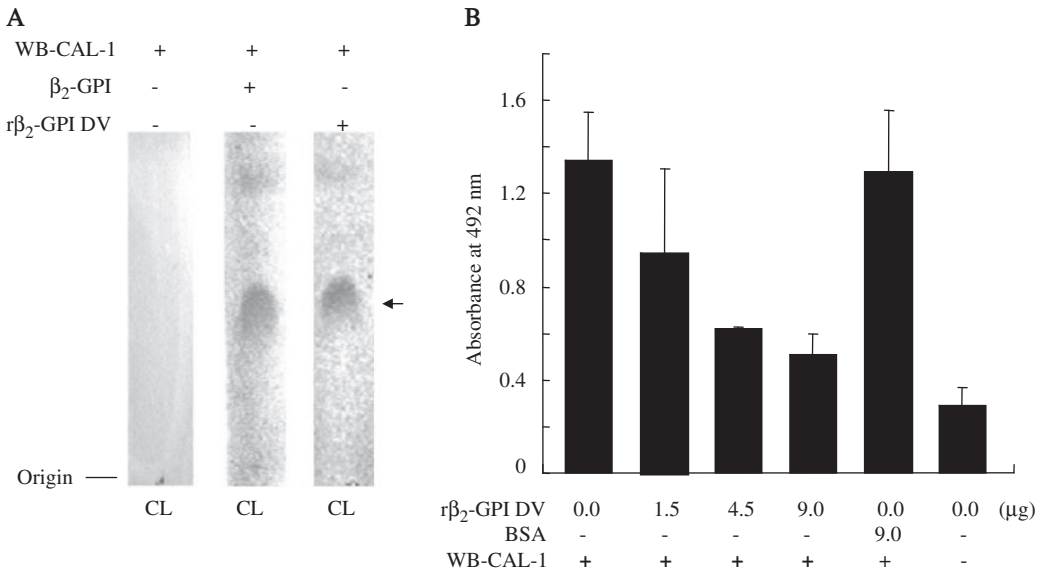

Fig. 2 Binding activity of rβ$_2$-GPI DV to CL. (A) CL (50 μg each plate) was spotted on HPTLC plate, developed with solution of chloroform/methanol (8 : 1, v/v), incubated with β$_2$-GPI/WB-CAL-1 and rβ$_2$-GPI DV/anti-His antibodies. (B) rβ$_2$-GPI DV competitively inhibit the formation of CL/β$_2$-GPI complex in ELISA. Observation wavelength in 492 nm. CL (50 μg/ml, 50 μl) was coated, β$_2$-GPI (1.5 μg/50 μl, each well), for competitive inhibition, rβ$_2$-GPI DV (pH = 7.4) was added as below: 0, 1.5 μg/50 μl, 4.5 μg/50 μl, 9 μg/50 μl, and BSA (9 μg/50 μl). Control is without rβ2-GPI DV and first antibody, WB-CAL-1. Data are indicated as the mean ± SD of triplicate samples.

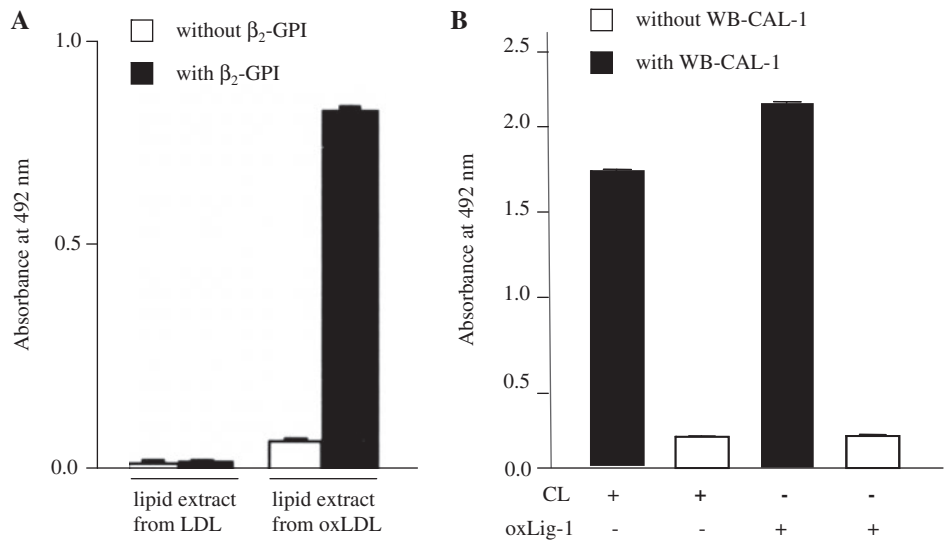

Fig. 3 Binding of β$_2$-GPI to lipid components in ELISA. (A) Binding of β$_2$-GPI to lipid extracts from native LDLs and oxLDLs in ELISA. Lipid extracts (50 μg/ml, 50 μl) was coated. Observation wavelength in 492 nm. (B) Binding of β$_2$-GPI to synthesized oxLig-1. CL/oxLig-1 (50 μg/ml, 50 μl) were coated, incubated with β$_2$-GPI (1.5 μg/50 μl). The plates were probed by WB-CAL-1, while control is without WB-CAL-1. Observation wavelength in 492 nm.

To investigate whether oxLig-1 binds to rβ_2-GPI DV, we synthesized oxLig-1 with 7-ketocholesterol and azelaic acid. The esterification reaction was catalysed by DCC and DMAP for 24 h. The synthesized oxLig-1 was isolated between 7-ketocholesterol and azelaic acid by silica-gel column chromatography (Fig. 4A). The yield was up to 80.4%. The structure of synthesized oxLig-1 was verified by ^{1}H and ^{13}C NMR spectroscopy, ^{1}H-spectroscopy, ^{1}H NMR of syn-oxLig-1 (500 MHz, $CDCl_3$): $\delta = 5.71$ (s, ^{1}H, H-6),

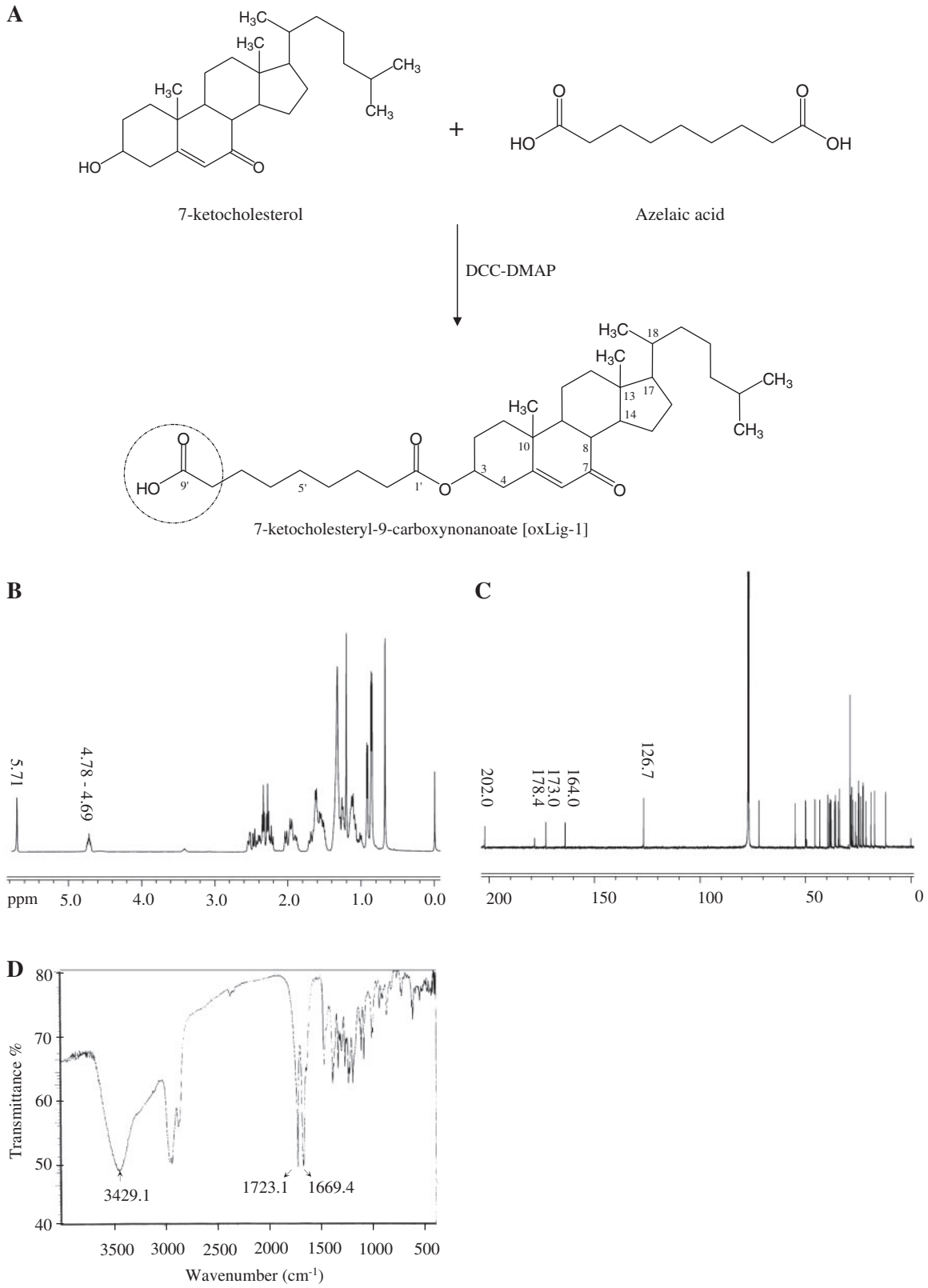

Fig. 4 Chemical synthesis of oxLig-1. (A) Synthesis and the structure of oxLig-1. (B) The 500-MHz ^{1}H NMR spectrum of oxLig-1. (C) The 125.8-MHz ^{13}C NMR of oxLig-1. (D) Infrared spectroscopy of oxLig-1.

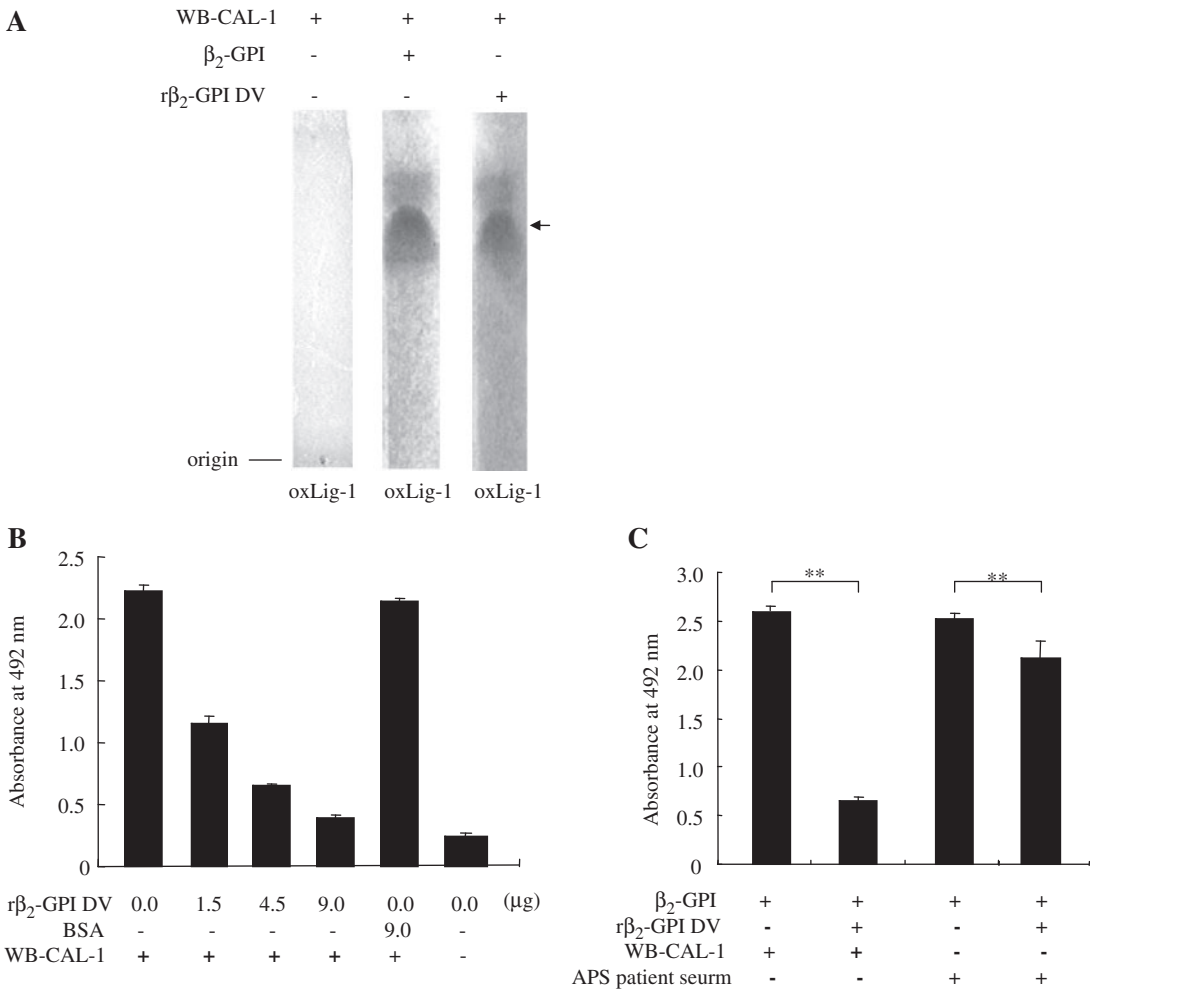

Fig. 5 Competitive binding inhibition of rβ$_2$-GPI DV to oxLig-1. (A) oxLig-1 (50 μg) was spotted on HPTLC plate, developed with a solution of chloroform/methanol (8 : 1, v/v), incubated with β$_2$-GPI/WB-CAL-1 and rβ$_2$-GPI DV/anti-His antibodies. (B) rβ$_2$-GPI DV competitively inhibits the formation of oxLig-1/β$_2$-GPI/WB-CAL-1 complex in ELISA. Observation wavelength in 492 nm. oxLig-1 (50 μg/ml, 50 μl, pH = 7.4) was coated, β$_2$-GPI (1.5 μg/50 μl, each well), for competitive inhibition, rβ$_2$-GPI DV was added as below: 0, 1.5 μg/50 μl, 4.5 μg/50 μl, 9 μg/50 μl and BSA (9 μg/50 μl). Control is without rβ$_2$-GPI DV and first antibody, WB-CAL-1. (C) rβ$_2$-GPI DV competitively inhibits the formation of oxLig-1/β$_2$-GPI/antoantibody (APS patient serum) complex in ELISA. OxLig-1 was coated, β$_2$-GPI (1.5 μg/50 μl, each well), for competitive inhibition, rβ$_2$-GPI DV (9 μg/50 μl) was added (**$P < 0.01$). Data are indicated as the mean ± SD of triplicate samples.

4.78-4.69 (m, ^{1}H, H-3) (Fig. 4B); ^{13}C NMR (125.8 MHz, $CDCl_3$): $\delta = 202.0$ (C-7), 178.4 (C-9′), 173.0 (C-1′), 164.0 (C-5′), 126.7 (C-6), 77.3, 77.0, 76.8, 72.0, 54.8, 50.0, 49.9, 49.4, 45.4, 43.2, 39.5, 38.7, 38.4, 37.8, 36.2, 36.0, 35.7, 34.5, 33.8, 28.9, 28.6, 28.0, 27.4, 26.3, 25.6, 24.9, 24.6, 23.9, 22.8, 22.6, 21.2, 18.9 (Fig. 4C). In addition, infrared spectroscopy of oxLig-1: wavenumbers = 3429, 2959, 2932, 2872, 2367, 1723, 1669, 1468, 1387, 1233, 1092, 1011, 944 (/cm) (Fig. 4D), showing that the characteristic absorption peak at 3429.1 corresponding to -OH of carboxyl (dashed circle). The structure of oxLig-1, a specific ligand for β$_2$-GPI was identified by NMR, as evidenced by harboring a carboxyl tail which could specially bind to the domain V of β$_2$-GPI.

The binding activity of the synthesized oxLig-1 to β$_2$-GPI was verified by ELISA. Interestingly, the binding activity of synthesized oxLig-1 to β$_2$-GPI was stronger than those of CL to β$_2$-GPI (Fig. 3B), suggesting that the chemically synthesized oxLig-1 has a biotical activity.

The rβ$_2$-GPI DV inhibits the formation of oxLig-1/β$_2$-GPI/anti-β$_2$-GPI antibody complex

As shown in Fig. 5A, the synthesized oxLig-1 bound to the rβ$_2$-GPI DV as well as to β$_2$-GPI in HPTLC immunostaining assay. It has been reported that the anti-β$_2$-GPI antibody, WB-CAL-1, can specifically recognize the domain IV of β$_2$-GPI, but not domain V (*11*). To assess whether rβ$_2$-GPI DV can inhibit the formation of oxLig-1/β$_2$-GPI/anti-β$_2$-GPI antibody complex, we established an oxLig-1/β$_2$-GPI/WB-CAL-1 complex model and performed a competitive binding inhibition experiment. The rβ$_2$-GPI DV significantly inhibits the formation of oxLig-1/β$_2$-GPI/WB-CAL-1 complex in a dose dependent manner (Fig. 5B). When the amount of rβ$_2$-GPI DV is up to 9 μg/50 μl, the inhibition level is similar to those of control group. To confirm the inhibitory specificity of rβ2-GPI DV, we carried out the binding inhibition experiments using 9.0 μg/50 μl BSA, and found the absorbance is not affected by treatment of BSA, showing that the binding of rβ2-GPI DV to CL or oxLig-1 is specific.

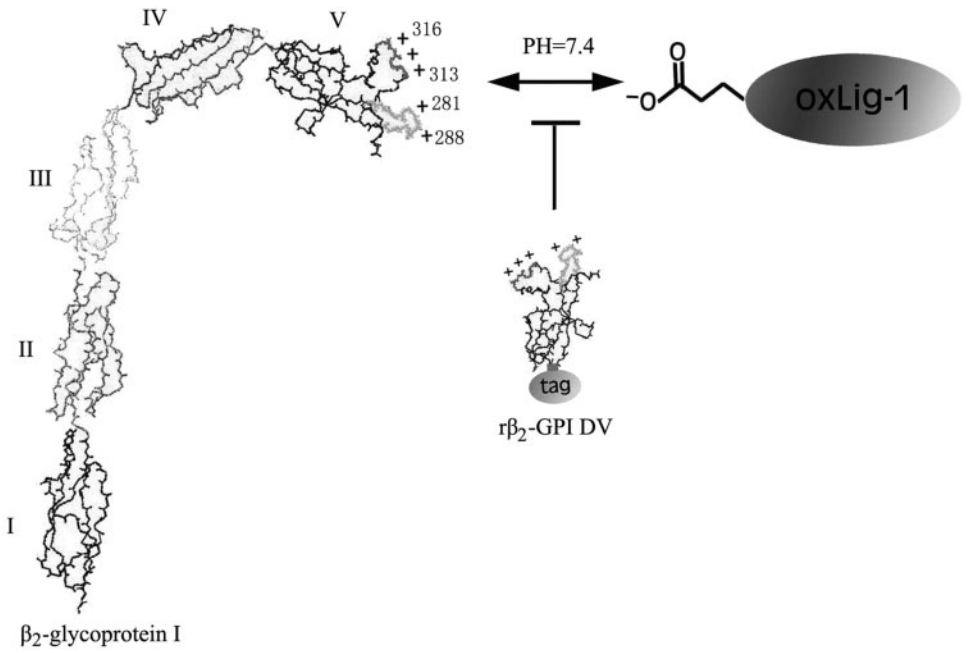

Fig. 6 Possible mechanisms of the suppression of the oxLig-1/β_2-GPI complex by rβ_2-GPI DV. The areas of positively charged amino acids, Cys281-Cys288 and Cys313-Trp316 on the domain V of β_2-GPI were essential for the binding to the carboxyl residue on oxLig-1.

To further investigate the role of rβ_2-GPI DV in the inhibition of the formation of oxLig-1/β_2-GPI/autoantibody complex, we used an APS patient serum instead of WB-CAL-1 antibody. The titer of anti β_2-GPI antibody in APS patient with episodes of arterial thrombosis was detected by anti β_2-GPI IgG ELISA kit (data not shown). Our findings showed that the rβ_2-GPI DV could also inhibit the formation of oxLig-1/β_2-GPI/autoantibody complex in competitive inhibition experiment (Fig. 5C). Taken together, our data showed rβ_2-GPI DV could inhibit the formation of oxLig-1/β_2-GPI/autoantibody complex through a competition with β_2-GPI.

Discussion

Our previous study showed that the formation of the oxLDL/β_2-GPI/autoantibodies complex is involved in the development of APS. From the analysis of crystal structure of β_2-GPI, the domains IV and I of the β_2-GPI could bind to the autoantibodies in the APS patient and the sites which oxLDL binds to β_2-GPI exist in domain V. To study the function of the domain V of β_2-GPI during the formation of oxLDL/β_2-GPI/autoantibodies complex, we expressed the rβ_2-GPI DV. In this study, prokaryotic expression vector pET-32a(+) was used to enhance solubility of recombinant target protein, and the host strains, Rosetta-gami, were used to make a exact folding of rβ_2-GPI DV. Although the domain V of β_2-GPI contains three disulfide bonds, in this paper, the rβ_2-GPI DV was successfully expressed in *E. coli* with a considerable yield and a biological function using a pET-32a(+) expression system.

In the present study, we synthesized oxLig-1 by the catalytic esterification of DCC/DMAP. The 80.4% of oxLig-1 was yielded by using a DCC/DMAP catalyser, which is higher than that by WSC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride]/DMAP (*19*), suggesting that the novel synthesized method using DCC/DMAP is high productive. Furthermore, the structure of the oxLig-1, a special ligand of β_2-GPI was identified by NMR. In NMR analysis of the structure of oxLig-1, we found oxLig-1 has a carboxyl tail as well as negative phospholipid, *e.g.* CL. It has been reported that the potential binding regions in domain V of β_2-GPI were Cys281-Cys288 (*14*, *15*) or Cys313-Trp316 (*16*, *17*). It is conceivable that the binding sites of carboxyl residue in oxLig-1 was the area consisting of the positively charged amino acids, Cys281-Cys288 or/and Cys313-Trp316 (Fig. 6). However, the binding site of the oxLig-1 to rβ_2-GPI DV needs our further determination.

It has been reported that the autoantibodies against to the complex oxLDL/β_2-GPI existed in the sera of APS patients (*4*). Indeed, the oxLDL/β_2-GPI/autoantibodies complex was taken up by macrophages via Fcγ receptor, resulting in foam cell formation and accelerating APS (*19*, *20*). Since the rβ_2-GPI DV could bind to the negative charged lipid ligands of oxLDL, but not bind to the autoantibodies (*8*, *10*), we hypothesized that rβ_2-GPI DV could inhibit the formation of the oxLDL/β_2-GPI/autoantibodies complex. Because oxLig-1 is a specific ligand for β_2-GPI binding to oxLDL in our previous study (*19*), we established oxLig-1/β_2-GPI/WB-CAL-1 complex model instead of oxLDL/β_2-GPI/autoantibodies complex. As we anticipated, in the present study we found that the rβ_2-GPI DV actually inhibited the formation of oxLig-1/β_2-GPI/WB-CAL-1 complex (Fig. 4C). Indeed the rβ_2-GPI DV could also inhibit the formation of the oxLig-1/β_2-GPI/autoantibodies complex (Fig. 4D).

The β_2-GPI binds to its special ligand, oxLig-1, via domain V (pH = 7.4), hence the rβ_2-GPI DV could inhibit the formation of oxLDL/β_2-GPI/autoantibodies complex (Fig. 5). In this regard, our present work provides an attractive opportunity toward a more effective strategy for APS therapy by preventing the formation of oxLDL/β_2-GPI/autoantibodies complex using rβ_2-GPI DV.

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Conflict of interest
None declared.

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