Domain V of $\beta_2$-Glycoprotein I Binds Factor XI/XIa and Is Cleaved at Lys$^{317}$-Thr$^{318}$

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The fifth domain (DV) of $\beta_2$-glycoprotein I (β2GPI) is important for binding a number of ligands including phospholipids and factor XI (FXI). β2GPI is proteolytically cleaved in DV by plasmin but not by thrombin, VIIa, tissue plasminogen activator, or uPA. Following proteolytic cleavage of DV by plasmin, β2GPI retains binding to FXI but not to phospholipids. Native β2GPI, but not cleaved β2GPI, inhibits activation of FXI by thrombin and factor XIIa, attenuating a positive feedback mechanism for additional thrombin generation. In this report, we have defined the FXI/FXIa binding site on β2GPI using site-directed mutagenesis. We show that the positively charged residues Lys$^{284}$, Lys$^{286}$, and Lys$^{287}$ in DV are essential for the interaction of β2GPI with FXI/FXIa. We also demonstrate that FXIA proteolytically cleaves β2GPI at Lys$^{317}$-Thr$^{318}$ in DV. Thus, FXI cleavage of β2GPI in vivo during thrombus formation may accelerate FXI activation by decreasing the inhibitory effect of β2GPI.

β2-Glycoprotein I (β2GPI) (also known as apolioprotein H, apoh) is a constituent of human plasma that circulates in free and bound forms associated with lipoproteins. β2GPI is a single chain glycoprotein containing 326 amino acids that comprise five complement control protein module-type repeats, also known as short consensus repeats or Sushi domains. Short consensus repeats are characterized by disulfide bridges joining the 1st–3rd and 2nd–4th cysteine residues (1–4). The first four domains of β2GPI structurally resemble each other, whereas DV has three internal disulfide bonds and an extra C-terminal loop encompassing residues Cys$^{266}$-Cys$^{268}$. The C-terminal loop is surface-exposed and susceptible to proteolytic cleavage (5, 6). The region defined by Cys$^{261}$-Cys$^{268}$ is critical for phospholipid and heparin binding and is highly conserved (5, 7–12). We have previously reported that β2GPI is proteolytically clipped between Lys$^{317}$ and Thr$^{318}$ in DV, abolishing its binding to anionic phospholipids but not heparin (5, 11). Cleavage at Lys$^{317}$-Thr$^{318}$ is generated in vitro by plasmin (13). Heparin greatly enhances the plasmin-mediated cleavage of the Lys$^{317}$-Thr$^{318}$ site in β2GPI at concentrations that can be achieved in vivo during anticoagulation therapy (11). The cleavage at Lys$^{317}$-Thr$^{318}$ is generated in vitro by plasmin and at extremely low efficiency by factor Xa (13) and in vivo in pathological states of increased fibrinolysis (13–15).

β2GPI is the principle antigenic target for antiphospholipid antibodies in patients with the antiphospholipid syndrome (APS) (16, 17). APS is a condition associated with recurrent arterial or venous thrombosis, complications of pregnancy including fetal loss and preeclampsia, and the presence of antiphospholipid antibodies (8, 18, 19). The binding of autoantibodies to β2GPI is now generally accepted as an important feature of APS, and a number of studies have shown that there is a significant correlation between thrombotic manifestations and the presence of anti-β2GPI antibodies (8, 20). The pathophysiological mechanisms by which autoantibodies exert their adverse effects remain unknown. However, β2GPI autoantibodies have been implicated in the aberrant activation of endothelial cells (21–23) by binding to β2GPI complexed with annexin II (24) or members of the toll-like receptor family (25). Additionally, autoantibody-bound β2GPI may activate platelets via interaction with the apolipoprotein E receptor 2 (26). We have recently reported that β2GPI binds factor XI (FXI) and inhibits its activation to factor XIa (FXIa) by thrombin and factor XIIa (27). β2GPI cleaved by plasmin at Lys$^{317}$-Thr$^{318}$ bound FXI as well as intact β2GPI but did not inhibit its activation by thrombin (27). This may be an important mechanism for regulation of the amplification pathway of the coagulation cascade, and autoantibodies in patients with APS may interfere with this inhibition, leading to thrombosis.

FXI is a unique zymogen that circulates in plasma as a disulfide-bound homodimer (28). FXIa catalyzes the activation of factor IX to its active form factor IXa (29). FXI/FXIIa binds to activated human platelets in the presence of high molecular weight kininogen (HK) and zinc or prothrombin and calcium ions in a specific and reversible manner (30–32). Activated platelets promote FXI activation by thrombin in the presence of HK or prothrombin, thereby initiating the intrinsic coagulation pathway independent of contact proteins and amplifying the generation of thrombin.

β2GPI proteolytically clipped at Lys$^{317}$-Thr$^{318}$ has been thought to be physiologically inactive as it loses its phospholipid binding properties. However, Koike and co-workers (33) have recently demonstrated that β2GPI proteolytically cleaved by plasmin further suppresses plasmin generation by binding
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to Glu-plasminogen via DV. This interaction suggests a negative feedback loop controlling extrinsic fibrinolysis (33). Increased levels of clipped β₂GPI (cβ₂GPI) have been reported in the plasma of patients with lupus anticoagulant and in patients with disseminated intravascular coagulation (13). Moreover, increased fibrinolysis and thrombin generation occurs in patients with disseminated intravascular coagulation and antiphospholipid antibodies (13–15).

In this study, we identified specific residues on β₂GPI involved in binding FXI/FXIIa and report that DV of β₂GPI is the major FXI/FXIIa binding site. Moreover, FXIIa cleaved β₂GPI at Lys817–Thr218 in DV. This cleavage of β₂GPI may represent a unique mechanism in the control of FXI activation and fibrinolysis.

EXPERIMENTAL PROCEDURES

Materials—Plasma-derived FXI, FXIIa, and HK were purchased from Calbiochem-Novabiochem. Substrate S2366 was from Chromogenix Instrumentation Laboratory SpA (Milano, Italy). Baculovirus vector pBacPak6 was obtained from Clontech Laboratories (Franklin Lakes, NJ). Spodoptera frugiperda (SF9) cells and serum-free medium SF900II were purchased from Invitrogen. Hirudin, polybrene (hexadimethrine bromide), human serum albumin, and bovine serum albumin (BSA) were purchased from Sigma. Plasma-derived native β₂GPI (nβ₂GPI) was purchased from Hematologic Technologies Inc. (Essen Junction, VT) or purified in our laboratory as previously described (16, 27).

Recombinant Human β₂GPI Preparations—Eight β₂GPI mutants were utilized in this study to define the binding site on β₂GPI for FXI. Recombinant full-length human β₂GPI (rhβ₂GPI) and domain deletion mutants of human β₂GPI were generated as described previously (34–36). The cDNAs encoding human β₂GPI were inserted into the baculovirus vector pBacPak6, and the nucleotide sequences of the cDNAs were confirmed by standard sequencing technology. SF9 cell monolayers were infected with the resultant constructs and were cultivated 3–5 days at 25 °C in serum-free medium. Affinity chromatography with a nickel column or a rabbit polyclonal anti-β₂GPI antibody was used to purify the resulting recombinant proteins in the conditioned medium. The purified proteins were analyzed by SDS-PAGE/immunoblot and subjected to automated Edman sequencing using Applied Biosystems (Foster City, CA). An oligonucleotide-directed N-terminal Sequencing analysis of the iodinated proteins revealed only one radioactive band, indicating that there was no contamination of FXI with FXIIa.

Binding of 125I-FXI to Mutants of β₂GPI—the binding of 125I-FXI to immobilized rhβ₂GPI was performed using Lockwell™ microtiter plates (Nunc, Roskilde, Denmark) as described (27, 36). Microtiter wells were coated with 100 μL of rhβ₂GPI, DI, DI-II, DI-III, DI-IV, DII-V, DV, mutant 2K, or mutant 3K of human β₂GPI or human serum albumin (3.12–200 nM) by incubation overnight at 4 °C. The plate was washed five times with PBS, 0.1% Tween 20 (PBST) using an automated microplate washer (Beckman Coulter Inc., Fullerton, CA). The wells were blocked with 2% BSA/PBST for 2 h at 25 °C. The plate was washed five times with PBST and then five times with PBS. 100 μL of 125I-FXI (0.56 nM) was added to the wells and incubated for 4–5 h at 25 °C. The wells were then washed five times with 0.5% BSA/PBS, air-dried, and counted in a γ-counter. The number of counts/min were measured and converted to the percentage of total binding by dividing by the counts/min bound in the absence of competitors. The IC50 was calculated by non-linear regression (one-site binding model, GraphPad Prism 3.03).

Saturation Binding of FXIIa to β₂GPI—Saturation binding of FXIIa to immobilized rhβ₂GPI or HK was performed using Lockwell microtiter plates, the wells of which were coated with 100 μL of HK, rhβ₂GPI, or BSA (100 nm) by incubation overnight at 4 °C. Plates were washed five times with PBST using a microplate washer. The wells were blocked with 2% BSA/PBST for 2 h at 25 °C and then washed five times with PBST and five times with PBS. 100 μL of various concentrations of THF (0.09–50 nm) in PBS then was added to individual wells and incubated for 3 h at 37 °C. The wells were washed five times with 0.5% BSA/PBS. 200 μL of FXIIa substrate (S2366, 600 μM) was then added to individual wells and incubated for 1 h at 25 °C, and then the optical density was measured at 405 nm using a microplate-scanning spectrophotometer (Power Wave, BIO-TEK Instruments Inc., Winoski, VT). The amount of FXIIa bound was derived from a standard curve constructed with the known concentrations of FXIIa.

Binding of FXIIa to Native, Recombinant, and Domain Deletion Mutants of β₂GPI—the binding of FXIIa to various preparations of immo- bilized β₂GPI was performed using Lockwell microtiter plates. The wells were coated with 100 μL of HK (single chain), nβ₂GPI, rhβ₂GPI, DI-IV, DII-V, DV of β₂GPI, or BSA (6.25–400 nm) by incubation overnight at 4 °C. The plate was treated as described above and 100 μL of FXIIa (10 nm) in 0.5% BSA/PBS was then added to individual wells and incubated for 3 h at 37 °C. The wells were washed five times with 0.5% BSA/PBS. FXIIa substrate (S2366, 600 μM) was then added to individual wells and the amount of FXIIa bound was derived as above.

Time Course of FXIIa Cleavage of β₂GPI—To investigate the time course of β₂GPI cleavage by FXIIa, nβ₂GPI (30 μM) was incubated with FXIIa (0.1 μM) in Tris-buffered saline buffer (pH 7.6) at 37 °C. At defined time points, 130 μL of aliquots were diluted 20-fold in heparin affinity column binding buffer (0.1 M Tris-HCl, 0.03 M NaCl (pH 8.0)) and then applied to heparin affinity columns (1 ml of cnβ₂GPI and intact nβ₂GPI were separated with a linear gradient (0.10–0.35 M NaCl, 0.01 M Tris-HCl (pH 8.0)) as described previously (11). The eluted peak area of cnβ₂GPI and intact nβ₂GPI was calculated using Primeview Evaluation software (Amersham Biosciences), and the amount of cnβ₂GPI and intact nβ₂GPI was expressed as a percentage of total nβ₂GPI binding to the heparin affinity column.

RESULTS

FXI/FXIIa Bind β₂GPI via Lysine Residues in DV—To investigate the FXI binding site on β₂GPI, we measured 125I-FXI binding to β₂GPI and various mutants coated on microplate wells. 125I-FXI bound to nβ₂GPI, rhβ₂GPI (27), domain deletion mutants DV, and DI-II-V but not to DI, DI-II, DI-III, or DI-IV (Fig. 1). These findings indicate that 125I-FXI binds to the C-terminal domain of β₂GPI. To further characterize the residues in DV that are critical for binding FXI, we tested β₂GPI containing point mutations in DV. The binding of FXIIa was negligible with Lys286 and Lys287 mutated to Glu (Fig. 1, 2K Mutant) and with Lys284, Lys286, and Lys287 mutated to Glu (Fig. 1, 3K Mutant). Thus the positively charged residues in DV of β₂GPI are critical for the interaction of FXI with β₂GPI.
Taken together, these results support the hypothesis that the activity of FXIa in the amidolytic assay with its chromogenic substrate (data not shown).

To confirm the interaction of DV with FXI, we used the panel of β₂GPI mutants in fluid phase to inhibit binding to immobilized rhβ₂GPI. The binding of ¹²⁵I-FXI to rhβ₂GPI was competitively inhibited in a dose-dependent manner by rhβ₂GPI, rDI-V, or DV with IC₅₀ values of 0.115, 0.167, 0.075, and 0.079 μM, respectively (Fig. 2). There was negligible inhibition with domain deletion mutants DI, DI-II, DI-III, and DI-IV at final concentrations of up to 2 μM (Fig. 2). Thus, the results confirm that DV contains the major FXI binding site on β₂GPI. To further define DV residues critical for binding, mutants 2K and 3K were tested in similar inhibition experiments. Neither mutant inhibited the binding of ¹²⁵I-FXI to rhβ₂GPI at concentrations of up to 2 μM (Fig. 2).

Interaction of FXIa with β₂GPI was assessed in direct binding experiments using FXIa enzymatic activity on the chromogenic substrate S2366 as described under “Experimental Procedures.” The Kₛ of FXIa binding to β₂GPI (3.86 ± 0.91 nM) was similar to HK (3.65 ± 0.20 nM) (Fig. 3). The Bₘₐₓ of FXIa binding to HK (0.14 ± 0.0042 pmol) was 3.18-fold greater than the Bₘₐₓ of FXIa binding to β₂GPI (0.045 ± 0.005 pmol). The Kₛ of FXIa binding to β₂GPI was much lower than that (Kₛ = 15.43 ± 1.00 nM) of FXIa binding to β₂GPI (27), indicating a higher affinity interaction with FXIa. Direct binding experiments demonstrated that FXIa bound to nβ₂GPI, rhβ₂GPI, rDI-V, and DV but not to rDI-IV or BSA (Fig. 4), confirming that FXIa also binds to DV of β₂GPI. Previous studies have shown that the peptide loop defined by Cys²⁸¹-Cys²⁸⁸ is critical for binding to phospholipids and heparin (9, 11, 27). Taken together, these results support the hypothesis that the peptide loop defined by Cys²⁸¹-Cys²⁸⁸ and containing Lys²⁸⁴, Lys²⁸⁶, and Lys²⁸⁷ may mediate multiple binding functions in vivo.

FXIa Enzymatic Activity Is Not Influenced by β₂GPI—As previously reported [27], β₂GPI did not influence the enzymatic activity of FXIa in the amidolytic assay with its chromogenic substrate (data not shown).

FXIa cleaves β₂GPI at Lys²¹⁵-Thr²¹⁸—Nβ₂GPI was incubated with FXIa at a molar ratio of 300:1 for up to 24 h. When applied to a heparin affinity column, the β₂GPI consisted of two elution peaks (Fig. 5A). This chromatogram has a profile similar to that seen when nβ₂GPI is subjected to plasmin cleavage (11, 13, 39). The amount of nβ₂GPI generated from nβ₂GPI increased over time, and greater than 50% nβ₂GPI had been cleaved by FXIa at 5 h of incubation (Fig. 5B). The amount of nβ₂GPI reached a plateau (80% cleavage of nβ₂GPI) at 24 h of incubation with FXIa (Fig. 5B).

N-terminal sequencing showed that there were two N termini in the nβ₂GPI preparations incubated with FXIa for 6 and 24 h (Table I). The affinity-purified nβ₂GPI preparation also had two N termini (Table I). The nβ₂GPI preparations incubated without FXIa for 0 and 24 h at 37°C and with FXIa at 0 time point revealed a single sequence with one N terminus (Table I). The results indicate that FXIa cleaved nβ₂GPI at
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DISCUSSION

We recently reported that β2-GPI binds FXI and inhibits its activation by thrombin and factor XIIa in the presence of dextran sulfate at concentrations lower than those normally found in human plasma (27). In this study, we established that the interaction of β2-GPI with FXI/FXIIa occurs in DV of β2-GPI. There was no binding of FXI/FXIIa to domain deletion mutants of β2-GPI that lacked DV. Furthermore, the peptide region spanning residues Cys281-Cys288 in DV is the FXI binding site. Positive-charged residues in this region at Lys284, Lys286, and Lys287 were found to be critical in the interaction of β2-GPI with FXI. The same lysine residues mediate binding to phospholipids and heparin (10, 11). In addition, FXIIa cleaves β2-GPI at Lys317-Thr318 within DV. The cleavage site on β2-GPI for FXIa is the same as that for plasmin (13–15). Functional studies demonstrate the significance of maintaining the integrity of the C-terminal loop in DV, because cleavage of DV at Lys317-Thr318 retains the binding of β2-GPI with FXI but attenuated or abolished the ability of β2-GPI to inhibit activation of FXI by thrombin (27).

β2-GPI consists of five complement control protein domains, the first four being more typical of the complement control protein family (1). DV terminates in a disulfide bridge and has a lysine surface patch that is predicted to be very mobile between Leu313 and Thr318, thus being poorly resolved on crystallographic studies (6, 40). The hydrophobic segment corresponding to residues Leu313-Trp316 is critical in binding with anionic phospholipids but not with heparin (11, 41).

The C-terminal loop and the peptide region Cys281-Cys288 in DV have been predicted to be involved in the interaction with lipid membrane surfaces (8, 40). This C-terminal loop is identical in human, bovine, and canine sequences and only differs in the first four being more typical of the complement control protein family (1). DV contains a positively charged lysine surface patch that is predicted to be very mobile between Leu313 and Thr318, which is a critical feature of the binding site for FXIa (11, 13). The positive-charged residues in this region at Lys284, Lys286, and Lys287 were found to be critical in the interaction of β2-GPI with FXI. The same lysine residues mediate binding to phospholipids and heparin (10, 11). In addition, FXIIa cleaves β2-GPI at Lys317-Thr318 within DV. The cleavage site on β2-GPI for FXIa is the same as that for plasmin (13–15). Functional studies demonstrate the significance of maintaining the integrity of the C-terminal loop in DV, because cleavage of DV at Lys317-Thr318 retained the binding of β2-GPI with FXI but attenuated or abolished the ability of β2-GPI to inhibit activation of FXI by thrombin (27).

Cleavage at Lys317-Thr318 produces two short polypeptide segments that remain linked to DV by disulfide bonds (5). This cleavage alters the spatial array of the three critical Lys residues and abolishes the ability of β2-GPI to bind anionic phospholipid surfaces (5). It has been reported that cleavage of β2-GPI at Lys317-Thr318 disturbs the nearby electrostatic environment (42). It is believed that the integrity of the 317/318 peptide bond is important in tethers the cluster of positively charged and hydrophobic residues in this region. Although the hydrophobic C-terminal loop is important in phospholipid binding, we have recently reported that a preparation of β2-GPI cleaved at Lys317-Thr318 by plasmin bound to FXI as well as rhβ2-GPI but did not inhibit thrombin activation of FXI (27).

Proteolytically cleaved β2-GPI at Lys317-Thr318 is found in the plasma of patients with lupus anticoagulant, disseminated intravascular coagulation, ischemic stroke, and in healthy individuals with lacunar infarct(s) (14, 15, 17, 33). It has been proposed that FXI plays an important role in the down-regulation of fibrinolysis (Fig. 6) (43). This is thought to occur as a
FIG. 6. \( \beta_2 \)GPI proteolytically clipped at Lys\(^{317}\)-Lys\(^{318}\) inhibits plasmin generation but not FXI activation. A schematic representation of the effect of \( \alpha_2 \)-GPI and \( \beta_2 \)GPI on FXI activation and plasmin generation is shown. V, activation; \( \beta_2 \)-GPI, inhibition. FDP, fibrin degradation products; sct-PA, single chain tissue plasminogen activator; tct-PA, two chain tissue plasminogen activator; PAI-I, plasminogen activator inhibitor-I; \( \alpha_2 \)-AP, \( \alpha_2 \)-antiplasmin; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFI\( \alpha \), activated TAFI; \( \gamma \)-2AP, attenuation of inhibition.

consequence of a FXI-dependent burst in thrombin generation, which results in the activation of thrombin-activatable fibrinolytic inhibitor, which inhibits the activation of plasminogen (43–45). The conversion of plasminogen to plasmin by two-chain tissue plasminogen activator is a key event in the fibrinolytic system, because plasmin is a critical protease in this system. Regulation of plasmin generation is important in \( in \ vivo \) fibrinolytic homeostasis. Proteolytically cleaved \( \beta_2 \)GPI at Lys\(^{317}\)-Thr\(^{318}\) has recently been demonstrated to inhibit plasmin generation by tissue plasminogen activator (35). The clipped form of \( \beta_2 \)GPI correlated with \( in \ vitro \) markers of fibrinolytic activity. The C-terminal hydrophobic loop of \( \beta_2 \)-GPI has been confirmed by heteronuclear magnetic resonance to be tightly fixed by electrostatic interaction with the lysine cluster at the phospholipid binding site while at the same time enhancing stability and neutralizing the positive charge in this region (42). Thus, the C-terminal loop of clipped \( \beta_2 \)GPI is more mobile than that of the intact molecule, possibly allowing the interaction of \( \beta_2 \)GPI with FXI/thrombin complexes, such that it binds FXI but does not inhibit its activation by thrombin (27).

\( in \ vivo \), it would appear that activated platelets provide an appropriate procoagulant surface for assembly of surface bound protease substrate complexes. FXI/FXIIa bind activated platelets in a saturable and reversible manner with \( K_\text{d} = 10 \) and 0.8 nM, respectively (46, 47). HK and zinc ions or prothrombin and calcium ions have been shown to promote this binding (30–32). \( \beta_2 \)GPI binds FXI and could possibly substitute for HK or prothrombin \( in \ vivo \) in the interaction of FXI with activated platelets. Thus, the clipping of \( \beta_2 \)GPI by FXIIa bound in a complex with FXIIa on activated platelets could abolish its phospholipid binding but not its FXI/FXIIa binding, a negative feedback that counters its inhibition of FXI activation by thrombin, because in the presence of activated platelets, thrombin is the preferred activator of FXI (48).

The interaction of \( \beta_2 \)GPI with FXI/FXIIa on the surface of activated platelets would facilitate the inhibition of FXI activation and the subsequent generation of \( \alpha_2 \)-GPI by FXIIa. The FXIIa cleavage of \( \beta_2 \)GPI in this complex would regulate the activation of FXI by thrombin, providing a negative feedback loop in FXI activation. Furthermore, \( \beta_2 \)GPI binds Glu-plasminogen-suppressing plasmin generation, thus providing a negative feedback loop controlling fibrinolysis (33). A schematic representation of the effect of \( \alpha_2 \)-GPI and intact \( \beta_2 \)GPI on this pathway is shown in Fig. 6.

Plasmin and FXIIa cleave \( \beta_2 \)GPI, but \( in \ vivo \), it is not clear which protease is responsible for the elevated levels of \( \beta_2 \)GPI demonstrated in certain patient populations. Even though 50% of the plasma \( \beta_2 \)GPI was cleaved at Lys\(^{317}\)-Thr\(^{318}\) in some of the patients with lupus anticoagulant, there was a minimal increase in plasmin inhibitor complex and D-dimer levels, indicating that plasmin is not the major protease \( in \ vivo \) responsible for this cleavage of \( \beta_2 \)GPI. Because FXIIa cleaves \( \beta_2 \)GPI \( in \ vivo \) as efficiently as plasmin and factor Xa is the only other plasma protease reported to cleave \( \beta_2 \)GPI with very poor efficiency by one group (13) but not confirmed by Horbach et al. (15), it is likely that FXIIa is the major \( in \ vivo \) protease responsible for this cleavage in patients with lupus anticoagulant.

In conclusion, we first have demonstrated that \( \beta_2 \)GPI binds FXI/FXIIa by its C-terminal domain and that lysine residues in the region CyS\(^{281}\)-CyS\(^{288}\) are critical in this interaction and in the inhibition of FXI activation by thrombin (27). Second, we have demonstrated that FXIIa cleaves \( \beta_2 \)GPI at Lys\(^{317}\)-Thr\(^{318}\) at its C terminus, which abolishes its inhibition of FXI activation by thrombin (27). We propose that \( \beta_2 \)GPI \( in \ vivo \) is proteolytically cleaved by FXIIa and that \( \beta_2 \)GPI represents a unique mechanism in the control of FXI activation and fibrinolysis.

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