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Prokaryotic Expression and Activity Analysis of 2-Glycoprotein I Domain V, Its Mutants, and Short Peptide Fragments: Post-print

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Abstract

2-glycoprotein I (2GP) is the primary antigen for antiphospholipid antibodies (aPL) in the serum of antiphospholipid syndrome (APS). 2GP binds to anionic phospholipid oxLDL through its fifth domain and is subsequently recognized by aPL, representing a critical event in the development of arterial thrombosis in APS. In this study, we constructed prokaryotic expression vectors encoding the fifth domain of 2GP (ß2GP -D), ß2GP -D mutants, and the Phe280-Ala320 fragment of ß2GP -D , induced their expression and purification, and elucidated the molecular mechanism of ß2GP -D binding to anionic phospholipids. The results demonstrated that maintaining a specific spatial conformation of the Cys281-Cys288 and Ser311-Lys317 segments in 2GPI-DV is a prerequisite for binding to CL, while the two disulfide bonds C245-C296 and C288-C326 play a role in maintaining the spatial conformation of these segments. Based on these findings, we further examined the binding activity of rDV (which possesses CL-binding biological activity) toward both oxLDL and oxLDL in APS serum, demonstrating that rDV exhibits biological activity consistent with native 2GP . The rß2GP -D obtained in this study, together with the established methodological system for oxLDL binding, lays the foundation for early laboratory diagnosis of APS.

Full Text

Prokaryotic Expression and Activity Analysis of the Fifth Domain of 2-Glycoprotein I and Its Mutants and Short Peptide Fragments

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Abstract

Beta-2-glycoprotein I (2GPI) is the primary antigen targeted by antiphospholipid antibodies (aPL) in antiphospholipid syndrome (APS). The binding of 2GPI to anionic phospholipids such as oxidized low-density lipoprotein (oxLDL) through its fifth domain, followed by recognition by aPL, represents a critical event in the pathogenesis of arterial thrombosis in APS. In this study, we constructed prokaryotic expression vectors encoding the fifth domain of 2GPI (2GPI-DV), a 2GPI-DV mutant, and the Phe280-Ala320 fragment of 2GPI-DV. Following induction and purification, we analyzed the molecular mechanism underlying 2GPI-DV binding to anionic phospholipids. Our results demonstrate that maintaining specific spatial configurations of the Cys281-Cys288 and Ser311-Lys317 segments in 2GPI-DV is a prerequisite for cardiolipin (CL) binding, with the Cys245-Cys296 and Cys288-Cys326 disulfide bonds playing crucial roles in stabilizing these conformations. Building upon these findings, we further examined the ability of biologically active rDV to bind oxLDL and oxLDL present in APS patient serum, confirming that rDV exhibits biological activity consistent with native 2GPI. The successful production of r 2GPI-DV and the establishment of a binding assay system for oxLDL detection lay the foundation for early laboratory diagnosis of APS.

Keywords: Antiphospholipid syndrome; Beta-2-glycoprotein I; Cardiolipin; Oxidized low-density lipoprotein; Prokaryotic expression

Introduction

Antiphospholipid syndrome (APS) is a multi-organ autoimmune disorder characterized clinically by arterial and venous thrombosis and spontaneous abortion [1]. Serologically, APS is defined by the presence of high titers of antiphospholipid antibodies (aPL). Research has revealed that the target antigens for aPL are not phospholipids themselves but rather a broad class of phospholipid-binding proteins, among which 2-glycoprotein I (2GPI) represents the primary antigen [2, 3].

2GPI is synthesized primarily in the liver but can also be expressed in other cell types. The mature human 2GPI has a molecular weight of approximately 50 kDa and consists of 326 amino acid residues organized into five domains (I, II, III, IV, and V) from the N-terminus to the C-terminus. The first four domains are highly homologous, each containing approximately 60 amino acid residues with four cysteine residues that form two disulfide bonds. In contrast, the fifth domain (C-terminus) has a distinct structure, comprising 82 amino acids with six cysteine residues forming three disulfide bonds: Cys245-Cys296, Cys281-Cys306, and Cys288-Cys326. Studies have identified two key regions in 2GPI-DV involved in binding negatively charged substances such as cardiolipin,

heparin, and anionic phospholipids: the C-terminally located positively charged Cys281-Lys-Asn-Lys-Glu-Lys-Lys-Cys288 region [4-7] and a hydrophobic loop formed by seven amino acids from Ser311 to Lys317 [8-10].

Notably, APS patients exhibit elevated serum levels of oxLDL. When 2GPI binds to anionic phospholipids on oxLDL through its DV domain, cryptic epitopes hidden in domain I [13] or domain IV [14] become exposed and are subsequently recognized by anti- 2GPI antibodies [15]. This forms a 2GPI/oxLDL/anti- 2GPI antibody ternary complex that is phagocytosed by macrophages, leading to foam cell formation—a key event in APS-associated arterial thrombosis [16]. Consequently, serum oxLDL levels are considered a major risk factor for arterial thrombosis in APS patients [17].

In this study, we constructed prokaryotic expression vectors encoding 2GPI-DV, a 2GPI-DV mutant, and the Phe280-Ala320 fragment of 2GPI-DV. Following induction and purification, we elucidated the molecular mechanism of 2GPI-DV binding to the anionic phospholipid cardiolipin (CL). Based on these findings, we obtained recombinant 2GPI-DV (r 2GPI-DV) with biological activity for binding anionic phospholipids, establishing a foundation for detecting oxLDL levels in APS patient serum and ultimately enabling clinical laboratory diagnosis of APS.

Materials and Methods

1.1 Materials The human 2GPI T-vector (CTA727-1), JM109 cloning strain, and Rosetta-gami (DE3) expression strain were maintained in our laboratory. OxLig-1 was synthesized and stored in our laboratory. The pET-32a-c(+) expression vector was purchased from Novagen. DNA gel extraction kits, plasmid extraction kits, isopropyl -D-1-thiogalactopyranoside (IPTG), tryptone, yeast extract, and agar were obtained from Bio Basic. LA Taq polymerase, DNA ligation kits, T-vector (TaKaRa PMD19-T simple vector), restriction enzymes EcoRI and KpnI, DNA Marker DL 2000, and Hind III digest DNA Marker were purchased from TaKaRa. ECL detection reagents and HisTrap affinity columns were from GE Healthcare. Anti-His antibody was from Tiangen Biotech. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Santa Cruz. Cardiolipin was from Sigma Chemical. Human 2GPI was from Yamasa, Japan. PCR primers and DNA sequencing services were provided by TaKaRa. Anti- 2GPI antibody was a gift from Professor Eiji Matsuura (Okayama University, Japan).

1.2 Methods

1.2.1 Gene Cloning of 2GPI-DV, 2GPI-DV Mutant, and 2GPI-DV Phe280-Ala320 Fragment Based on the 2GPI cDNA sequence published

in GenBank, primers were designed using Primer5.0 software following standard PCR primer design principles.

(1) 2GPI-DV primer design: - Forward primer (Primer 1, 45 bp): 5' -CGG GGTACC GACGACGACGAC GGACGGACCTGTCCCAAGCCAGAT-3'- Reverse primer (Primer 2, 32 bp): 5'-CG GAATTC TTAGCATGGCTTTACATCGGATGC-3'

Primer 1 contains a KpnI site, protective bases, an enterokinase (EK) cleavage site, and the 5' coding sequence of 2GPI at its 5' end. Primer 2 contains an EcoRI site, protective bases, and the complementary sequence of the 3' coding region of 2GPI at its 5' end.

(2) 2GPI-DV mutant (mDV) primer design: - Forward primer (Primer 3): 5'-GTCATCGGCCATGGGACGACGACGACGCATCTggcAAAGTACCTGTG-3'- Reverse primer (Primer 4): 5'-AGCTCACGGGATCCTTAggcTGGCTTTACATCGG-3'

Primer 3 contains an NcoI site, protective bases, an EK cleavage site, and the 5' coding sequence of 2GPI-mDV (with lowercase letters indicating mutation sites). Primer 4 contains a BamHI site, protective bases, and the complementary sequence of the 3' coding region of 2GPI-mDV (with lowercase letters indicating mutation sites).

(3) 2GPI-DV anionic phospholipid-binding active center fragment (cDV) primer design: - Forward primer (Primer 5, 52 bp): 5' -CATGCCATGGGACGACGACGACTTCTGCAAAAATAAGGAAAAGAAGTGTAGC-3'- Reverse primer (Primer 6, 40 bp): 5'-CGGGATCCGCCTGAGCCACCTGCATCAGTTTTCCAAAAAG-3'

Primer 5 contains an NcoI site, protective bases, an EK cleavage site, and the 5' coding sequence of 2GPI-cDV. Primer 6 contains a BamHI site, protective bases, and the complementary sequence of the 3' coding region of 2GPI-cDV.

Using the human 2GPI T-vector maintained in our laboratory as template, PCR was performed with primer pairs 1/2 and 5/6. PCR conditions were: 94°C for 1 min; 30 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 30 s; final extension at 72°C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis, purified using a gel extraction kit, and ligated into pMD19-T simple vector. The ligation products were transformed into JM109 competent cells, selected on ampicillin (50 g/mL) plates, and positive clones were identified by PCR. Plasmids were extracted from positive clones and sequenced by TaKaRa. Sequences were aligned with human 2GPI in GenBank using Sequencher software. Correct recombinant plasmids were designated pMD19T-DV, pMD19T-mDV, and pMD19T-cDV.

1.2.2 Construction and Identification of Recombinant Expression Vectors Recombinant plasmids pMD19T-DV, pMD19T-mDV, and pMD19T-cDV were amplified in E. coli JM109. Plasmid DNA and pET32a-c(+) vector were

double-digested with EcoRI/KpnI (for DV) or NcoI/BamHI (for mDV and cDV). Digested DV, mDV, and cDV fragments were recovered and ligated using a TaKaRa ligation kit at 16°C for 1 h. Ligation products were transformed into JM109 competent cells and plated on ampicillin (50 g/mL) agar. After 16 h incubation, positive colonies were selected for colony PCR, expanded, and plasmids were extracted for double-digestion verification with EcoRI/KpnI or NcoI/BamHI.

1.2.3 Induction and Purification of rDV, rmDV, and rcDV Proteins in E. coli Verified expression vectors were transformed into Rosetta-gami competent cells and plated on LB agar containing tetracycline (12.5 g/mL), ampicillin (50 g/mL), and kanamycin (15 g/mL). After overnight culture at 37°C, positive colonies were expanded to OD600nm 0.5, induced with 0.5 mmol/L IPTG, and cultured at 37°C for 6 h. Cells were harvested by centrifugation at 6,000 r/min for 10 min, lysed by sonication on ice, and centrifuged at 4°C, 10,000 r/min for 10 min. The supernatant was filtered through a 0.45 m membrane and purified using a nickel affinity column.

1.2.4 Western Blot Analysis of Expression Products Purified rDV, rmDV, and rcDV proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% BSA for 1 h. After five washes with TBST, membranes were incubated overnight at 4°C with anti-His antibody on a rocking platform. Following washing, HRP-conjugated goat anti-mouse secondary antibody was applied for 1 h. After final washes, ECL detection was performed with a 5-min exposure in a darkroom, and images were captured using a gel imaging system.

1.2.5 HPTLC Detection of Recombinant Protein Binding to CL Cardiolipin (50 g/plate) was spotted on silica plates and developed with chloroform:methanol (8:1). Plates were blocked with 1% gelatin at 37°C for 1 h, then incubated with rDV, rmDV, or rcDV at 37°C for 1 h. After incubation with anti-His primary antibody (37°C, 1 h) and HRP-conjugated goat anti-mouse secondary antibody (37°C, 1 h), plates were washed with PBST and developed for 10 min using a solution containing 5 mL 4-methoxy-1-naphthol in methanol (3 mg/mL), 25 mL Tris-HCl (pH 7.4), and 3.5 L 30% H2O2.

1.2.6 ELISA Detection of Recombinant Protein Binding to CL Microtiter plates were coated with CL (50 g/mL, 50 L/well) and blocked with 1% gelatin at 37°C for 1 h. After washing three times with PBST (200 L/well), plates were incubated with rDV, rmDV, or rcDV (50 g/mL, 50 L/well) at 37°C for 1 h. Following washing, anti-His antibody (1:1,000 dilution) was added at 37°C for 1 h, then HRP-conjugated goat anti-mouse secondary antibody (1:1,000 dilution) at 37°C for 1 h. After washing, OPD substrate was added and absorbance was measured at 492 nm using a microplate reader.

1.2.7 ELISA Detection of r 2GPI-DV Binding to oxLDL and oxLDL in APS Patient Serum Microtiter plates were coated with r 2GPI-DV protein or natural 2GPI protein (n 2GPI, 100 g/mL, 50 L/well), dried with a cold hair dryer, and blocked with 1% gelatin (100 L/well) at 37°C for 1 h. After four washes with PBST (200 L/well), plates were incubated at 37°C for 1 h with oxLDL, LDL, or APS patient serum (50 g/mL, 50 L/well). Following washing, anti-apoB antibody (50 L/well) was added at 37°C for 1 h, then HRP-conjugated goat anti-mouse secondary antibody (1:1,000, 50 L/well) at 37°C for 1 h. After extensive washing with PBST, OPD substrate was added and absorbance was read at 492 nm.

Results

2.1 Gene Cloning of 2GPI-DV, 2GPI-DV Mutant, and 2GPI-DV Anionic Phospholipid-Binding Fragment PCR products for rDV, rmDV, and rcDV were analyzed by agarose gel electrophoresis, showing bands consistent with the expected sizes from GenBank [Figure 1: see original paper]. The target genes were cloned into pMD19-T simple vector to generate pMD19T-DV, pMD19T-mDV, and pMD19T-cDV. Plasmids were extracted and sequenced by TaKaRa. Sequence alignment using Sequencher software confirmed that the target fragments contained no mutations.

2.2 Construction and Identification of Recombinant Expression Vectors pET32a-DV, pET32a-mDV, and pET32a-cDV pMD19T-DV was double-digested with KpnI and EcoRI, while pMD19T-mDV and pMD19T-cDV were digested with NcoI and BamHI to obtain the target gene fragments. These were ligated into similarly digested pET32a-c(+) expression vector. The resulting expression vectors pET32a-DV, pET32a-mDV, and pET32a-cDV were transformed into JM109 cloning cells and plated on ampicillin-containing medium. Positive clones were selected, plasmids were extracted, and double-digestion with KpnI/EcoRI or NcoI/BamHI confirmed correct insertion of the target fragments into pET-32a-c(+) [Figure 2: see original paper].

2.3 Induction and Purification of rDV, rmDV, and rcDV Proteins in E. coli Expression vectors pET32a-DV, pET32a-mDV, and pET32a-cDV were transformed into Rosetta-gami expression cells. After IPTG induction, whole-cell protein SDS-PAGE analysis revealed prominent bands at approximately 29 kDa, 29 kDa, and 26 kDa [Figure 3: see original paper], indicating successful expression of rDV, rmDV, and rcDV proteins in E. coli. All three proteins were expressed in soluble form. Cells were harvested, sonicated, and centrifuged, and the supernatant was filtered and purified using nickel affinity chromatography. SDS-PAGE analysis of purified proteins showed removal of most contaminating proteins, with purity exceeding 90% as determined by Bandscan software [Figure 4: see original paper].

2.4 Western Blot Analysis of rDV, rmDV, and rcDV Proteins Western blot analysis using anti-His antibody demonstrated specific binding to His-tagged rDV, rmDV, and rcDV proteins, with bands appearing at the expected molecular weights [Figure 5: see original paper].

2.5 HPTLC Analysis of rDV, rmDV, and rcDV Protein Binding to CL Using n 2GPI as a positive control, HPTLC analysis revealed that rDV, like n 2GPI, produced specific spots at the same position, whereas rmDV and rcDV showed no spots [Figure 6: see original paper]A. This confirms that rDV protein can specifically bind CL and possesses biological activity, while rmDV and rcDV lack this activity.

2.6 ELISA Analysis of rDV, rmDV, and rcDV Protein Binding to CL ELISA analysis using n 2GPI as a positive control demonstrated that rDV could specifically bind CL, whereas rmDV and rcDV did not exhibit CL-binding activity and [Figure 6: see original paper]B.

2.7 ELISA Analysis of r 2GPI-DV Protein Binding to oxLDL and oxLDL in APS Patient Serum Having established that rDV, but not its mutants or short peptides, binds to the phospholipid CL, we next investigated whether rDV could bind oxLDL and oxLDL present in APS patient serum. Microtiter plates coated with n 2GPI/r 2GPI-DV were incubated with Cu^2 -oxidized oxLDL, native LDL, or APS patient serum. After washing, anti-apoB antibody was added, followed by OPD development. The results showed that r 2GPI-DV could bind Cu^2 -oxidized oxLDL but not LDL and [Figure 7: see original paper]A. Furthermore, r 2GPI-DV, like n 2GPI, could bind oxLDL in patient serum and [Figure 7: see original paper]B. These data demonstrate that r 2GPI-DV protein possesses biological binding activity consistent with native 2GPI.

Discussion

2GPI has a molecular weight of approximately 50 kDa and consists of 326 amino acid residues. X-ray crystallography analysis reveals that 2GPI comprises five short consensus repeat (SCR) domains arranged in a fishhook-like structure, with the fifth SCR domain being structurally distinct from the first four. Domains I-IV each contain approximately 60 amino acid residues with four cysteines forming two disulfide bonds. Domains I, III, and IV contain seven -sheets, domain II has six, while domain V comprises 82 amino acids with three disulfide bonds and four -sheets. This domain plays a crucial role in 2GPI binding to negatively charged substances.

Two regions in 2GPI-DV are thought to cooperate in binding anionic phospholipids. The first is the Cys281-Lys-Asn-Lys-Glu-Lys-Lys-Cys288 region, which

readily binds negatively charged molecules due to its high positive charge density [18,19]. The second is the Ser311-Lys317 region, where the Leu313-Ala-Phe-Trp316 segment contains four highly conserved hydrophobic amino acids forming a hydrophobic loop that facilitates lipid binding [20,21].

2GPI binding to anionic phospholipids through its fifth domain is fundamental to both its physiological functions and pathological involvement. To elucidate the molecular mechanism of this interaction and deepen our understanding of 2GPI function, we first expressed and purified 2GPI-DV (Ser244-Cys326) using a prokaryotic expression system. HPTLC and ELISA analyses confirmed that the purified DV recombinant protein possessed biological activity for binding to the anionic phospholipid CL. To identify the minimal functional peptide capable of CL binding, we designed primers to amplify a peptide fragment containing both the C281-C288 and Ser311-Lys317 functional regions—the 40-amino-acid Phe280-Ala320 fragment. After construction, expression, and purification, this fragment (rcDV) showed no CL-binding activity. Analysis revealed that 2GPI-DV contains three disulfide bonds (Cys245-Cys296, Cys281-Cys306, Cys288-Cys326), while our short peptide fragment (cDV) disrupted proper formation of the Cys245-Cys296 and Cys288-Cys326 disulfide bonds, preventing the Cys281-Cys288 and Ser311-Lys317 regions from maintaining their required spatial configuration. We hypothesized that proper spatial conformation of these functional segments is a prerequisite for anionic phospholipid binding. To test this, we mutated the cysteines essential for disulfide bond formation (Cys245 and Cys326) to glycine, then expressed and purified this mutant (mDV). As predicted, rmDV lost CL-binding activity, confirming that maintaining the spatial configuration of the Cys281-Cys288 and Ser311-Lys317 segments is essential for CL binding, with the Cys245-Cys296 and Cys288-Cys326 disulfide bonds playing important roles in stabilizing these conformations.

Having established the molecular mechanism of rDV binding to anionic phospholipids and demonstrated that rDV (but not its short peptide or mutant) binds CL, we next examined whether r 2GPI-DV could bind oxLDL. Using Cu^2 -oxidized oxLDL and native LDL as controls, ELISA confirmed that r 2GPI-DV exhibits oxLDL-binding activity similar to native 2GPI. Since APS patients have elevated serum oxLDL levels, detecting 2GPI-DV-oxLDL binding could serve as a diagnostic method. We tested serum from three APS patients with arterial thrombosis, and ELISA demonstrated that r 2GPI-DV could detect oxLDL in APS serum, consistent with n 2GPI.

In summary, this study elucidated the molecular mechanism of rDV binding to anionic phospholipids and obtained a recombinant domain with biological activity for binding anionic phospholipids, establishing a foundation for detecting oxLDL levels in APS patient serum and enabling early laboratory diagnosis of APS.

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