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Plasma gelsolin facilitates interaction between β_2 glycoprotein I and $\alpha_5\beta_1$ integrin

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Abstract

Antiphospholipid syndrome (APS) is characterized by thrombosis and the presence of antiphospholipid antibodies (aPL) that directly recognizes plasma β_2-glycoprotein I (β_2GPI). Tissue factor (TF), the major initiator of the extrinsic coagulation system, is induced on monocytes by aPL *in vitro*, explaining in part the pathophysiology in APS. We previously reported that the mitogen-activated protein kinase (MAPK) pathway plays an important role in aPL-induced TF expression on monocytes. In this study, we identified plasma gelsolin as a protein associated with β_2GPI by using immunoaffinity chromatography and mass spectrometric analysis. An *in vivo* binding assay showed that endogenous β_2GPI interacts with plasma gelsolin, which binds to integrin $\alpha_5\beta_1$ through fibronectin. The tethering of β_2GPI to monoclonal anti-β_2GPI autoantibody on the cell surface was enhanced in the presence of plasma gelsolin. Immunoblot analysis demonstrated that p38 MAPK protein was phosphorylated by monoclonal anti-β_2GPI antibody treatment, and its phosphorylation was attenuated in the presence of anti-integrin $\alpha_5\beta_1$ antibody. Furthermore, focal adhesion kinase, a downstream molecule of the fibronectin-integrin signalling pathway, was phosphorylated by anti-β_2GPI antibody treatment. These results indicate that molecules including gelsolin and integrin are involved in the anti-β_2GPI antibody-induced MAPK pathway on monocytes and that integrin is a possible therapeutic target to modify a prothrombotic state in patients with APS.

Keywords: β_2GPI • gelsolin • integrin • TF • APS

Introduction

Antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of antiphospholipid antibodies (aPL). Although the original concept of aPL considers that those antibodies were directed against anionic phospholipids, evidence shows that phospholipid-binding plasma proteins such as β_2-glycoprotein I (β_2GPI) [1–3] and prothrombin [4] are the dominant antigenic targets recognized by aPL in patients with APS.

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Among the aPL found in patients with APS, antibodies directing to cardiolipin-β_2GPI complex (aCL/β_2GPI), also called anticardiolipin antibodies or anti-β_2GPI antibodies, have been the best studied in their clinical or biological properties in the last two decades [5]. β_2GPI is a single-chain glycoprotein containing 326 amino acids and contains a high proportion of proline and cysteine residues and is heavily glycosylated [6]. β_2GPI is a member of the complement control protein repeat or short consensus repeat (SCR) superfamily and is composed of five homologous motifs of approximately 60 amino acids designated as SCR or as sushi domains. Each motif contains four conserved half cysteine residues, related to the formation of two internal disulphide bridges. While the first four domains are typical, the fifth domain of β_2GPI is a modified form containing 82 amino acid residues and six half cysteines. The tertiary structure of β_2GPI revealed a highly glycosylated protein with an elongated fishhook-like arrangement

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of the globular SCR domains [7, 8]. β_2GPI binds to solid phase phospholipids through a major phospholipid binding site located in the fifth domain, C281KNKEKKC288 close to the hydrophobic loop [9].

The aCL/β_2GPI recognize the epitopes that appear on β_2GPI only when β_2GPI interacts with anionic phospholipids [10]. The location of the exact epitopic sites for aCL/β_2GPI on β_2GPI molecule has been focus of intensive debate. ACL/β_2GPI have been shown to recognize different epitopes located in all five domains of β_2GPI. Domain IV or I were reported as candidates for major epitopic location by using a series of deletion mutant proteins of β_2GPI [11]. Recently, de Laat *et al.* showed that pathogenic aCL/β_2GPI bind a cryptic epitope on domain I of β_2GPI, which is accessible for aCL/β_2GPI only after conformational change, and is induced by the binding of β_2GPI to a negatively charged surface *via* a positive-charge patch in domain V [12, 13]. Moreover, our group demonstrated that epitopic structures recognized by aCL/β_2GPI are cryptic and that three electrostatic interactions between domain IV and V (D^{193}-K^{246}, D^{222}-K^{317} and E^{228}-K^{308}) are involved in their exposure [14]. This hypothesis is also supported by our previous data showing that replacement of one single amino acid at position 247 of β_2GPI, which is important for the interaction between domain IV and V, can alter the antigenicity of β_2GPI for pathogenic autoantibodies [14, 15].

Recently, great interest has arisen on the binding of aCL/β_2GPI to endothelial cells or other procoagulant cells and how this binding mediates cell dysfunctions that potentially induce the clinical manifestations of the APS. A number of *in vitro* studies have shown that procoagulant cells, treated with aCL/β_2GPI, are activated and express procoagulant molecules such as tissue factor (TF) [16, 17]. Further research has focused on the signal transduction mechanisms implicated in the increased expression of pro-coagulants substances in response to aPL. The adapter molecule myeloid differentiation protein (MyD88)-dependent signalling pathway and the nuclear factor κB (NF-κB) have been involved in endothelial cell activation by aPL [18–21]. We [22] and others [23–26] showed clear evidence that the p38 mitogen-activated protein kinase (MAPK) pathway of cell activation plays an important role in aPL-mediated cell activation. Such cell activation by aCL/β2GPI might require an interaction between β_2GPI and a specific cell surface receptor. The Toll-like receptor (TLR) family may mediate a role in the interaction of the β_2GPI-aCL/β_2GPI complex on the endothelial cell surface [18]. Annexin II, also known as Annexin A2, is an endothelial cell receptor for tPA and plasminogen, and suggested to interact with the β_2GPI-aCL/β_2GPI complex on the endothelial cell surface mediating cell activation [27, 28]. Some members of low-density lipoprotein receptor family, such as LDL-R related protein, megalin, the very-low density lipoprotein receptor, were shown to bind to β_2GPI [29]. However, no evidence has shown a direct interaction between β_2GPI and TLRs. Annexin II does not span the cell membrane thus cannot induce cell activation unless the presence of an unknown 'adaptor' is present. β_2GPI was required to be chemically dimerized to bind to any of LDL receptors [29]. In addition, no information has been available regarding β_2GPI on monocytes. In fact, monocytes are more potent to produce TF compared with endothelium, therefore the investigation of β_2GPI-aCL/β_2GPI interaction on monocytes are essential to explore the pathophysilogy of APS.

In this study, we identified a plasma gelsolin as a novel protein associated with β_2GPI by using affinity purification and liquid chromatography with mass spectrometry (LC-MS) analysis, and we showed functional interaction of plasma gelsolin with β_2GPI.

Materials and methods

Cell culture

RAW264.7 and HEK293T cell lines were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Gibco BRL, Paisley, UK). To remove β_2GPI, the culture medium was changed to serum-free DMEM for 16 hrs before the assay.

Cloning of cDNAs and plasmid construction

The signal sequence region and other region of human β_2GPI cDNA lacking the signal sequence (β_2GPI[ss-]) were amplified by PCR from human B cell cDNA (CLONTECH Laboratories, Inc., Mountain View, CA, USA). The resulting fragment containing β_2GPI(ss-) was ligated into the EcoR I and Sal I sites of p3xFLAG CMV7.1 vector (Sigma Chemical Co.). The fragment containing the human β_2GPI signal sequence was ligated into the BamH I and Pst I sites of pBluescript II SK^+ vector (pBS-Sig) (Stratagene, La Jolla, CA, USA). The β_2GPI(ss-) cDNA fragment with 3xFLAG was ligated into the Pst I and Sal I sites of pBS-Sig. The Sig-3xFLAG-β_2GPI fragment was then ligated into pcDNA3 (Invitrogen, Carlsbad, CA) (pcDNA3-Sig-3xFLAG-β_2GPI) or into pCAG-puro vector which contains a puromycin-resistant gene in pCAGGS vector provided by Dr. J. Miyazaki (Osaka University). The plasma gelsolin cDNA was obtained from ATCC (#MGC-39262, Manassas, VA, USA) and ligated into pcDNA3.

Proteins and antibodies

Recombinant human β_2GPI was purified as described previously [11]. FLAG-β_2GPI was collected from the culture supernatant of HEK293T cells transiently transfected with pcDNA3-Sig-3xFLAG-β_2GPI using FuGENE6 (Roche, Branchburg, NJ). Expression of all constructs was performed in conditioned serum-free Opti-MEM (Gibco BRL). Furthermore, a stable cell line expressing FLAG-β_2GPI was generated by transfection with pCAG-I-puro vector encoding FLAG-tagged-β_2GPI cDNA. The culture supernatant of FLAG-β_2GPI-expressing cells was collected after 4 days of culture and then filtered (0.22 μm). The antibodies used in this study were as follows: mouse monoclonal anti-β_2GPI antibody (WBCAL1; aCL/β_2GPI [30], and MAB1066, Chemicon International Inc., Temecula, CA, USA), mouse monoclonal anti-gelsolin antibody (clone 2, BD Transduction Laboratories, San Jose, CA, USA), rat monoclonal anti-integrin $\alpha_5\beta_1$ antibody (MAB1984, Chemicon International Inc.), mouse monoclonal anti-integrin β_1 antibody (Ha2/5, BD), rabbit polyclonal anti-p38 MAP kinase antibody (#9212, Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit polyclonal anti-phospho-p38

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MAP kinase antibody (#9211, Cell Signaling Technology, Inc.), mouse monoclonal anti-focal adhesion kinase (FAK) antibody (clone 77, BD), mouse monoclonal anti-FAK(pY397)-phospho-specific antibody (clone 18, BD), mouse anti-β-actin (AC15, Sigma Chemical Co.) and mouse monoclonal anti-FLAG (M2) (Sigma Chemical Co.). EZ-Link Sulfo-NHS-Biotin Reagent was used as a biotinylation reagent. IgG with aPL activity was purified from sera of six patients with APS diagnosed by Sapporo criteria. Control IgG was isolated from normal human serum. Protein concentrations were determined by Bradford method. Consent forms for this study were signed by all of the patients and healthy donors.

Purification of the β_2GPI-related proteome

NHS-activated Sepharose 4 Fast Flow (0.5 ml) (Amersham Biosciences AB, Sweden) washed with 100 mM HCl was mixed with 250 μg of anti-FLAG (M2) mAb (250 μg) in coupling buffer (0.2 M $NaHCO_3$-NaCl, pH 8.3), and the mixture was rotated for 2 hrs at room temperature for conjugation. The unconjugated antibody was removed from the resin by washing with 500 mM ethanolamine (pH 8.3) and 0.1 M CH_3COOH (pH 4.0), and the resin was then equilibrated with PBS. RAW264.7 cells were cultured under an atmosphere of 5% CO_2 at 37°C in serum-free DMEM for 16 hrs. After incubation, 5×10^7 cells were collected, suspended with 1 ml of PBS, and incubated for 2 hrs at 4°C with 0.5 ml of culture supernatant (FLAG-β_2GPI) after transfection with pcDNA3-Sig-3xFLAG-β_2GPI. The cells were then washed twice with 1 ml of PBS, suspended with 1 ml of PBS, and incubated for 2 hrs at 4°C after addition of the membrane-impermeable cross-linker 3,3′-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce) was added to a final concentration of 1 mM. Then Tris-HCl (pH 7.5) was added as a stop solution to a final concentration of 10 mM. The cells were then harvested, washed with PBS, lysed in 10 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, aprotinin (10 mg/ml), leupeptin (10 mg/ml), 1 mM PMSF, 400 mM Na_3VO_4, 400 mM EDTA, 10 mM NaF and 10 mM sodium pyrophosphate (buffer A), and centrifuged at 16,000 $\times$ *g* for 10 min. at 4°C. The resulting supernatant was applied to the M2 column of 100 μl bed volume and the column was then washed with buffer A. HA-peptide (Roche) was loaded to the column to remove non-specific binding and then bound proteins were eluted with two volumes of FLAG peptide (Sigma Chemical Co.). The eluents from the column were concentrated by precipitation with TCA and subjected to in-solution digestion for LC-MS/MS analysis.

In-solution digestion of purified proteins

Proteins were precipitated with 10% TFA and washed with acetone twice. Precipitated proteins were dissolved in 100 mM Tris-HCl and 7 M guanidium hydroxide (pH 8.0), diluted with 100 mM Tris-HCl (pH 8.0) to 1 M guanidium hydroxide, and then digested with Lys-C endopeptidase (500 ng) for 16 hrs at 37°C. The resulting peptides were desalted with a C18 disc settled microtip, dried, and dissolved in 0.1% TFA/2% acetonitrile.

MS and database searching

Peptides were analysed by using a quadrupole time of flight hybrid mass spectometer (Q-tof2, Waters) equipped with an Agilent HP1100 nanoflow pump with a laboratory-made nano-spray stage and ESI column. C18 beads (L-column, 3 μm) were packed in the spray tip and used as a nano-ESI column (5 cm in length, 100 μm in id). The sample was loaded to the ESI column at a flow rate of 800 nl/min with mobile phase A (0.1% formic acid/2% acetonitrile) and eluted with a linear gradient of 5 to 35% B (0.1% formic acid/90% acetonitrile) at a flow rate of 200 nl/min. CID spectra were acquired automatically in the data-dependent scan mode in which the two highest peaks were selected for precursor ions. All MS/MS spectra were processed by a MASCOT distiller for generation of peak list files and were subjected to a database search by the MASCOT algorithm (Matrix Science, London) against the non-redundant National Center for Biotechnology Information (nrNCBI) database. Search parameters were set as follows: Lys-C/P was selected as an enzyme allowing one miscleavage, oxidized methionine and pyroglutamine derived from the amino terminus of glutamine were selected as variable modifications, and the mass tolerance was 0.3 Da for precursor ions and 0.3 Da for MS/MS ions.

Transfection, immunoprecipitation and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method or lipofection method. After 48 hrs, the cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000 $\times$ *g* for 10 min. at 4°C, and the resulting supernatant was incubated with antibodies for 2 hrs at 4°C. Protein G-Sepharose (Amersham Biosciences AB) that had been equilibrated with the same solution was added to the mixture, which was then rotated for 1 hr at 4°C. The resin was separated by centrifugation, washed four times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with the primary antibodies with horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin G (1:10,000 dilution, Promega Corporation, Madison, WI, USA) and an enhanced chemiluminescence system (ECL, Amersham Biosciences, Little Chalfont, UK).

Binding assay

The binding between phospholipid-β_2GPI complex and plasma gelsolin was confirmed by an ELISA. Non-irradiated microtitre plates (Sumilon type S, Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of 50 μg/ml cardiolipin (Sigma Chemical Co.) and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of Tris-buffered saline (TBS) containing 1% fatty acid-free bovine serum albumin (BSA, A-6003, Sigma Chemical Co.) and $CaCl_2$ (BSA-Ca). After 3 washes in TBS containing 0.05% Tween 20 (Sigma Chemical Co.) and $CaCl_2$ (TBS-Tween-Ca), 50 μl of 10 μg/ml β_2GPI in BSA-Ca was added to half of the wells in the plates and the same volume of BSA-Ca alone (as a sample blank) was added to the other half of the wells. After 1-hr incubation at 37°C, plates were washed and 50 μl of plasma gelsolin (Sigma Chemical Co.) (0–10 μg/ml) in BSA-Ca was added in duplicate. Plates were incubated for 1 hr at room temperature, followed by incubation with mouse monoclonal anti-gelsolin antibody, alkaline phosphatase conjugated goat antimouse IgG and substrate. The optical density of wells coated with cardiolipin alone was subtracted from that of wells containing cardiolipin-β_2GPI complex. All procedures were done in the presence of 0, 1 or 2 mM $CaCl_2$.

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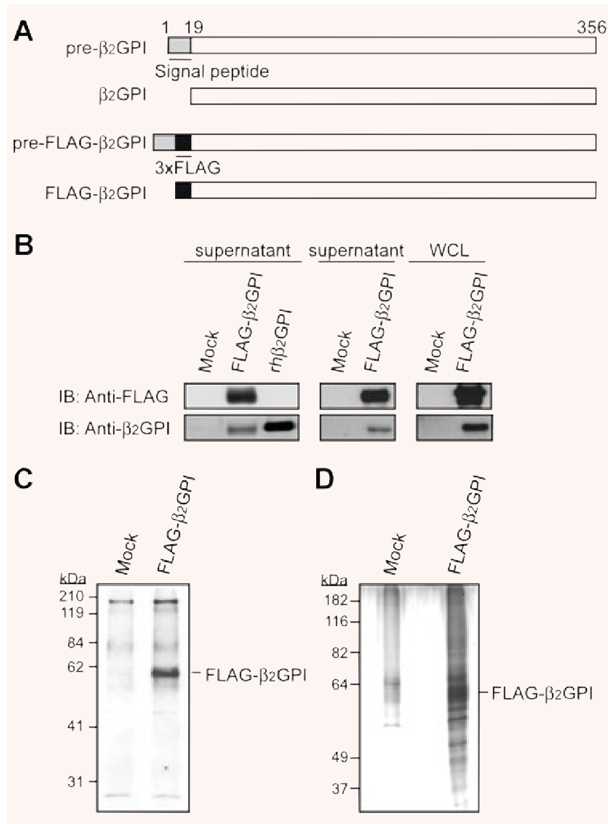

Fig. 1 Immunoaffinity purification of the β_2GPI-related proteome. (**A**) Schematic representation of β_2GPI. Grey box: signal peptide, black box: FLAG-tag. (**B**) Expression of secretory β_2GPI and intracellular β_2GPI. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI. Cells were lysed and subjected to immunoblot analysis with anti-FLAG or anti-β_2GPI antibody. (**C**) Pull-down analysis of biotinylated cell surface proteins binding to FLAG-β_2GPI. RAW264.7 cell surface proteins were biotinylated using EZ-Link Sulfo-NHS-Biotin Reagent and then the cells were cross-linked with FLAG-β_2GPI using 3,3′-Dithiobis(sulfosuccinimidylpropionate). The cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. The resulting precipitates were subjected to SDS-PAGE and visualized with HRP-conjugated streptavidin. (**D**) Silver staining of β_2GPI-associated proteins. The β_2GPI-associated proteins purified by the procedure indicated in (**C**) were detected by silver staining.

Flowcytometric analysis

Surface aCL/β_2GPI and gelsolin binding on RAW264.7 cells was analysed using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with the CellQuest program. RAW264.7 cells were cultured with serum-free DMEM for 16 hrs. The cultured cells were washed with PBS including 2% BSA and 0.1% NaN_3 and treated with 50 μg/ml of β_2GPI at room temperature for 10 min., followed by exposure to primary antibodies for 30 min. on ice. After washing twice, cells were stained with Alexa488-labeled goat antimouse IgG antibody (Invitrogen) for 30 min. on ice. After washing twice, cells were analysed using FACSCalibur.

Luciferase assay

Stable κB luciferase reporter-expressing RAW264.7 cells were inoculated into a 24-well dish at 1×10^6 cells/500 μl of cell culture medium and stimulated as indicated [31]. After stimulation at 37°C for 4 hrs, the cells were harvested and lysed in 50 μl of cell culture lysis reagent (Promega Corporation), and then luciferase activity was measured using 20 μl of lysate and 100 μl of luciferase assay substrate (Promega Corporation). The luminescence was quantified with a luminometer (Berthold Japan, Tokyo, Japan).

Results

Immunoaffinity purification of β_2GPI-associated proteins

To isolate β_2GPI-binding proteins, we constructed FLAG-tagged human β_2GPI (Fig. 1A). Since β_2GPI binds to anionic phospholipids *via* a lysine-rich motif on domain V at the carboxy-terminus, we decided to preserve the intact structure at the carboxy-terminus. β_2GPI is a secretory apolipoprotein that is mainly produced in the liver and secreted to plasma. β_2GPI cDNA encodes a protein of 345 amino acids including a hydrophobic amino-terminal signal sequence (19 amino acid residues) that is lacking in the mature form of β_2GPI. Thus, the FLAG-tag sequence was placed between the signal peptide sequence and mature protein (Fig. 1A). An expression vector encoding FLAG-β_2GPI was transfected into HEK293T cells, and the culture supernatant and whole cell lysate were analysed by immunoblotting using anti-FLAG or anti-β_2GPI antibody. Immunoblot analysis showed that FLAG-β_2GPI was detected by anti-FLAG and anti-β_2GPI antibody from both the cell lysate and culture supernatant (Fig. 1B).

To confirm that β_2GPI binding proteins exist on the cell surface of RAW264.7 cells, we performed a pull-down assay using biotinylated cell surface proteins from RAW264.7 cells and FLAG-tagged β_2GPI. The cell surface proteins of RAW264.7 cells were biotinylated, incubated with FLAG-β_2GPI, and then biotinylated cell surface membrane proteins were chemically cross-linked with FLAG-β_2GPI. Cells were lysed, and the lysates were immunoprecipitated with anti-FLAG antibody to purify biotinylated proteins cross-linked to FLAG-β_2GPI and visualized with HRP-conjugated streptavidin. Smeared proteins other than FLAG-β_2GPI were reproducibly found (Fig. 1C). Next, we performed large-scale immunoaffinity chromatography with an anti-FLAG pull down assay. RAW264.7 cells were cultured with FLAG-β_2GPI and then the lysate of RAW264.7 cells was used for affinity chromatography with anti-FLAG antibody-conjugated Sepharose beads. The purified fraction eluted using FLAG-peptides was subject to SDS-PAGE and detected with silver staining. Silver staining indicated that a large number of smeared proteins interact with β_2GPI (Fig. 1D).

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A

Start-stop	Charge	Sequence	Delta mass	Mascot score
2-10	2	VVEHPEFLK	-0.05	26
11-24	3	AGKEPGLQIWRVEK	-0.08	22
127 - 142	3	HVVPNEVVVQRLFQVK	-0.10	55
534 - 546	2	SGALNSNDAFVLK.	-0.06	58
547 - 564	2	TPSAAYLWVGAGASEAEK	-0.06	20

Fig. 2 Identification of β2GPI-binding protein. (**A**) Identified peptide sequences of gelsolin by MS analysis. Five peptides corresponding to mouse gelsolin were identified. (**B**) Assigned b- or y- ions from amino acid sequence of 127 to 142 of mouse gelsolin are represented. (**C**) MS/MS spectrum of peptide 127 to 142 of mouse gelsolin. Fragment ions corresponding to b- and y- ions from identified sequence (124 to 142) are indicated.

Identification of β2GPI-associated proteins by LC-MS

Proteins immunopurified with anti-FLAG (M2)-conjugated Sepharose were directly digested with Lys-C endopeptidase and analysed by an online-nanoLC-ESI-quadrupole time of flight hybrid mass spectrometer. Obtained MS/MS data were searched against the non-redundant National Center for Biotechnology Information (nrNCBI) database MASCOT algorithm. Many proteins with a significant MASCOT score were identified, but most of them were intracellular proteins such as molecular chaperones and ribosomal proteins that were abundant and seem to be contaminants (Table S1). These proteins were omitted from the list of identified protein, and proteins expressed on the membrane and/or cell surface were approved as candidates for β2GPI-binding protein. Eventually, we found five peptides corresponding to gelsolin, and two of the five peptides had reliable MASCOT scores (more than 31, $P < 0.05$,) (Fig. 2A). The MS/MS data of the peptide (127–142 amino acid residue) with the highest MASCOT score are shown in Fig. 2B and C. Gelsolin has two types of localization pattern: one isoform localizes in the cytosol to regulate formation of actin fibre, whereas the other isoform, which has a signal peptide, localizes outside the cell or on the cell surface. We focused on plasma gelsolin as a β2GPI-binding protein because gelsolin interacts with fibronectin and integrin, which can transduce intracellular signalling in collaboration with several types of kinase such as MAPK and FAK.

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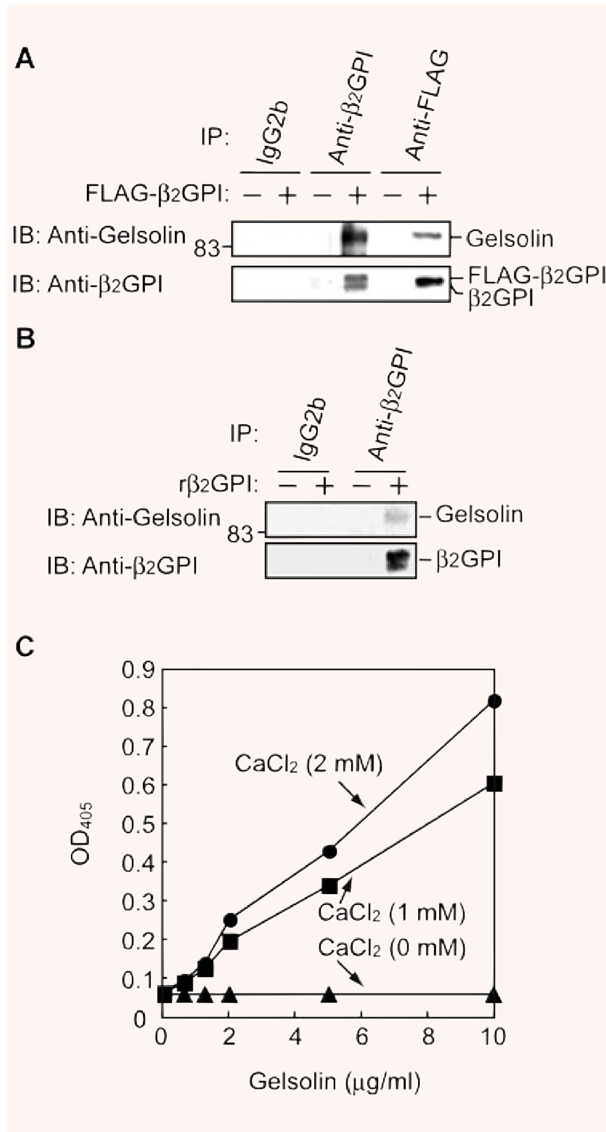

Fig. 3 Biochemical interaction between β_2GPI and plasma gelsolin. (**A**) Interaction between gelsolin and FLAG-β_2GPI. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI and plasma gelsolin. Proteins secreted from transfected cells were subjected to immunoprecipitation with an antibody as indicated, and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-FLAG antibody. (**B**) Interaction between endogenous gelsolin and recombinant human β_2GPI. HEK293T cells were transfected with expression plasmids encoding gelsolin. Gelsolin secreted from transfected cells was mixed with human recombinant β_2GPI and subjected to immunoprecipitation with an antibody as indicated, and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-β_2GPI antibody. (**C**) The binding between phospholipid-bound β_2GPI and plasma gelsolin was confirmed by ELISA as described in the 'Materials and methods' section.

Interaction of β_2GPI with plasma gelsolin

To confirm the binding between β_2GPI and gelsolin, we examined the interaction between β_2GPI and gelsolin using HEK293T cells. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI and plasma gelsolin. The cell lysates were immunoprecipitated with anti-β_2GPI (WBCAL1; monoclonal aCL/β_2GPI) or FLAG antibodies and then immunoblotted with anti-gelsolin or β_2GPI antibodies (MAB1066). Immunoprecipitation and immunoblot analysis revealed that FLAG-tagged β_2GPI specifically interacted with gelsolin (Fig. 3A). To further verify the interaction between recombinant β_2GPI and endogenous gelsolin, we examined the interaction with endogenous gelsolin. The supernatant including endogenous plasma gelsolin secreted from cultured RAW264.7 cells was mixed with human recombinant β_2GPI and subjected to immunoprecipitation with antibodies as indicated (WBCAL1 or Mock), and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-β_2GPI antibody (MAB1066). An *in vitro* pull-down assay showed that plasma gelsolin directly binds to β_2GPI (Fig. 3B). Direct binding of plasma gelsolin to cardiolipin-β_2GPI complex in a calcium-dependent fashion was confirmed by ELISA (Fig. 3C).

Plasma gelsolin enhances the localization of β_2GPI on the cell surface

It has been reported that plasma gelsolin binds to fibronectin, which belongs to the family of extracellular matrix (ECM) proteins and plays important roles in cellular adhesion, proliferation, differentiation and migration [32]. First, we confirmed the expression of β_2GPI (recombinant human β_2GPIs or FLAG-tagged β_2GPIs) on the cell surface by flowcytometric analysis. When RAW264.7 cells were incubated with β_2GPI at 37°C for 1 hr, interaction of β_2GPI on the cell surface was observed (Fig. 4A and B). To determine whether gelsolin affects the expression of β_2GPI on the cell surface, we examined the expression level of β_2GPI on the cell surface. The expression level of β_2GPI on the cell surface was enhanced in the presence of gelsolin compared to that in the absence of gelsolin (Fig. 4C). It has been shown that fibronectin, which binds to gelsolin, associates with the extracellular domain of the integrin family. To determine whether the cell surface expression of β_2GPI depends on integrin, we examined the expression level of β_2GPI with anti-integrin $\alpha_5\beta_1$ antibody as an inhibitory antibody. Anti-integrin $\alpha_5\beta_1$ antibody inhibited the expression of β_2GPI on the surface of RAW264.7 cells (Fig. 4D and E). Furthermore, to determine whether anti-integrin $\alpha_5\beta_1$ antibody affects the expression of β_2GPI without addition of recombinant β_2GPI, RAW264.7 cells cultured with serum-free medium were incubated with anti-integrin $\alpha_5\beta_1$ antibody, and then the cells were stained by aCL/β_2GPI. Flowcytometric analysis showed that without addition of recombinant β_2GPI, anti-integrin $\alpha_5\beta_1$ antibody does not affect the staining by anti-β_2GPI antibody (WBCAL1) (Fig. 4F). These findings indicate that gelsolin

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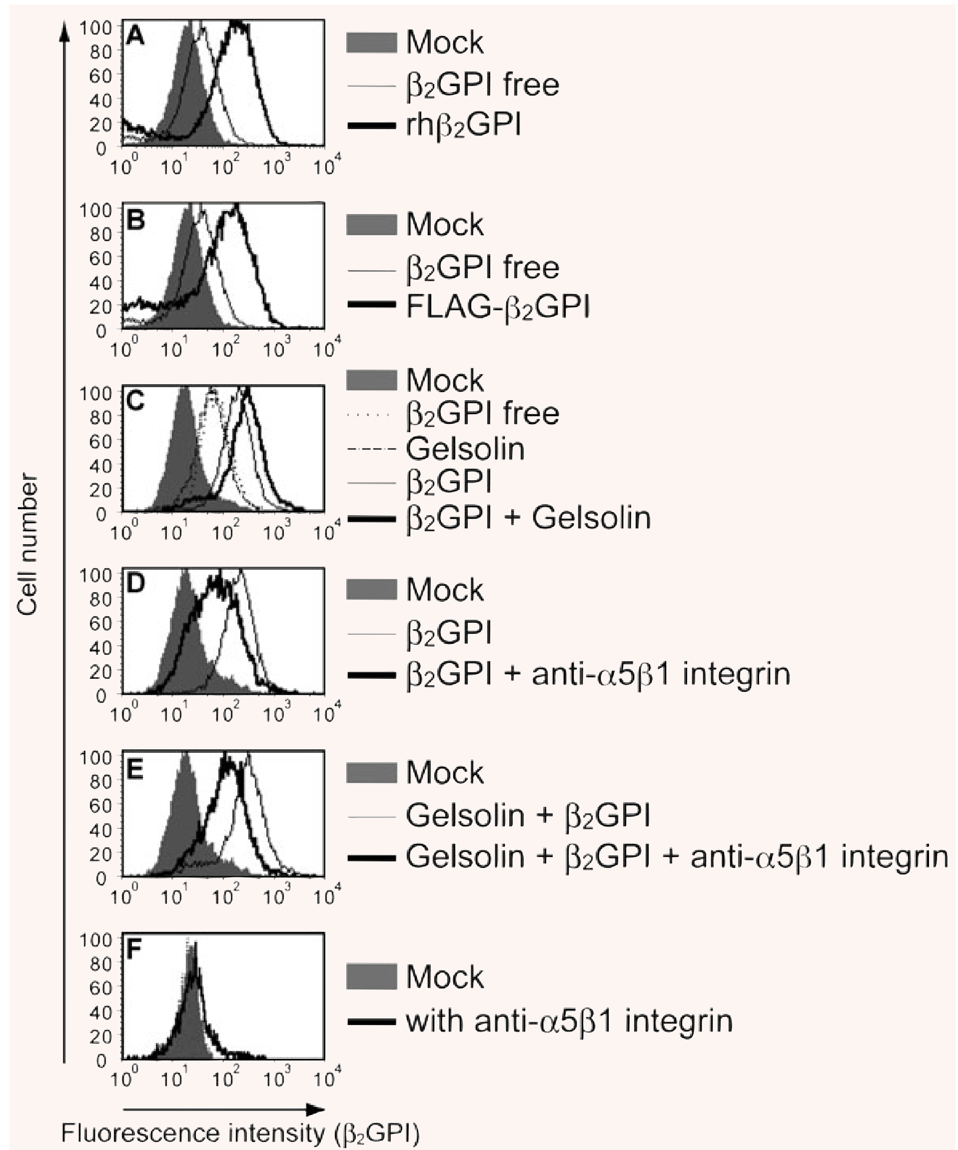

Fig. 4 Interaction of β_2GPI and plasma gelsolin on cell surface. (**A** and **B**) β_2GPI binding to the cell surface. RAW264.7 cells cultured with serum-free medium were incubated with or without recombinant human β_2GPI or FLAG-β_2GPI, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI (WBCAL1). Mock has no primary antibody. Binding to the cell surface by recombinant human β_2GPI (**A**) showed almost the same intensity as that by secreted FLAG-β_2GPI (**B**). (**C**) Gelsolin affects the binding of β_2GPI to the cell surface. RAW264.7 cells were incubated with or without recombinant FLAG-β_2GPI and gelsolin, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI. (**D** and **E**) Binding of β_2GPI to the cell surface was inhibited by anti-integrin $\alpha_5\beta_1$ antibody. RAW264.7 cells were incubated with or without recombinant FLAG-β_2GPI, gelsolin and anti-integrin $\alpha_5\beta_1$ antibody, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI. **(F)** RAW264.7 cells cultured with serum-free medium were incubated with anti-integrin $\alpha_5\beta_1$ antibody, and then the cells were stained by aCL/β_2GPI.

enhanced the cell surface expression of β_2GPI and that the interaction is mediated by integrin on the cell surface.

Intracellular signalling *via* aCL/β_2GPI antibody is dependent on integrin $\alpha_5\beta_1$

We previously reported that p38-MAPK was phosphorylated in RAW264.7 cells stimulated by human monoclonal aCL/β2GPI [22]. To determine whether a cell surface complex including gelsolin activates RAW264.7 cells, we investigated the phosphorylation of p38-MAPK. Stimulation to RAW264.7 cells by aCL (WBCAL1) showed that p38-MAPK phosphorylation was not induced by plasma gelsolin alone but was induced by aCL/β_2GPI stimulation and was further enhanced by plasma gelsolin plus aCL/β_2GPI stimulation (Fig. 5A). However, anti-integrin $\alpha_5\beta_1$ antibody attenuated phosphorylation of p38-MAPK by plasma gelsolin plus aCL/β_2GPI stimulation (Fig. 5A). These findings indicate that aCL/β_2GPI caused phosphorylation of p38-MAPK in collaboration with gelsolin and integrin on the cell surface. Furthermore, to determine the effect on downstream molecules such as focal adhesion kinase FAK, the phosphorylation of FAK by aCL/β_2GPI was investigated. Stimulation of aCL/β_2GPI and plasma gelsolin resulted in an increased level of phosphorylation of FAK, whereas anti-integrin $\alpha_5\beta_1$ antibody attenuated the phosphorylation of FAK (Fig. 5B). Taken together, the results suggest that anti-β_2GPI

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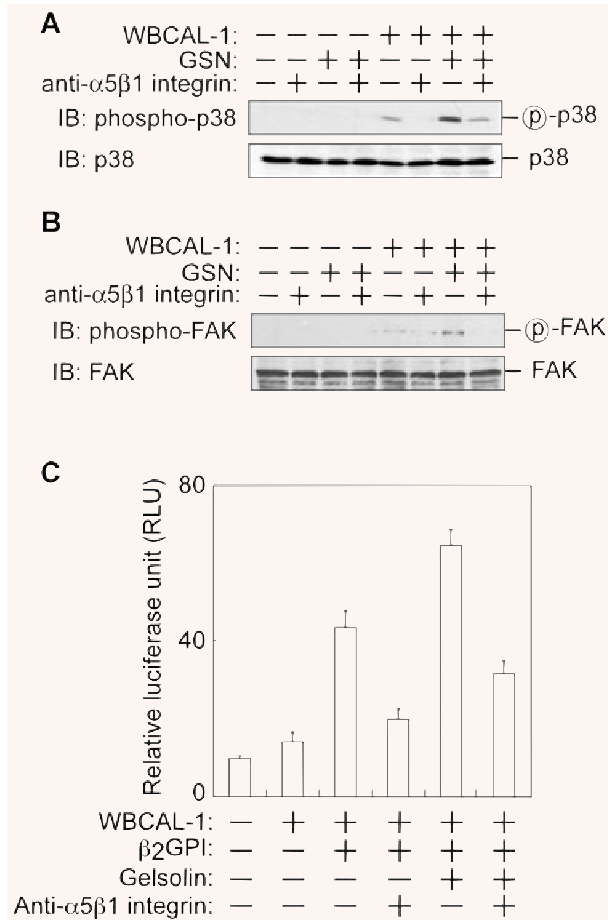

Fig. 5 Change in intracellular signalling by gelsolin and aCL/β_2GPI. (**A**) Gelsolin and integrin affect phosphorylation of p38 MAPK by anti-β_2GPI antibody. RAW264.7 cells were incubated for 2 hrs as indicated after serum-free culture for 16 hrs and then stimulated with aCL/β_2GPI (WBCAL1) for 30 min., and then phosphorylation of p38 MAPK was determined by immunoblot analysis using specific antibodies against total-p38 and phospho-p38. (**B**) Anti-integrin $\alpha_5\beta_1$ antibody inhibits phosphorylation of FAK by aCL/β_2GPI. RAW264.7 cells were stimulated with aCL/β_2GPI for 10 min. and then phosphorylaion of FAK was determined by immunoblot analysis. (**C**) aCL/β_2GPI increases NF-κB activity. RAW264.7 cells stably expressing κB luciferase reporter were inoculated into a 24-well dish and stimulated as indicated. After stimulation at 37°C for 4 hrs, κB luciferase activity was measured.

antibody affects the integrin signalling including its downstream signal molecule FAK, followed by activation of p38-MAPK.

It has been reported that the p38-MAPK pathway is linked to the NF-κB pathway [33]. To determine whether aCL/β_2GPI antibody functions with gelsolin, we examined its effect on relative luciferase activity by NF-κB. The transcriptional activity of NF-κB was further increased by stimulation with the combination of gelsolin and one of the aCL/β_2GPI antibodies, WBCAL1, whereas it was inhibited by anti-integrin $\alpha_5\beta_1$ antibody (Fig. 5C). These findings indicate that aCL/β_2GPI causes the engagement of integrin with gelsolin, resulting in activation of the p38-MAPK pathway and NF-κB pathway.

Discussion

We identified plasma gelsolin as a novel β_2GPI-binding protein on the cell surface of monocytes. Affinity purification using an anti-FLAG-β_2GPI-conjugated column clarified that β_2GPI interacts with gelsolin on monocytes, and then the binding of β_2GPI with gelsolin was confirmed by immunoprecipitation. Moreover, flowcytometric analysis demonstrated that gelsolin enhances the affinity of β_2GPI on the cell surface. Gelsolin is expressed as two isoforms, cytoplasmic gelsolin and plasma gelsolin, which are encoded by a single gene and produced by alternative translation [34]. Both gene products of gelsolin have six homologous repeats (S1–S6), each of which contains 120–130 amino acid residues, and plasma gelsolin has an extra 23 amino acid residues at the amino-terminus [35, 36]. Cytoplasmic gelsolin is known as an actin-depolymerizing factor and plays a crucial in removal of actin released by tissue injury. Plasma gelsolin has another function as a carrier protein for bioactive mediators such as lysophosphatidic acid (LPA), lipopolysaccharide (LPS), amyloid β protein (Aβ) and platelet-activating factor (PAF) to protect cells from exposure to excess stimulation [37–41]. Plasma gelsolin also interacts with fibronectin and especially colocalizes at a region where inflammation arises [32]. Fibronectin forms a dimeric glycoprotein in plasma and a dimeric or multimeric form that interacts with integrin $\alpha_5\beta_1$ on the cell surface. Fibronectin is involved in cell adhesion, morphological change and migration processes, including wound healing, blood coagulation, host defense and metastasis.

Integrins are heterodimeric membrane proteins composed of an α chain and a β chain. Each specific integrin induces a variety of responses in different cell types. Integrin α5 chain undergoes post-translational cleavage to yield disulfide-linked light and heavy chains that join with the β_1 chain to form a fibronectin receptor [42]. Integrins provide dynamic, physical links between the ECM such as fibronectin and cytoskeletons. In addition to adhesion, integrins are known to participate in cell surface-mediated signalling in concert with other cell surface receptors, including growth factor receptors such as epidermal growth factor, lysophosphatidic acid or thrombin, and are involved in proliferation, survival, morphological change, migration and gene expression. Ligation of ECM to integrins triggers assembly of cytoskeleton proteins (such as tallin, actin and paxillin) and intracellular tyrosine kinase FAK and results in a large variety of signal transduction events. Integrin-mediated signals is likely to be necessary in normal cells, such as human umbilical vein endothelial cells or mammary epithelial cells, to block apoptosis *via* the Akt pathway and activate cells *via* the MAPK pathway [43–45]. In

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Ntera2 neuronal cells, $\alpha_5\beta_1$-mediated adhesion to fibronectin decreased apoptosis. Previous studies have shown that expression of $\alpha_5\beta_1$ promotes apoptosis of human hematopoietic cell lines, monocyte-differentiated HL-60 cell lines and mouse macrophage RAW264.7 cell lines [46–48]. However, it has been reported that fibronectin could not mediate the binding of β_2GPI to the cell surface in endothelial cells [27, 49]. We confirmed direct interaction of phospholipid-bound β_2GPI and gelsolin by ELISA, and the binding was found only in the presence of calcium. The interaction of β_2GPI with gelsolin in our study suggests that engagement of β_2GPI by anti-β_2GPI antibody caused the complex formation including gelsolin, fibronectin and integrin $\alpha_5\beta_1$, followed by activation of the p38-MAPK pathway and NF-κB pathway.

Zeisel *et al*. reported that FAK and myeloid differentiation protein 88 (MyD88) pathways were inter-linked and initiate a pro-inflammatory response through NF-κB activation [50]. In a previous study, we demonstrated that the p38 MAPK-dependent signalling pathway participates in aPL-mediated TF expression. A specific inhibitor of p38 MAPK decreased TF mRNA expression induced by aCL/β_2GPI stimulation, indicating a crucial role of the p38 MAPK pathway in APS. Raschi *et al*. reported that a dominant-negative form of TNF-receptor-associated factor 6 (TRAF6) and MyD88 abrogated NF-κB activation induced by monoclonal aCL/β_2GPI, suggesting that aCL/β_2GPI reacts to β_2GPI associated with a member of the TLR or interleukin-1 receptor family. The present study demonstrated that gelsolin is a scaffolding protein that links β_2GPI and integrin/fibronectin and that integrin is also important for activation of the p38 MAPK and NF-κB pathways by aCL/β_2GPI.

Several inhibitors for integrins have been developed and investigated in animal models of inflammatory diseases, and some of these inhibitors (*e.g.* anti-integrin $\alpha IIb\beta_3$, anti-integrin $\alpha_4\beta_7$) are used clinically as anti-platelet agents or anti-inflammatory bowel disease agents [51]. Recently, arginine-glycine-aspartic acid (RGD) peptides that bind to integrin $\alpha v\beta_3$, $\alpha v\beta_5$ or $\alpha_5\beta_1$ have become available for the treatment of inflammatory arthritis [52]. This is a first report to prove how the β_2GPI-aCL/β_2GPI interaction on monocyte surface occurs with its partner molecule, gelsolin. Despite the fact that we could not show direct procoagulant alteration of cells by aCL/β_2GPI with integrin $\alpha_5\beta_1$ blockade in this study, either on monocytes or on endothelial cells, our findings provide a clue for establishing specific treatments by down-regulating the p38 MAPK pathway *via* integrin $\alpha_5\beta_1$ and therapeutic benefits for patients with autoimmmune diseases, including APS.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Peptide sequences of β_2 GPI-associated proteins identified by MS analysis.

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