β_2-Glycoprotein I Is Proteolytically Cleaved *In Vivo* upon Activation of Fibrinolysis

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Summary

β_2-glycoprotein I (β_2GPI) is a plasma glycoprotein with unknown physiological function(s). In *in vitro* experiments it has been demonstrated that β_2GPI has both anticoagulant properties, such as the inhibition of factor X and prothrombin activation and procoagulant properties, such as the inhibition of the anticoagulant activity of activated protein C. Besides this, β_2GPI bound to cardiolipin is recognized by antiphospholipid antibodies (aPL).

In this study we demonstrate that β_2GPI is very sensitive for cleavage between Lys317 and Thr318 by plasmin, resulting in two immunologically different cleaved forms. *In vitro* experiments show that these plasmin cleaved forms of β_2GPI bind to negatively charged phospholipids with much lower affinity compared to intact β_2GPI. Similar to plasmin, trypsin and elastase can also induce this proteolytical cleavage in β_2GPI, whereas thrombin and factor Xa do not cleave β_2GPI. The *in vivo* occurrence of the proteolytical cleavage was demonstrated by the finding that in plasmas of patients with disseminated intravascular coagulation (DIC) and in plasmas of patients treated with streptokinase, significant amounts of cleaved β_2GPI (up to 12 μg/ml) are present. During the development of DIC, the increase in levels of cleaved β_2GPI is accompanied by a 70% decrease in the levels of intact β_2GPI, whereas in streptokinase treated patients levels of intact β_2GPI stay within the normal range.

This study demonstrates for the first time that during *in vivo* activation of fibrinolysis β_2GPI is cleaved, which results in the formation of a form of β_2GPI with much lower affinity for negatively charged phospholipids. Plasmin is most likely responsible for this modification.

Introduction

The presence of antiphospholipid antibodies (aPL) in plasmas of patients with systemic lupus erythematosus (SLE) is strongly correlated with a history of venous and arterial thrombosis, recurrent fetal loss and thrombocytopenia (1-4). This clinical-serological entity is called the antiphospholipid syndrome (APS). Till 1990, it was generally accepted that aPL are directed against negatively charged phospholipids and that aPL could be divided into different subclasses named anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC), based on the detection method used. This idea changed by the work of different groups, who pointed to the importance of phospholipid binding plasma proteins, such as β_2-glycoprotein I (β_2GPI), for the binding of aPL (5-8).

β_2GPI, also known as apolipoprotein H, is a plasma glycoprotein, which consists of 326 amino acids, has a molecular mass of approximately 50 kD and has five potential glycosylation sites (9). The protein comprises 5 repeating domains of about 60-80 amino acids, in which cysteine, proline and tryptophane residues appear to be highly conserved. Each domain resembles the so called short consensus repeats (SCR) or Sushi domains (10, 11).

Although there is considerable insight in the structure of β_2GPI, little is known about the physiological function of the protein in human plasma. The plasma concentration of β_2GPI is approximately 160 μg/ml (3.8 μM) of which 40% is bound to lipoproteins, especially chylomicrons and VLDL (12, 13). Besides binding to lipoproteins, β_2GPI can also bind to negatively charged surfaces (14-16) such as heparin, negatively charged phospholipids and DNA. *In vitro*, β_2GPI shows a variety of anticoagulant properties. β_2GPI inhibits contact activation (17-19), it inhibits tenase and prothrombinase activity on activated human platelets or phospholipid vesicles (20-22) and it inhibits ADP-induced platelet aggregation (14, 16). β_2GPI also has procoagulant activities, because it was shown that β_2GPI can inhibit the anticoagulant activity of activated protein C (23, 24). However, the physiological importance of β_2GPI as a regulator of coagulation is disputable, because individuals without detectable levels of β_2GPI do not suffer from clotting or bleeding complications (25, 26).

In this study, we describe that during purification of β_2GPI from human plasma using heparin-Sepharose affinity chromatography, proteolytical cleavage of β_2GPI, between residues Lys317 and Thr318, occurs. We found that plasmin, trypsin and elastase can cause this cleavage *in vitro*. With monoclonal antibodies raised against cleaved and intact β_2GPI, levels of the different forms of β_2GPI were measured in plasmas of healthy controls, in plasmas of patients with disseminated intravascular coagulation (DIC) and in plasmas of patients treated with streptokinase. From the results it can be concluded that β_2GPI is cleaved *in vivo* upon activation of fibrinolysis and that plasmin is most likely to be responsible for this *in vivo* modification of β_2GPI.

Methods

Materials

The different Sepharoses used and horseradish peroxidase labeled sheep anti-M13 antibody (α-M13-PO) were from Pharmacia Biotech (Uppsala, Sweden). High binding ELISA plates (strip plate, cat.no. 9102) and regular 96-well ELISA plates were from Costar (Cambridge, MA, USA). PNGase F was from New England Biolabs (Beverly, MA, USA). BCA protein assay kit was from Pierce (Rockford, IL, USA). MaxiSorp tubes were from Nunc (Roskilde, Den-

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mark). Protifar was from Nutricia (Zoetermeer, The Netherlands). E. coli strain XL1-Blue and helper phage VCS-M13 were from Stratagene (La Jolla, CA, USA). Polyclonal rabbit anti-β_2GPI-antibodies (PoAb) were from Behring (Amersfoort, The Netherlands). (Peroxidase labeled) rabbit anti mouse immunoglobulins (RAMPO or RαM) and peroxidase labeled swine anti rabbit immunoglobulins (SWARPO) were from Dako (Glostrup, Denmark). Soybean Trypsin Inhibitor (SBTI), benzamidin, polybrene, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine were from Sigma (St. Louis, MO, USA). PVDF Immobilon-P Transfer membranes were from Millipore Corporation (Bedford, MA, USA). S2251 was from Chromogenix (Mölndal, Sweden). Streptokinase (Streptase®) was from Hoechst (Amsterdam, The Netherlands). Alkaline phosphatase conjugated goat anti human IgG was from Tago (Burlingame, CA, USA). Trypsin was from Worthington Biochemical Corp. (Freehold, NJ, USA). Elastase and automated STA APTT kit was from Boehringer Mannheim (Mannheim, Germany). Nyocard D-dimer kit was from Nycomed UK Ltd (Birmingham, UK).

Blood Collection

Blood samples were collected by venipuncture using evacuated tubes containing 3.8% trisodium citrate (0.129 M) as the anticoagulant (9:1, v/v). To obtain platelet poor plasma the samples were centrifuged twice at 2000 × g for 10 min at 4° C. Plasma samples were stored at -50° C until use.

Healthy Controls and Patients

Healthy controls. Plasmas of 65 healthy controls (40 females and 25 males) were used in this study. The mean age was 32 years (range 23-51 years).

Sepsis/DIC patients. 15 sepsis/DIC patients were recruited from a primary medical-surgical intensive care unit. Their median age was 60 years (range 23-80 years). Patients were identified as having sepsis on the basis of temperature (>38.3° C or <35.0° C) with a systolic blood pressure 20 mmHg below their usual level. The sepsis syndrome was verified by positive blood cultures and/or a specific site of infection. Mean platelet count was $130*10^9$/l (range $18*10^9$-$372*10^9$) (27). All sepsis patients included in this study suffered from disseminated intravascular coagulation (DIC), as monitored by the presence of a prolonged activated partial thromboplastin time (APTT), prolonged prothrombin time (PT), prolonged thrombin time (TT) and elevated levels of D-dimer and F1+2.

Streptokinase treated patients. Plasmas of four patients who were treated with streptokinase ($1.5*10^6$ units) after acute myocardial infarction were included in this study. Samples were taken 1 h after streptokinase administration.

An almost drowned person. Multiple plasma samples were taken from a person (male, age 22 years) who was hospitalized immediately after being rescued from drowning. The patient developed severe DIC.

Activated Partial Thromboplastin Time (APTT)

APTT clotting times were measured in citrated plasma using the automated STA APTT kit, according to instructions of the manufacturer.

Levels of D-dimer

D-dimer levels were measured in citrated plasma using NyoCard D-dimer kit, according to the instructions of the manufacturer.

Proteins

β_2GPI was purified from freshly frozen citrated human plasma (obtained from the local blood bank) as described before (3), with a few modifications. Briefly, human plasma was quickly thawed at 37° C and 40 μg/ml Soybean Trypsin Inhibitor (SBTI), 1 mM benzamidin, 1 mM EDTA and 0.0025% polybrene were added to the plasma. The plasma was successively applied to a DEAE-Sephadex A50 column, protein G-Sepharose column and a S-Sepharose column. Afterwards, eluted proteins were applied to a heparin-Sepharose column. Bound phospholipid contaminations were eluted using 20 mM Tris/50 mM NaCl/0.1% Triton X-100 (pH 8.3). The column was washed with the same Tris buffer without Triton X-100 and bound proteins were eluted with a linear salt gradient from 50 mM to 350 mM NaCl in 20 mM Tris (pH 8.3). The peak fractions were analyzed by SDS-PAGE, and the presence of β_2GPI was demonstrated after the proteins were transferred to a PVDF Immobilon-P Transfer membrane by blotting with rabbit anti-human β_2GPI-antibodies. Protein concentration was determined using a BCA protein assay kit according to the manufacturer's instructions with bovine serum albumin (BSA) as a standard.

Plasminogen [purified from freshly frozen citrated human plasma, as described elsewhere (28)] was a kind gift from Dr. Peter A. Kr. von dem Borne (University Hospital Utrecht, The Netherlands). Plasminogen was activated by using a plasminogen-streptokinase complex (29). Plasmin concentration was determined using a BCA protein assay kit according to the manufacturer's instructions with BSA as a standard. The final preparation was homogeneous by SDS-PAGE.

Factor X was purified from human plasma with a monoclonal anti-factor X-antibody as described by Hackeng et al. (30). Factor X was activated by incubation with immobilized Russell viper venom as described by Bock et al. (31).

PNGase F Treatment

To remove all N-linked carbohydrate side chains from β_2GPI, the protein (150 μg/ml) was incubated overnight at 37° C with PNGase F under reducing conditions, according to the instructions of the manufacturer.

N-terminal Sequence Analysis

β_2GPI was subjected to 12% SDS-PAGE followed by Western blotting, according to instructions from the Sequence Centre Utrecht (Centre for Biomembranes and Lipid Enzymology, Department Biochemistry of Lipids, Utrecht, The Netherlands). The protein band was cut from the blot and the N-terminal sequence was determined by automated Edman degradation using a gas phase sequenator (Applied Biosystems model 476A) by the Sequence Centre Utrecht.

Production and Purification of Monoclonal Antibodies against β_2GPI

BALB/c mice were immunized with a 1:1 mixture of β_2GPI^{*1} and β_2GPI^{intact} according to standard methods. Spleen cells were fused with Ag 8.653 myeloma cells. Fusion and hybridoma selection were performed according to standard procedures. Culture supernatants were screened for the presence of specific antibodies by an ELISA in which β_2GPI was used as antigen. Bound antibodies were detected with peroxidase-labeled rabbit antibodies against mouse immunoglobulins (RAMPO). Positive clones were subcloned by limiting dilution and tested. This resulted in two clones (from different fusions), one named NIK-13A10 and one named OCK-4F3. MoAbs were purified from culture medium by passing the medium through a 3.5 ml RαM (rabbit antibodies against mouse immunoglobulins)-Sepharose column. 9 mg RαM was coupled to 3.5 ml CNBr-activated Sepharose according to the instructions of the manufacturer. Bound proteins were eluted with 3M KSCN in TBS (50 mM Tris/150 mM NaCl). Antibody containing fractions were combined, dialyzed against TBS and applied to a protein G-Sepharose FF-column (10 ml) for concentration. Bound proteins were eluted with 0.1 M glycine (pH 2.7) and antibody containing fractions were pooled and dialyzed against TBS. Purified antibodies were stored at -20° C until use.

Selection of Phage-antibodies against β_2GPI

The phage display library used for this study was constructed as described before (32) and was kindly provided by Dr. J. de Kruif (Department of Immunology, Utrecht University, The Netherlands). Phages which express human single chain Fv antibody fragments against β_2GPI^{intact} on the surface were selected from the phage display library using a selection procedure as described before (32), in which β_2GPI^{intact} (50 μg/ml in TBS, pH 7.4) was coated on

MaxiSorp tubes overnight at 4° C. The selected phages, that recognize coated β_2GPI^{intact} were used to infect bacteria. *E. coli* strain XL1-Blue were infected with the eluted phage antibodies and grown as described before (33). The assembling of new phage particles by the infected XL1-Blue strain was accomplished using helper phage VCS-M13. Phage antibodies were isolated from the XL1-Blue culture by polyethylene glycol (PEG)/NaCl precipitation. After the last precipitation the phage containing pellet was dissolved in 3 ml PBS/1% BSA and stored at 4° C.

After each selection round, obtained phage antibodies were screened for their specific binding to β_2GPI^{intact} using an ELISA, in which β_2GPI^{intact} (10 μg/ml in TBS, 100 μl/well) was coated on high binding 96 wells ELISA plates. Wells were blocked with TBS/3% BSA/0.1% Tween-20 (150 μl/well) for 2 h at 37° C and wells were incubated with phage antibodies at various dilutions for 1.5 h at 37° C. Afterwards bound phage antibodies were detected with 1:2500 diluted horseradish peroxidase labeled sheep anti-M13 antibody (α-M13-PO) for 1.5 h at 37° C and developed with staining solution (100 μl/well) consisting of 0.4 mg/ml o-phenylenediamine (OPD) and 0.002% H_2O_2 in 100 mM phosphate/50 mM citric acid buffer (pH 5.0). The reaction was stopped by the addition of 1 M H_2SO_4 (50 μl/well) and absorbance was measured at 490 nm in a multiscan photometer (Vmax reader, Molecular Devices, CA, USA). Dilutions were made in TBS/3% BSA/0.1% Tween-20 and after each incubation step, the wells were washed three times with TBS/0.1% Tween-20.

To obtain highly specific anti-β_2GPI^{intact}-phage antibodies, the selection procedure was repeated three times. After the last selection round, individual colonies were used to prepare monoclonal phage antibodies, as described before (32). This procedure resulted in a monoclonal anti-β_2GPI^{intact}-phage antibody, designated phage-F3.

Specificity of Different Monoclonal anti-β_2GPI-antibodies for Cleaved and Intact β_2GPI Preparations

Recognition of cleaved and intact β_2GPI by phage-F3 antibodies, murine MoAbs 13A10, 4F3 and 2B2 (the latter was a generous gift of Dr. A. Tincani, Spedali Civili, Brescia, Italy) or polyclonal rabbit anti-β_2GPI-antibodies (PoAb) was tested in an ELISA. High binding ELISA plates were coated with increasing concentrations of purified β_2GPI (100 μl/well) in TBS. Wells were blocked with 150 μl/well blocking buffer (TBS/3% BSA/0.1% Tween-20) for 2 h at 37° C. Afterwards wells were incubated with 50 μl/well phage-F3 antibodies (1:50 diluted), 13A10 (3 μg/ml), 4F3 (3 μg/ml), 2B2 (3 μg/ml) or PoAb (3 μg/ml) for 1.5 h at 37° C. Bound phage-F3 antibodies were incubated with α-M13-PO (1:2500 diluted), bound MoAbs with RAMPO (1:1000 diluted) and bound PoAbs with peroxidase labeled swine anti rabbit antibodies (SWARPO, 1:1000 diluted) for 1.5 h at 37° C. Staining procedure was performed with staining solution (100 μl/well) consisting of 0.4 mg/ml o-phenylenediamine (OPD) and 0.002% H_2O_2 in 100 mM phosphate/50 mM citric acid buffer (pH 5.0). The reaction was stopped by the addition of 1 M H_2SO_4 (50 μl/well) and absorbance was measured at 490 nm. Between every incubation, wells were washed three times with TBS/0.1% Tween-20. All dilutions were made in blocking buffer.

Purification of IgG from Plasma

From the plasma of a SLE-patient with high levels of anti-β_2GPI-IgG (122%) and aCL-IgG (>100 GPL), without anti-β_2GPI-IgM and aCL-IgM antibodies (3) total IgG was purified with protein G-Sepharose according to the manufacturer's instructions.

Binding of Patient IgG to Immobilized β_2GPI

The binding of purified patient IgG to immobilized β_2GPI was tested as described before (3), using the ELISA for the detection of anti-β_2GPI-antibodies in plasma. Briefly, β_2GPI was coated on high binding ELISA plates. After blocking aspecific binding sites, wells were incubated with 100 μg/ml purified patient IgG, followed by incubation with alkaline phosphatase conjugated goat antihuman IgG. Colour development was initiated by the addition of p-nitrophenyl phosphate and was stopped by the addition of 2.4 M NaOH. Absorbance was measured at 405 nm in a multiscan photometer.

Detection of β_2GPI Levels in Plasma with ELISA

Total β_2GPI (cleaved and intact β_2GPI) levels in plasma were detected with an ELISA in which 100 μl/well of a solution containing 3 μg/ml MoAb 2B2 (in 15 mM $Na_2CO_3.10H_2O$, 35 mM $NaHCO_3$, pH 9.6) was coated on 96-wells ELISA plates overnight at 4° C. Afterwards, wells were blocked with TBS/3% BSA/0.1% Tween-20, followed by successive incubations with 1:1000 diluted plasmas (50 μl/well, 1.5 h at 37° C) and 50 μl/well PoAb (3 μg/ml; 1 h at 37° C). Bound proteins were detected with 1:1000 diluted SWARPO (50 μl/well) and developed using OPD. Purified β_2GPI^{intact} (5 ng/ml-1 μg/ml) was used as a standard.

For the selective detection of β_2GPI^{intact} a similar approach was used, in which phage-F3 antibodies (1:50 diluted) instead of PoAb and α-M13-PO (1:2500 diluted) instead of SWARPO were used.

For the selective detection of β_2GPI^{*1} levels in plasma, 96-wells ELISA plates were coated with MoAb 13A10 instead of MoAb 2B2. After blocking, wells were incubated with 50 μl/well 1:5 diluted plasmas for 1.5 h at 37° C, followed by 50 μl/well 3 μg/ml PoAb for 1 h at 37° C. Bound antibodies were detected with 100 μl/well 1:1000 diluted SWARPO and developed using OPD. Purified β_2GPI^{*1} (10 ng/ml-1 μg/ml) was as a standard.

Dilutions were made in TBS/3% BSA/0.1% Tween-20 and after each incubation, wells were washed three times with TBS/0.1% Tween-20.

Proteolytical Cleavage of β_2GPI

In order to investigate the proteolytical cleavage of β_2GPI, the effect of plasmin on β_2GPI was studied in solution and when β_2GPI was immobilized on high binding ELISA plates.

1. The effect of plasmin on β_2GPI in solution. β_2GPI^{intact} (200 μg/ml) was incubated with 0.2 μM plasmin in TBS at 37° C. At various time intervals, 10 μl samples were taken from the incubation mixture, treated with PNGase F and subjected to 12% SDS-PAGE under reducing conditions. The proteins were visualized by Coommassie Brilliant Blue staining.

2. The effect of plasmin, trypsin, elastase, factor Xa and α-thrombin on immobilized β_2GPI. β_2GPI^{intact} (5 μg/ml) was coated on high binding 96-wells ELISA plates as described before. Afterwards, the wells were incubated with increasing concentrations of plasmin (0-0.2 μM), trypsin (0-0.5 μM), elastase (0-0.5 μM), factor Xa (0-50 nM) or α-thrombin (0-0.5 μM) (a generous gift of Dr. Walter Kisiel, University of New Mexico, Albuquerque, NM, USA) in TBS/3% BSA/3 mM $CaCl_2$ for 90 min at 37° C. The different forms of β_2GPI on the surface were measured using MoAb 4F3, MoAb 13A10 or phage-F3, followed by RAMPO or anti-M13-PO, as described before.

Preparation of Phospholipid Vesicles

Phospholipid vesicles containing 20% phosphatidylserine, 40% phosphatidylcholine and 40% phosphatidylethanolamine were prepared according to Brunner et al. (34), with some modifications as described by Van Wijnen et al. (35). The phospholipid content of the fractions was determined by phosphate analysis (36).

Binding of β_2GPI to Phospholipids

Binding of β_2GPI to PS/PC/PE vesicles was tested in a solid phase binding assay. High binding ELISA plates were coated with PS/PC/PE vesicles (25 μM in TBS; 50 μl/well) overnight at 4° C. Wells were blocked with 150 μl/well TBS/0.5% gelatine for 2 h at 37° C. After blocking, wells were incubated with the different β_2GPI preparations (50 μl/well) for 1.5 h at 37° C, followed by incubation with MoAb 2B2 (3 μg/ml; 50 μl/well; 1.5 h at 37° C). Afterwards, the wells were incubated with RAMPO (1:1000 diluted) followed by staining procedure usin OPD. All samples were diluted in TBS/0.5% gelatine and after each

incubation step, wells were washed three times with TBS. Non-specific binding was determined in wells where phospholipids were absent.

Binding of Human anti-β_2GPI-antibodies to Cardiolipin Bound β_2GPI

To determine whether anti-β_2GPI-antibodies recognize β_2GPI which is bound to coated cardiolipin (CL), an aCL-ELISA was performed as described previously (3), with a few modifications. Briefly, CL (5 μg/well) was coated on high binding 96-well ELISA plates. Wells were blocked with blocking buffer (TBS/0.1% gelatine/0.5 mg/ml purified horse-IgG) and incubated with 100 μg/ml purified patient IgG in the presence of the different forms of 2GPI (0-50 μg/ml) for 1.5 h at 37° C. Binding of human-IgGs was visualized as described before. All dilutions were made in blocking buffer and after each incubation the wells were washed three times with TBS.

Results

Purification and Characterization of β_2GPI from Normal Human Plasma

The final step in our purification of β_2GPI from plasma is the elution of β_2GPI from a heparin-Sepharose column with a linear salt gradient. This procedure results in three separate peaks at 115 mM (β_2GPI-1, fraction 16-26), 150 mM (β_2GPI-2, fraction 33-36) and 200 mM NaCl (β_2GPI-3, fraction 47-56), respectively (Fig. 1). The mutual ratio of the three peaks varied between purifications.

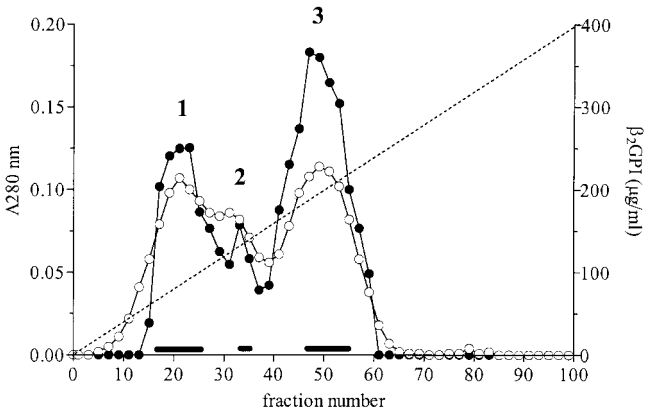

Fig. 1 Heparin-Sepharose chromatography of β_2GPI. The dotted line indicates the linear salt gradient from 50-350 mM NaCl. Closed symbols (●) correspond to the right y-axis and show levels of β_2GPI (μg/ml), as measured with ELISA. Open symbols (○) correspond to the left y-axis and show protein levels, as measured as the absorption at 280 nm. The horizontal bars indicate which fractions are pooled for further experiments: β_2GPI-1 = fraction 16-26, β_2GPI-2 = fraction 33-36 and β_2GPI-3 = fraction 47-56

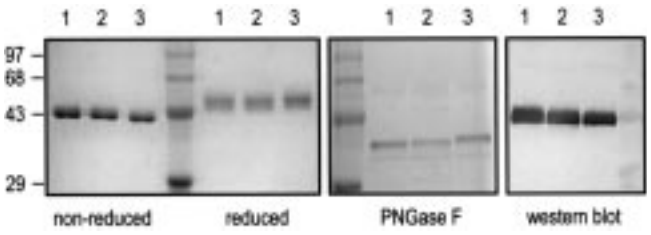

Fig. 2 SDS-PAGE and Western blot analysis of the different β_2GPI preparations. Purified β_2GPI preparations were separated by SDS-PAGE under non-reducing conditions, under reducing conditions and after PNGase F treatment, and visualized by Coommassie Brilliant Blue staining. Binding of rabbit anti-β_2GPI-antibody to purified β_2GPI was analyzed on Western blot. Lane 1: β_2GPI-1, lane 2: β_2GPI-2, lane 3: β_2GPI-3. Molecular markers (in kDa) are shown on the left

The three protein preparations were analyzed by SDS-PAGE under (non) reducing conditions, after PNGase F treatment (which removes all N-linked carbohydrate side chains) and by Western blotting (Fig. 2). Under non-reducing and reducing conditions the three protein preparations showed a triplet of a molecular weight of about 42 kDa and 51 kDa, respectively. Western blotting analysis showed that all three protein preparations reacted with polyclonal rabbit anti human β_2GPI antibodies. After treatment with PNGase F under reducing conditions the three β_2GPI preparations showed a single band with a molecular weight of approximately 38 kDa for β_2GPI-1 and β_2GPI-2, whereas for β_2GPI-3 the molecular weight was about 1 kDa higher. The enzyme PNGase F is represented by the band with a molecular weight of 36 kDa.

N-terminal sequence analysis of the three β_2GPI preparations revealed a single sequence for β_2GPI-3 (Gly-Arg-Thr-Cys-Pro-Lys), consistent with normal intact β_2GPI (9). Analysis of β_2GPI-1 and β_2GPI-2 revealed for both preparations two different N-terminal amino acid sequences, with similar molar concentrations, indicating that in these β_2GPI preparations two N-termini are present. One sequence is identical to the sequence found for β_2GPI-3. The other sequence (Thr-Asp-Ala-Ser-Asp-Val) is identical to that which appears when β_2GPI is cleaved in the fifth domain between Lys317 and Thr318, as described by Hunt et al. (37).

In conclusion, heparin-Sepharose affinity chromatography results in three different forms of β_2GPI: two "cleaved" forms β_2GPI-1 and β_2GPI-2, which are designated as β_2GPI^{*1} and β_2GPI^{*2}, respectively, and one intact form β_2GPI-3, which is designated as β_2GPI^{intact}.

Characterization of the Monoclonal anti-β_2GPI-antibodies

The specificity of MoAbs 13A10, 4F3, 2B2 and phage-F3 for the three β_2GPI forms was tested in an ELISA in which purified β_2GPI was coated on high-binding 96 wells plates. As shown in Fig. 3, MoAb 13A10 only recognized β_2GPI^{*1}, phage-F3 only recognized β_2GPI^{intact}, whereas MoAb 4F3 recognized all three forms of β_2GPI with comparable affinity. β_2GPI^{*2} is not recognized by MoAb 13A10 nor by phage-F3. MoAb 2B2 also recognized all three forms of β_2GPI (data not shown). Unfortunately, we were unable to raise specific MoAb for β_2GPI^{*2}. With the different anti-β_2GPI-antibodies, ELISAs were developed with which plasma levels of total β_2GPI, β_2GPI^{intact} and β_2GPI^{*1} were measured.

Levels of Cleaved and Intact β_2GPI in Plasmas

1. Healthy controls. Mean levels (± SD) of total β_2GPI and β_2GPI^{intact} in plasmas of healthy individuals were 142 ± 30 μg/ml and 161 ± 31 μg/ml, respectively. Only trace amounts of β_2GPI^{*1} (0.1 ± 0.05 μg/ml) were detectable (Fig. 4).

2. Sepsis/DIC patients and patients treated with streptokinase. In plasmas of the patients with sepsis/DIC levels of total β_2GPI (73 ± 38 μg/ml) and β_2GPI^{intact} (65 ± 44 μg/ml) were significantly decreased ($p < 0.0001$ and $p < 0.0001$, respectively; student's t-test) compared to the healthy control population (Fig. 4). Levels of β_2GPI^{*1} (1.1 ± 1.2 μg/ml) were significantly increased ($p < 0.0001$, student's t-test) compared to the healthy control population. In plasmas of four streptokinase treated patients levels of total β_2GPI (165 ± 23 μg/ml) and β_2GPI^{intact} (157 ± 15 μg/ml) were similar to their respective levels in the control population, whereas levels of β_2GPI^{*1} (10 ± 2 μg/ml) were

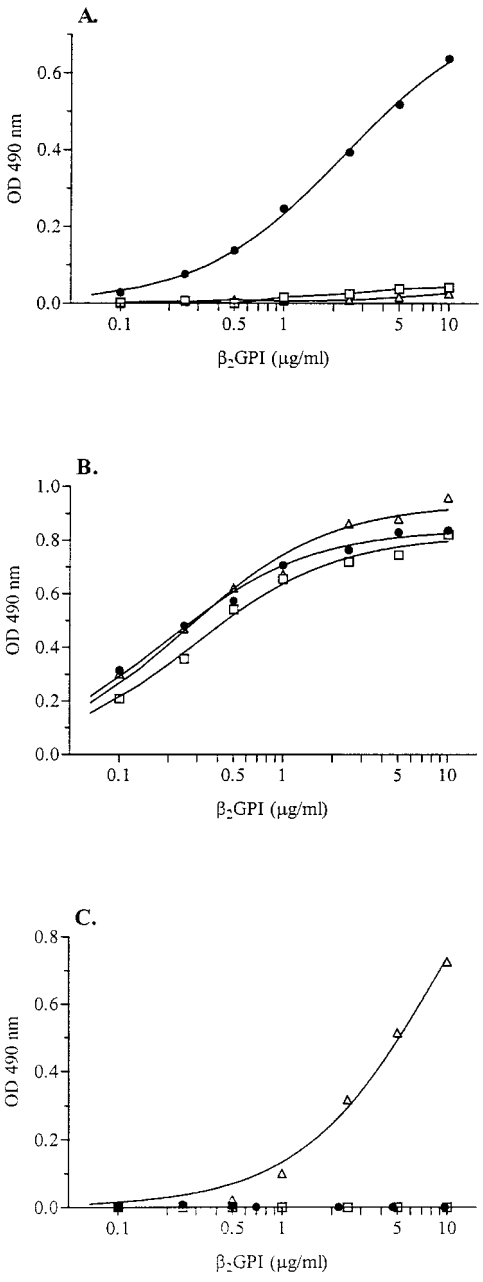

Fig. 3 Recognition of the different β_2GPI forms by MoAb 13A10 (A), MoAb 4F3 (B) and phage F3 (C). Increasing concentrations of purified β_2GPI^{*1} (●), β_2GPI^{*2} (□) and β_2GPI^{*intact} (△), were coated on high binding 96-wells ELISA plates and were assayed for antibody recognition

approximately 100 times increased ($p < 0.0001$, student's t-test) compared to the control population (Fig. 4).

3. A patient rescued from drowning. APTT clotting times, levels of D-dimers (as measure of the fibrinolytic activity) and levels of total β_2GPI, β_2GPI^{*1} and β_2GPI^{intact} were measured in plasma samples of a patient who was hospitalized immediately after being rescued from drowning. Within 10 h after hospitalization the APTT prolonged up to 120 s, paralleled by an increase of plasma D-dimer levels up to 24 mg/ml (Fig. 5A). During the next 5 days both APTT clotting times and D-dimer levels gradually decrease to normal values. Simultaneous with this activation of the coagulation and fibrinolysis, there was a decrease in levels of total β_2GPI and β_2GPI^{intact} to about 30% of the value at admission to the hospital, and an increase in levels of β_2GPI^{*1}

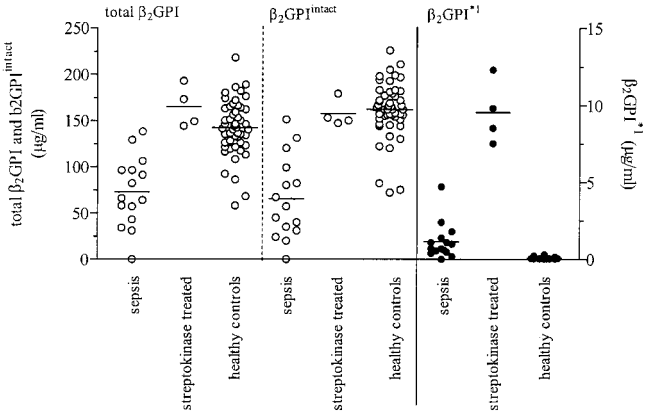

Fig. 4 Plasma levels of total β_2GPI, β_2GPI^{intact} and β_2GPI^{*1} in sepsis patients (n = 15), streptokinase treated patients (n = 4) and healthy controls (n = 65). Open symbols (○) correspond to the left y-axis and closed symboils (●) correspond to the right y-axis. Horizontal bars indicate mean β_2GPI levels

(Fig. 5B). When coagulation and fibrinolytic activities were normalizing, there was an increase in levels of total β_2GPI and β_2GPI^{intact} and a decrease of levels of β_2GPI^{*1}, returning to levels found at hospitalization.

Proteolytical Cleavage of β_2GPI

To investigate which enzymes are responsible for the cleavage of β_2GPI^{intact}, purified β_2GPI^{intact} (200 μg/ml) was incubated with plasmin (0.2 μM), a protease with known specificity for Lys residues. At different time points samples were taken, treated with PNGase F and subjected to SDS-PAGE. Purified β_2GPI^{*1} was used as a control. As shown in Fig. 6, during plasmin incubation a protein band with a molecular weight of approximately 1 kDa below β_2GPI^{intact} appeared, indicating that cleaved β_2GPI (β_2GPI^{*1} and/or β_2GPI^{*2}) is formed. Within 60 min incubation more than 50% of the β_2GPI^{intact} was cleaved. N-terminal sequence analysis confirmed that this plasmin treated β_2GPI is cleaved between residues Lys317 and Thr318. In addition, heparin-Sepharose chromatographic analysis of the plasmin cleaved β_2GPI product suggests that the plasmin cleaved form of β_2GPI is similar to β_2GPI^{*2} (data not shown).

We also tested the effect of plasmin on β_2GPI^{intact} bound to a plastic surface. β_2GPI coated on an ELISA plate, was incubated with increasing concentrations of plasmin for 90 min. Subsequently, coated β_2GPI was incubated with MoAbs with specificity for the different forms of β_2GPI. As shown in Fig. 7, the recognition of coated β_2GPI by MoAb 4F3 decreases to 55% (at the highest plasmin concentration) of the originally coated β_2GPI, indicating that upon plasmin incubation part of the coated β_2GPI is removed from the plastic surface. The recognition by phage-F3 antibodies totally disappeared after incubation with 0.15 μM plasmin, indicating that with this plasmin concentration, coated β_2GPI is totally cleaved by plasmin. However, cleavage of coated β_2GPI^{intact} by plasmin does not result in the formation of β_2GPI^{*1}, because MoAb 13A10 did not recognize the plasmin treated protein. These results strongly suggest that cleavage of coated β_2GPI by plasmin results in the formation of β_2GPI^{*2} and not in the formation of β_2GPI^{*1}.

We also tested the proteolytical effect of trypsin, elastase, α-thrombin and factor Xa on β_2GPI coated to an ELISA plate (data not shown). Both trypsin and elastase had similar effect as plasmin, whereas α-thrombin and activated factor Xa did not cause a cleavage of β_2GPI.

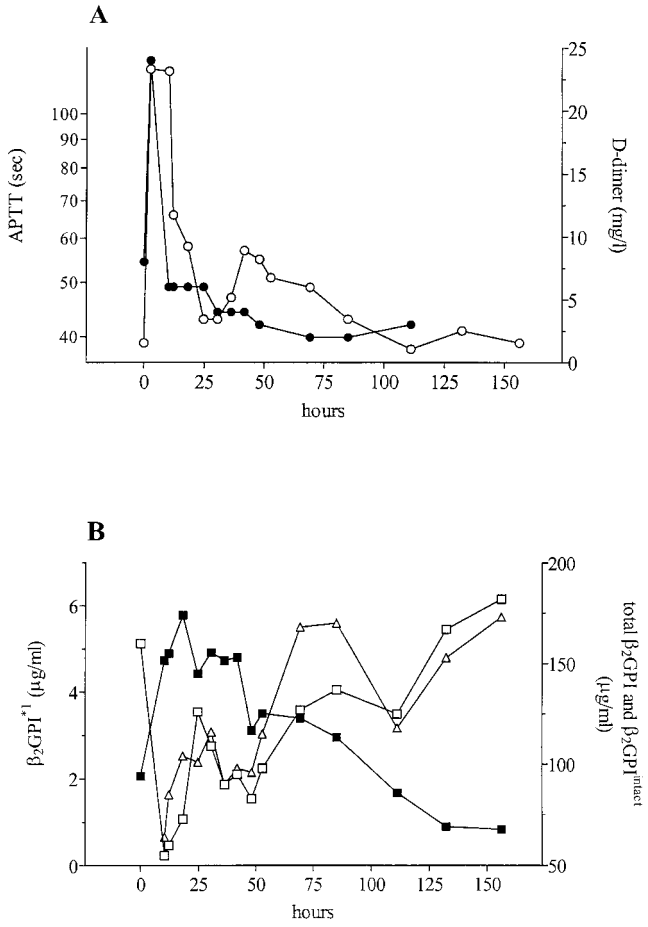

Fig. 5 (A) APTT and D-dimer levels and (B) levels of different forms of β_2GPI in plasmas of a patient who was rescued from drowning. Plasmas were taken immediately after hospitalization (t = 0) at various times. (A) Closed circles (●) show levels of D-dimer (right y-axis) and open circles (○) indicate APTT clotting time (left y-axis). (B) Closed squares (■) indicate levels of β_2GPI^{*1} (left y-axis), open squares (□) indicate levels of total β_2GPI (right

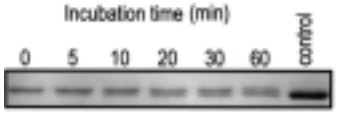

Fig. 6 Cleavage of β_2GPI^{intact} by plasmin in solution. β_2GPI^{intact} (200 μg/ml) was incubated with 0.2 μM plasmin and at various times samples were taken. After PNGase F treatment, samples were subjected to SDS-PAGE and visualized by Coommassie Brilliant Blue staining. Purified β_2GPI^{*1} served as a control

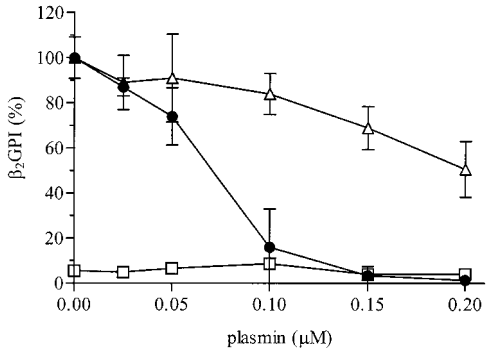

Fig. 7 Plasmin cleavage of immobilized β_2GPI^{intact}. Wells of high binding ELISA plates were coated with β_2GPI^{intact} and were incubated with increasing concentrations plasmin (0-0.2 μM) for 90 min at 37° C. Afterwards, coated β_2GPI was either detected by MoAb 13A10 (□), which recognizes only β_2GPI^{*1}, MoAb 4F3 (△), which recognizes all three forms of β_2GPI or phage F3 (●), which recognizes only β_2GPI^{intact}. Measured OD490 nm values are expressed as percentage of originally coated β_2GPI^{intact}. Results are expressed as mean ± SD of three individual experiments

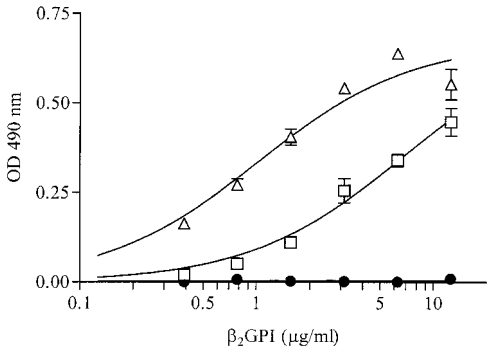

Fig. 8 Binding of different forms of β_2GPI to immobilized phospholipids. PS/PC/PE (20%/40%/40%) vesicles were immobilized on high binding 96-well plates and incubated with increasing concentrations β_2GPI^{intact} (△), β_2GPI^{*2} (□) or β_2GPI^{*1} (●). Afterwards, bound β_2GPI was detected with MoAb 2B2. Results are expressed as mean ± SD of three individual experiments

Characteristics of the Different β_2GPI Preparations

Binding of β_2GPI to phospholipids. Binding of the three different β_2GPI preparations to PS/PC/PE (20%/40%/40%) was studied in a solid-phase binding assay, in which PS/PC/PE vesicles were coated on high binding ELISA plates. Fig. 8 shows binding curves of β_2GPI^{*1}, β_2GPI^{*2} and β_2GPI^{intact} to immobilized PS/PC/PE. Analysis of the specific binding revealed a β_2GPI concentration at which half maximal binding was observed of 0.02 μM (1 μg/ml) for β_2GPI^{intact} and 0.13 μM (6.6 μg/ml) for β_2GPI^{*2}. For β_2GPI^{*1} this concentration could not be determined, because no saturation of binding occurred with the β_2GPI concentrations used.

Binding of human anti-β_2GPI-IgG to β_2GPI. The recognition of the three different β_2GPI preparations by anti-β_2GPI-IgG from a SLE-patient was tested in an ELISA using total IgG purified from the plasma of a patient with high anti-β_2GPI-antibody titres. As shown in Fig. 9A, patient anti-β_2GPI-antibodies bind to all three β_2GPI preparations, immobilized directly to the ELISA plates, indicating that the epitope for anti-β_2GPI-antibodies is present on all three forms of β_2GPI.

We next studied the binding of patient anti-β_2GPI-antibodies to the different β_2GPI preparations bound to cardiolipin. Fig. 9B shows that the amount of bound anti-β_2GPI-antibodies reflects the amount of β_2GPI bound to the cardiolipin coated well.

Discussion

In 1990 it was shown that the presence of a plasma protein, identified as β_2GPI, was required for the binding of aPL to negatively charged phospholipids (6, 7). At that time β_2GPI was already known as a natural anticoagulant in *in vitro* experiments. It inhibits the contact ac-

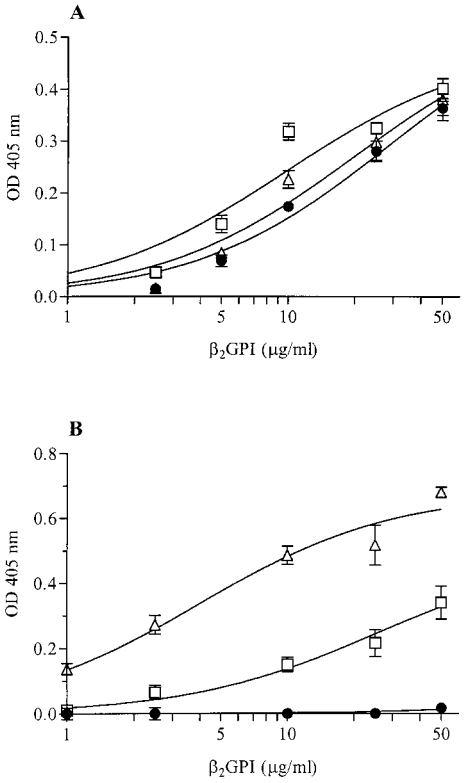

Fig. 9 Recognition of different forms of β_2GPI by human anti-β_2GPI-antibodies. (A) Increasing concentrations purified β_2GPI^{intact} (△), β_2GPI^{*2} (□) or β_2GPI^{*1} (●) were coated on high binding 96-wells ELISA plates and were incubated with 100 μg/ml purified patient IgG. Bound anti-β_2GPI-antibodies were detected with alkaline phosphatase conjugated goat anti-human-IgG. (B) High binding 96-wells ELISA plates were coated with cardiolipin (5 μg/well) and were was incubated with 100 μg/ml purified patient-IgG, in the presence of increasing concentrations β_2GPI^{intact} (△), β_2GPI^{*2} (□) or β_2GPI^{*1} (●). Bound anti-β_2GPI-antibodies were detected with alkaline phosphatase conjugated goat anti-human-IgG. Results are expressed as mean ± SD of three individual experiments

tivation of the intrinsic coagulation pathway, both tenase and prothrombinase activity and ADP-induced platelet aggregation (14, 16-22). Apart from these anticoagulant effects, β_2GPI also inhibits the activity of APC, which is one of the most important natural anticoagulants (23, 24). Whether β_2GPI is an important anticoagulant or procoagulant factor in vivo is still uncertain, because persons with a β_2GPI deficiency lack the clinical symptoms of bleeding or thrombosis (25, 26).

In this study we have found that purified β_2GPI is very sensitive for proteolytical cleavage, resulting in β_2GPI which is cleaved in the fifth domain between Lys317 and Thr318. In purified systems, it was found that plasmin, trypsin and elastase are able to cause this specific cleavage. Upon proteolytical cleavage, the phospholipid binding affinity of β_2GPI decreases (Fig. 8), which may have consequences for the proposed function(s) of β_2GPI. It is thought that β_2GPI inhibits pro- and anticoagulant reactions by shielding of the negatively charged phospholipid surface, which is essential as a template for most coagulation reactions. After plasmin digestion β_2GPI might be released from these catalytic surfaces, which then results in continuation of different pro- and anticoagulant reactions. To prevent unwanted cleavage of β_2GPI during purification, pretreatment of plasma with perchloric acid precipitation is advised. Such treatment will protect β_2GPI against proteolytical cleavage.

Although we initially discovered the proteolytical cleavage of β_2GPI during purification (Fig. 1), we next demonstrated that under certain pathological conditions proteolytically cleaved forms of β_2GPI can be detected in plasma of patients (Fig. 4). Clinical syndromes in which these cleaved forms of β_2GPI circulate were patients suffering from DIC and patients treated with streptokinase. After induction of DIC, illustrated by a prolonged APTT and increase in D-dimer levels in a patient who was rescued from drowning, the plasma level of cleaved β_2GPI increased up to 6 μg/ml (Fig. 5). Concomitant with the formation of cleaved β_2GPI a drop of intact β_2GPI was noticed. The drop of intact β_2GPI in plasma exceeds the increase in cleaved β_2GPI. The simultaneous occurrence of an increase of cleaved β_2GPI and a decrease of intact β_2GPI, might suggest that the cleavage of β_2GPI induces a clearance of β_2GPI. However, in plasmas of the patients treated with streptokinase strongly increased levels of cleaved β_2GPI are present, in absence of a drop in levels of intact β_2GPI. This suggests that cleavage of β_2GPI itself is not the explanation for clearance of β_2GPI in patients with DIC. In previous reports, it has been suggested that β_2GPI is involved in the clearance of negatively charged phospholipid liposomes (38-42). It was described that *in vitro* β_2GPI binds to different kinds of particles expressing negatively charged phospholipids, like apoptotic bodies and oxidised LDL. Therefore, a simple explanation for the disappearance of β_2GPI from the circulation in patients with DIC is that β_2GPI binds to negatively charged phospholipids, which are exposed abundantly on apoptotic bodies. These bodies are known to be present in patients with sepsis/DIC (43-46).

The observation of high levels of cleaved β_2GPI in streptokinase treated patients and the observation that *in vitro* plasmin is able to cleave β_2GPI, suggests that *in vivo* plasmin is also responsible for the proteolytic cleavage. Our *in vitro* experiments showed that cleavage of β_2GPI by plasmin results in the formation of β_2GPI^{*2} (Fig. 6-7), which is immunological different from the cleaved form of β_2GPI (β_2GPI^{*1}) that we demonstrated *in vivo* (Fig. 4). These findings can point to the presence of an unknown factor in plasma which *in vivo* immediately converts plasmin cleaved β_2GPI^{*2} into β_2GPI^{*1}. This assumption is strengthened by the observation that during purification of β_2GPI from human plasma, both cleaved forms of β_2GPI are formed (Fig. 1). Unfortunately, the lack of a specific antibody against β_2GPI^{*2} made it impossible to measure the presence of β_2GPI^{*2} directly in plasma.

Based on N-terminal sequence analysis and molecular weight determination, however, β_2GPI^{*1} and β_2GPI^{*2} were found to be identical (Fig. 2). At this time, we can only speculate about the differences between β_2GPI^{*1} and β_2GPI^{*2}. It is possible that the difference between the cleaved forms of β_2GPI is caused by a modification in glycosylation. Alternatively, the immunological differences between β_2GPI^{*1} and β_2GPI^{*2}, however, may result from differences in tertiary structure. Another explanation might be that the differences between β_2GPI^{*1} and β_2GPI^{*2} are caused by the presence of different β_2GPI allotypes.

In conclusion, we have demonstrated for the first time that *in vivo* β_2GPI is cleaved between Lys317 and Thr318 during fibrinolysis and that *in vitro* plasmin could induce this specific proteolytical cleavage. The proteolytical cleavage of β_2GPI is not required for the observed clearance of β_2GPI from the circulation in patients with DIC. β_2GPI is probably removed from the circulation by binding to negatively charged phospholipids, which are exposed on apoptotic cell particles.

After finishing this study, a study from Ohkura et al. (47) was published in which comparable proteolytical effect of plasmin on β_2GPI was demonstrated. However, the authors did not measure the *in vivo* occurrence of the intact and cleaved forms of β_2GPI.

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