# Investigating the binding interaction between human factor XIII and fibrinogen

## Abstract

Venous thrombosis occurs when pathological clotting arises in the venous circulation. Factor XIII (FXIII) crosslinks fibrin, increasing the mechanical stability of clots. FXIII binds to fibrinogen, which accelerates enzymatic activation of FXIII. Recent work implicated FXIII-fibrinogen binding as a determinant of thrombus size. Murine studies suggest that fibrinogen γ-chain residues 390-396, which are highly conserved in human fibrinogen, mediate this binding. We hypothesized that mutating human γ-chain residues 390-396 would disrupt FXIII-fibrinogen binding. We expressed the native γ-module (residues γ148-411, γ-mod$^{Nat}$) and a γ-module with residues 390-396 mutated to alanines (γ-mod$^{390\text{-}396A}$). γ-mod$^{390\text{-}396A}$ exhibited decreased affinity for FXIII relative to γ-mod$^{Nat}$ in an enzyme-linked immunosorbent assay, suggesting that γ-chain residues 390-396 mediate FXIII-fibrinogen binding in humans. Future studies will use γ-mod$^{Nat}$ as "bait" to probe FXIII for the residues involved in fibrinogen binding. Identification of the binding residues on FXIII may yield a potential target for reducing venous thrombosis.

## Introduction

As many as 1,000,000 cases of venous thrombosis and pulmonary embolism, collectively termed venous thromboembolism, arise every year in the United States with a mortality rate of 30% within 30 days of incidence.[1] Venous thrombosis occurs when pathologic clotting arises in venous circulation.[2] These intravenous clots, termed venous thrombi, are mainly composed of red blood cells and fibrin.[2] Coagulation is activated in vein valve pockets, leading to thrombin-mediated conversion of soluble plasma fibrinogen into insoluble fibrin. Fibrin then polymerizes, forming the framework of the clot. Venous thrombi can detach from the endothelium and occlude lung vasculature in a process called pulmonary embolism.

Recently, the Wolberg laboratory determined that FXIII activity contributes to venous thrombosis.[3] FXIII is a protransglutaminase that circulates as a tetramer composed of two catalytic A subunits and two inhibitory B subunits (FXIII-$A_2B_2$). FXIII is activated during coagulation when thrombin, a serine protease, cleaves an activation peptide from the FXIII-A subunits.[4,5] This reaction is followed by calcium-mediated dissociation of the regulatory FXIII-B

subunits.[6] Activated FXIII covalently crosslinks fibrin, strengthening the clot and rendering it more resistant to lysis.[7] FXIII crosslinking of fibrin also promotes red blood cell retention in venous thrombi and consequently, determines venous thrombus size.[3,8]

FXIII and fibrinogen circulate as a tightly-bound complex ($K_D$ ~10 nM[9]), and fibrinogen delivers bound FXIII to nascent clots. The majority of FXIII in plasma is bound to fibrinogen.[10] FXIII-fibrinogen binding accelerates FXIII activation by promoting FXIII-B dissociation and thrombin-mediated cleavage of FXIII-A activation peptide.[11] However, the residues that mediate FXIII-fibrinogen binding are currently unknown. Aleman *et al.* found that mice with residues 390-396 on the γ-chain of fibrinogen mutated to alanines (Fibγ$^{390\text{-}396A}$) showed delayed FXIII activation relative to wild-type. This finding suggests that fibrinogen residues 390-396 facilitate FXIII binding in blood.[3] Consistent with this observation, Fibγ$^{390\text{-}396A}$ fibrinogen displays 6-fold reduced binding affinity for FXIII compared to wild-type fibrinogen and a corresponding 5-fold decrease in FXIII activation rate.[3] In venous thrombosis models, Fibγ$^{390\text{-}396A}$ mice produce smaller thrombi with reduced red blood cell content, similar to FXIII-A-deficient mice.[3] Interestingly, Fibγ$^{390\text{-}396A}$ mice do not exhibit a bleeding phenotype. Collectively, these findings suggest that the FXIII-fibrinogen interaction is a potential target for reducing venous thromboembolism while limiting the risk for bleeding.

The goal of my project was to define the key residues for binding on both fibrinogen and FXIII. Because γ-chain residues 390-396 are highly conserved between mouse and human, we hypothesized that these residues also mediate FXIII-fibrinogen binding in humans. My findings indicate that when γ-chain residues 390-396 are mutated to alanines, the γ-module of fibrinogen has decreased affinity for FXIII-$A_2B_2$ compared to wild-type γ-module and that FXIII-$A_2$ and

FXIII-$A_2B_2$ do not crosslink the 390-396A mutant γ-module as effectively as wild-type γ-module.

## Materials and Methods

### Materials and Instruments

Full-length human fibrinogen cDNA was provided by Dr. Susan Lord (University of North Carolina at Chapel Hill). *Taq* polymerase was obtained from New England BioLabs, Inc. (Ipswich, MA). Forward and reverse primers for the γ-modules (fibrinogen γ-chain residues 148-411) were purchased from Eurofins Genomics (Huntsville, AL). FastDigest® Restriction Enzymes (*BamHI, NdeI, HindIII, NcoI*) and GeneJET PCR Purification and Gel Extraction Kits were obtained from Thermo Scientific (Pittsburgh, PA). Polyclonal anti-human fibrinogen was from Dako (Carpinteria, CA). Human FXIII-$A_2B_2$, thrombin, and anti-human FXIII-A antibody were from Enzyme Research Laboratories (South Bend, IN). SureBlue™ TMB Microwell Peroxidase Substrate (1-Component) was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Recombinant FXIII-$A_2$ was a generous gift of Novo Nordisk (Bagsværd, Denmark). QuikChange Site-Directed Mutagenesis Kit was from Agilent Technologies (Cedar Creek, TX). Mutagenesis primers and anti-human FXIII-B antibody were from Sigma-Aldrich (St. Louis, MO). Corifact® Human FXIII concentrate was from CSL Behring (King of Prussia, PA). Centrifugal filters (5 kDa cut-off) were purchased from EMD Millipore (Bellerica, MD). FeBABE was from Dojindo Molecular Technologies, Inc. (Rockville, MD).

### Expression of γ-module in *E. coli*

Expression of γ-module in *E. coli* was based on a method established by Medved *et al.*[12] Polymerase chain reaction (PCR) of γ-module DNA utilized a forward primer that contained

restriction enzyme sites for *BamHI* and *NdeI* directly upstream of the γ-module start codon and a reverse primer containing a site for *HindIII* following the stop codon (Figure 1). PCR was carried out with a Techne Progene FRROG05Y Thermocycler. γ-module DNA was isolated from the initial PCR product in agarose gel, which was stained with ethidium bromide (0.5 μg/mL). Next, the PCR product was digested with *BspHI* and *HindIII*, and pET28b vector was digested with with *NcoI* and *HindIII* for 2 hours at 37°C. The digested insert and vectors were ligated, and DH5α *E. coli* was transformed with the resultant plasmid. Transformed *E. coli* were grown on agar plates with kanamycin, which selects for *E. coli* with the pET28b vector. Following *in vivo* amplification of vectors, a colony PCR technique was performed to amplify the plasmid DNA and identify positive colonies. DNA from positive colonies was sequenced by Eton Biosciences (Durham, NC) to screen for point mutations. Positive DNA from the colony PCR was used to transform Rosetta™ Cells (Novagen, Darmstadt, Germany). γ-module expression was induced with isopropyl β-D-1-thiogalactopyranoside. After expressing protein overnight, the protein was isolated, re-solubilized, and re-folded *in vitro* because *E. coli* releases the γ-module in inclusion bodies.[12]

Enzyme-Linked Immunosorbent Assay (ELISA) of FXIII and γ-module

A high-binding 96-well microplate was coated with human FXIII-$A_2B_2$ and incubated for 1 hour at room temperature. After incubation, the wells were washed with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 150 mM NaCl (HBS) containing 1 mM $Ca^{2+}$ (HBS plus $Ca^{2+}$) and incubated with Cappel horseradish peroxidase-goat-anti-human fibrinogen antibody at 4°C overnight to deplete the antibody of anti-FXIII activity. Additional wells were coated with either 10 μg/mL FXIII or bovine serum albumin in HBS plus $Ca^{2+}$ and incubated for 1 hour. Wells were washed with wash buffer and blocked with HBS plus $Ca^{2+}$ for

90 minutes. After washing again, γ-module (3.2 nM - 50 μM) was added to the wells and incubated for 1 hour. Wells were then washed with HBS plus $Ca^{2+}$, and anti-FXIII activity depleted antibody was added and incubated for 60 minutes. Finally, the wells were washed with HBS plus $Ca^{2+}$, SureBlue™ substrate was added, and reactions were monitored at 600 nm on a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA). We measured the maximum rate of change in absorbance ($V_{max}$) and correlated $V_{max}$ with FXIII-γ-module binding affinity.

Crosslinking of γ-module by FXIII

To examine the kinetics of γ-module crosslinking by FXIII-$A_2B_2$ (0.077 mg/mL, 240 nM, final) or recombinant FXIII-$A_2$ (0.040 mg/mL, 240 nM, final), FXIII was incubated with γ-module (0.4 mg/mL, final) at room temperature and FXIII activation was triggered with thrombin (5 nM, final) and calcium (5 mM, final). All reactions were performed in Tris-buffered saline (40 mM Tris base, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4). Reactions were stopped by the addition of sodium dodecyl sulfate (SDS) loading dye and boiling for 5 minutes. To determine the rate of FXIII solution phase activation the reactions were repeated without γ-module. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, probing for fibrinogen and FXIII-A.

Site-directed mutagenesis of γ-module and tethered cleavage

Primers were designed to induce cysteine substitutions at residues 389, 393, and 397 of the γ-module, termed γ-$mod^{F389C}$, γ-$mod^{T393C}$, and γ-$mod^{G397C}$, respectively. Using the QuikChange Kit from Agilent, mutated DNA was transformed into Rosetta™ Cells. Expression of these constructs utilized the aforementioned γ-module expression protocol. Size exclusion chromatography with a Sephadex G-75 stationary phase was used to purify γ-module products

for use in tethered cleavage chemistry. Purified γ-mod$^{G397C}$ was dialyzed into metal removal buffer (30 mM 3-(N-morpholino)propanesulfonic acid, 4 mM ethylenediaminetetraacetic acid (EDTA), pH 8.2) to scour metal ions from the protein for 24 hours at 4°C. FXIII-$A_2B_2$ (isolated from Corifact® Human FXIII concentrate by size exclusion chromatography) was concurrently dialyzed against cutting buffer (50 mM 3-(N-morpholino)propanesulfonic acid, 120 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10% glycerol, pH 8.0) for 24 hours at 4°C. A 5 kDa cut-off centrifugal filter was used to buffer exchange γ-mod$^{G397C}$ from metal removal buffer to conjugation buffer (30 mM 3-(N-morpholino)propanesulfonic acid, 100 mM NaCl, 1 mM EDTA, 5% glycerol, pH 8.2). The cleaving reagent, the iron chelate of bromoacetamidobenzyl-EDTA (FeBABE), was suspended in conjugation buffer, and the FeBABE solution was added to the γ-module and incubated for 1 hour at 37°C. FeBABE-conjugated γ-module was buffer exchanged into cutting buffer with a new 5 kDa cut-off centrifugal filter, and this solution was mixed with the FXIII in cutting buffer solution for 30 minutes, followed by a 10-minute incubation on ice. FeBABE cleavage was triggered with the sequential addition of ascorbic acid (40 mM ascorbic acid, 10 mM EDTA, pH 7.0) and hydrogen peroxide (40 mM hydrogen peroxide, 10 mM EDTA). After 30 seconds of cleavage, reactions were quenched with an equal volume of sample buffer (1:1 solution of SDS sample buffer and 80% glycerol). Samples were boiled for 5 minutes, and cleavage products were identified with SDS-PAGE and Western blotting for FXIII-A and FXIII-B subunits.

## Results

FXIII shows reduced binding affinity for γ-mod$^{390\text{-}396A}$ compared to γ-mod$^{Nat}$

Previous findings implicate murine fibrinogen γ-chain residues 390-396 in FXIII binding.[7] Since γ-chain residues 390-396 are highly conserved between mouse and human, we hypothesized that these residues also mediate FXIII-fibrinogen binding in humans. To test this hypothesis, we isolated human γ-module (fibrinogen γ-chain residues 148-411) constructs with (γ-mod$^{390\text{-}396A}$) and without (γ-mod$^{Nat}$) the 390-396A mutation and compared binding of these constructs to FXIII with an ELISA. We calculated dissociation constants ($K_D$) for FXIII-γ-module binding and found that, compared to γ-mod$^{Nat}$, γ-mod$^{390\text{-}396A}$ had reduced affinity for FXIII-$A_2B_2$ (10.07 versus 90.08 μM, respectively, Figure 2). These data suggest human γ-chain residues 390-396 contribute to FXIII-fibrinogen binding.

Solution phase-activated FXIII crosslinks γ-mod$^{Nat}$ more readily than γ-mod$^{390\text{-}396A}$

FXIII-$A_2B_2$ binding to fibrinogen accelerates enzymatic activation. Accordingly, Aleman et al. observed delayed FXIII-$A_2B_2$ activation and fibrin crosslinking in clots formed from plasma from mice expressing γ390-396A fibrinogen.[3] To investigate the role of these residues in human FXIII activation and crosslinking, we incubated γ-mod$^{Nat}$ and γ-mod$^{390\text{-}396A}$ with thrombin, calcium, and recombinant FXIII-$A_2$ or FXIII-$A_2B_2$. We then detected γ-module crosslinked species using SDS-PAGE and by Western blotting with anti-human fibrinogen antibody. These experiments showed that both recombinant FXIII-$A_2$ and FXIII-$A_2B_2$ crosslinked γ-mod$^{Nat}$ more readily than γ-mod$^{390\text{-}396A}$ (Figure 3A-D).

To test whether the reduced crosslinking seen in γ-mod$^{390\text{-}396A}$ was due to reduced FXIII activation in these reactions, we re-probed blots from the crosslinking experiments for FXIII-A to detect FXIII activation via a band shift associated with activation peptide cleavage. Activation

of both FXIII-$A_2B_2$ and recombinant FXIII-$A_2$ was identical in the γ-mod$^{Nat}$ and γ-mod$^{390\text{-}396A}$ crosslinking reactions (4A-D). Subsequent solution-phase FXIII assays without γ-module indicated identical FXIII activation to that observed in the crosslinking reactions for both FXIII-$A_2B_2$ and recombinant FXIII-$A_2$ (Figure 5A-B). The identical activation rates observed in the presence and absence of fibrinogen suggest FXIII activation is occurring in solution in the crosslinking assays as it is in the solution-phase experiments. Consequently, the effects of the reduced binding affinity of FXIII with γ-mod$^{390\text{-}396A}$ compared to γ-mod$^{Nat}$ do not manifest in this assay. Therefore, the decrease in crosslinking of γ-mod$^{390\text{-}396A}$ compared to γ-mod$^{Nat}$ may be a result of disrupted protein structure in the γ-mod$^{390\text{-}396A}$ that hinders FXIII activity.

FeBABE Cleavage of FXIII

FeBABE reagent cleaves peptide chains within a 12-angstrom radius and is used in tethered cleavage chemistry. Although we have localized binding of FXIII to fibrinogen γ-chain residues 390-396, the FXIII residues mediating this interaction remain unknown. To map the residues on FXIII that bind to fibrinogen, we conducted tethered cleavage chemistry of FXIII with FeBABE. By conjugating FeBABE reagent to γ-module near residues 390-396, we anticipated that cleavage would occur at FXIII residues located near this motif. The reactions with FeBABE-γ-mod$^{G397C}$ conjugates yielded unique bands compared to control reactions (Figure 6A). Unique bands appeared at approximately 27, 35, 38, 45, 50, and 57 kDa when probing for FXIII-A, suggesting that FeBABE reagent cleaved this subunit of FXIII. Additional control reactions containing triggered FeBABE-FXIII conjugates produced a similar band pattern, indicating that FeBABE conjugation to FXIII-A subunits likely occurred during the initial experiment and that the cleaved fragments do not provide information about the FXIII-γ-module binding surface (Figure 6B).

## Discussion

Given the importance of FXIII-fibrinogen interactions in murine thrombosis, studying these interactions in human proteins may reveal new targets to reduce venous thromboembolism. We generated human fibrinogen γ-module in its native form and with alanine mutations to the proposed FXIII-binding region (residues 390-396) and compared the binding affinities of the constructs to FXIII via ELISA. With calculated $K_D$ values of 10.07 μM and 90.08 μM respectively, γ-mod$^{Nat}$ and γ-mod$^{390\text{-}396A}$ both showed significantly decreased FXIII affinity compared to full-length fibrinogen ($K_D$ ~ 10 nM[5]). Thus, although γ-module bound FXIII in my experiments, the data suggest other components of full-length fibrinogen are important for FXIII-fibrinogen binding or that the γ-module is not in its native conformation. Nonetheless, the higher affinity of γ-mod$^{Nat}$ versus γ-mod$^{390\text{-}396A}$ to FXIII supports my hypothesis that γ-chain residues 390-396 contribute to FXIII-fibrinogen interactions in humans.

The crosslinking assays indicate that FXIII cannot crosslink γ-mod$^{390\text{-}396A}$ as efficiently as γ-mod$^{Nat}$. The band pattern of FXIII activation peptide cleavage is identical in crosslinking reactions and solution-phase reactions without γ-module. Given that crosslinking reactions do not utilize pre-incubated FXIII and γ-module, these results suggest that FXIII was not bound to either γ-module when activated in the crosslinking experiments. The inefficiency of γ-mod$^{390\text{-}396A}$ crosslinking relative to γ-mod$^{Nat}$ crosslinking, therefore, cannot be attributed to hindered FXIII activation due to deficient FXIII binding. Instead inefficient crosslinking might be the result of the 390-396A mutation disrupting γ-module structure in a way that impairs crosslinking.

Given the identical cleavage patterns of FeBABE-FXIII conjugated protein in the final control and the experimental reactions, any cleavage of FXIII is likely due to FeBABE conjugation to a free cysteine on FXIII-$A_2B_2$ and not because of module-conjugated FeBABE

mediating cleavage at the binding site. Excess FeBABE likely remained in the γ-module solution after conjugation and mixed with FXIII, subsequently conjugating to FXIII cysteine residues during the FXIII-γ-mod$^{G397C}$ incubation. These results indicate that the cleavage products of the reaction do not represent the fibrinogen binding residues of FXIII. Further experiments will more stringently buffer exchange free non-γ-module-conjugated FeBABE reagent out of the reaction.

In future studies, we will reassess my approach to use FeBABE to identify the FXIII residues that bind fibrinogen. The γ-module has proven problematic to work with in that it is difficult to purify and has a much lower affinity for FXIII than the full-length protein. We are exploring the use of a yeast expression system that could dramatically increase γ-module yields and purity.[13] Alternatively, we will conduct studies with full-length fibrinogen rather than the γ-module. For these experiments, we will introduce chemical crosslinks between FXIII and fibrinogen, which will link the binding domains together. By then utilizing protein cleavage, SDS-PAGE, Western blotting, and mass spectrometry, we will analyze the cleavage products for a fragment containing peptides from both FXIII and fibrinogen.[14] We hypothesize that the cleavage fragment containing both FXIII and fibrinogen residues will contain the respective binding domains for each protein. Better understanding of the FXIII-fibrinogen interaction may provide targets for new anti-thrombotic therapies.

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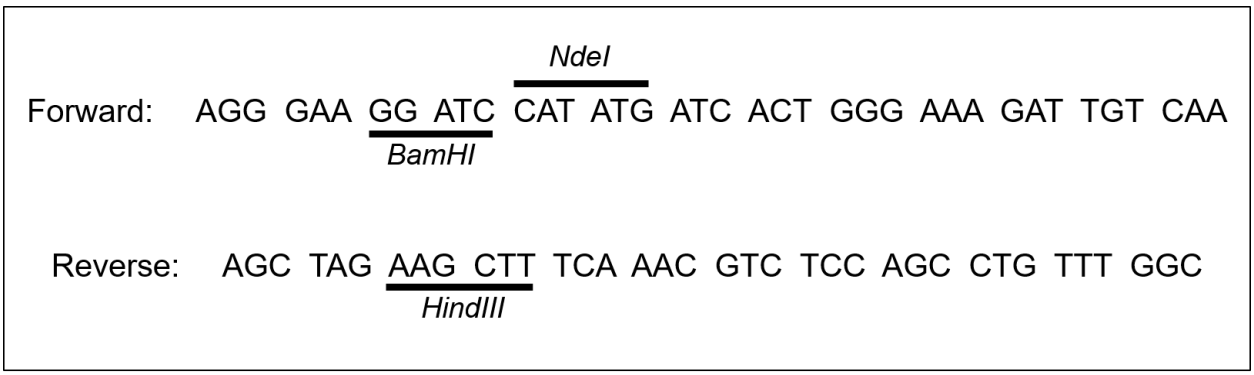


**Figure 1. Forward and reverse primers used in γ-module PCR.** The forward primer contained restriction enzyme sites for *BamHI* and *NdeI*, and the reverse primer contained a restriction enzyme site for *HindIII*.

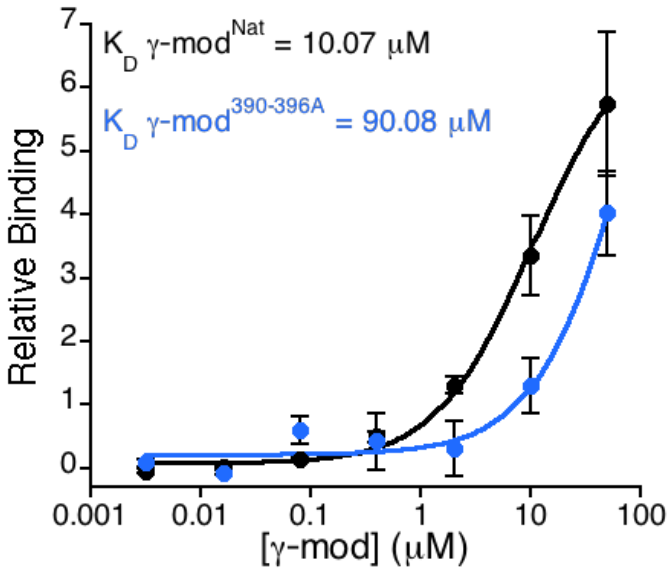


**Figure 2. FXIII-$A_2B_2$ shows reduced affinity for γ-mod$^{390-396A}$ compared to γ-mod$^{Nat}$.** FXIII-γ-module binding was measured by ELISA. Briefly, microplates were coated with 10 μg/mL FXIII-$A_2B_2$ and blocked with HBS plus $Ca^{2+}$ plus 1% bovine serum albumin**.** γ-mod$^{Nat}$ and $^{390-396A}$ were added to the wells, and binding was detected with a peroxidase-conjugated anti-fibrinogen antibody and peroxidase substrate. Data are means ± standard error of n = 3 experiments.

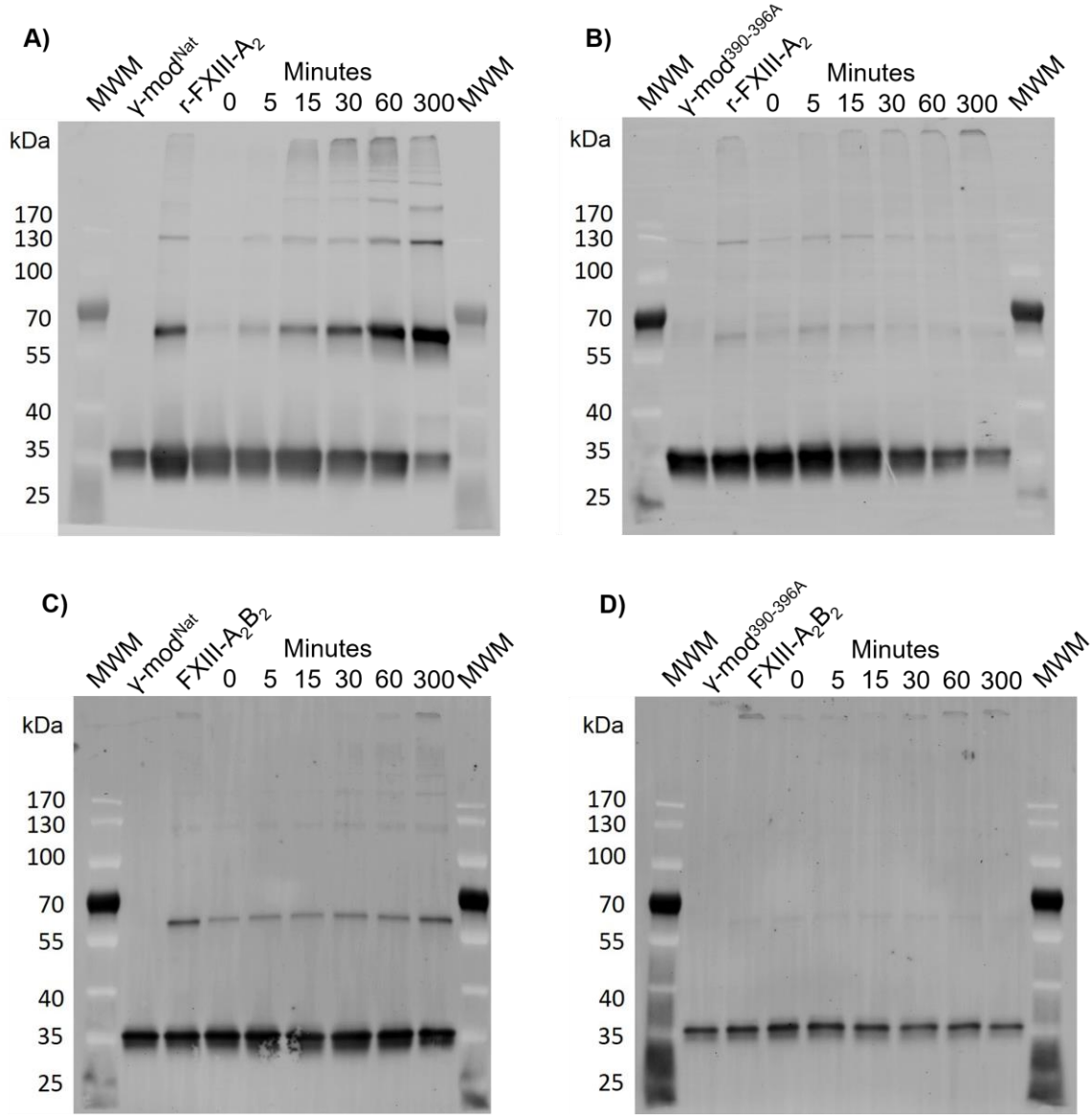


**Figure** 3**. FXIII-mediated crosslinking of γ-mod$^{Nat}$ and γ-mod$^{390-396A}$.** Crosslinking of γ-module by recombinant(r)-FXIII-$A_2$ (A, B) or FXIII-$A_2B_2$ (C, D) was measured by incubating γ-mod$^{Nat}$ (A, C) or γ-mod$^{390-396A}$ (B, D) with FXIII in the presence of thrombin (5 nM) and $CaCl_2$ (5 mM). Timed samples were analyzed by SDS-PAGE and Western blotting with anti-human fibrinogen antibody. Control lanes marked as γ-mod$^{Nat}$ or γ-mod$^{390-396A}$ contain only γ-module without FXIII and thrombin.

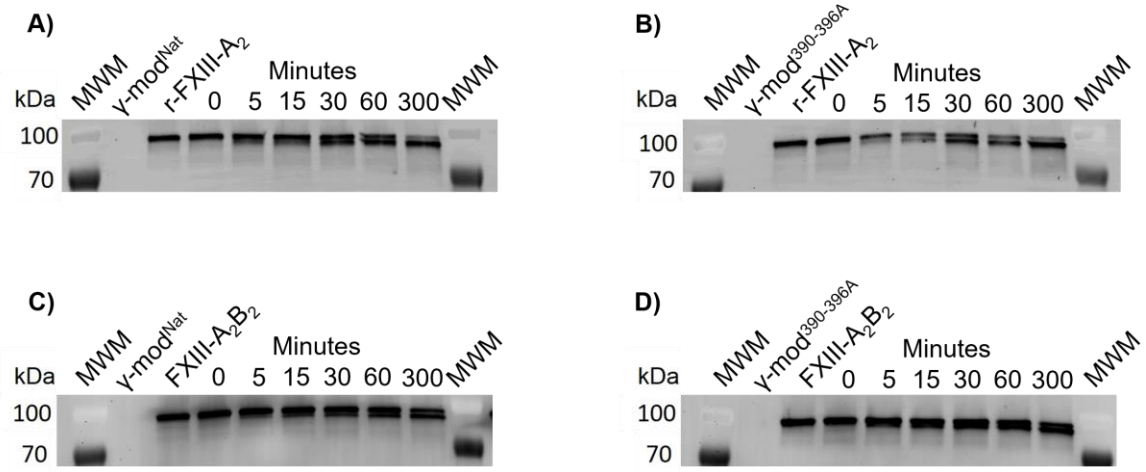


**Figure 4. FXIII-A subunit activation during crosslinking.** Activation rates of r-FXIII-$A_2$ (A, B) and FXIII-$A_2B_2$ (C, D) during crosslinking were measured by incubating γ-mod$^{Nat}$ (A, C) or γ-mod$^{390\text{-}396A}$ (B, D) with FXIII in the presence of thrombin (5 nM) and $CaCl_2$ (5 mM). FXIII activation was detected by re-probing membranes from crosslinking reactions with an anti-human FXIII-A antibody. FXIII activation corresponds with a molecular weight shift following activation peptide cleavage. Control lanes marked as γ-mod$^{Nat}$ or γ-mod$^{390\text{-}396A}$ contain only γ-module without FXIII and thrombin.

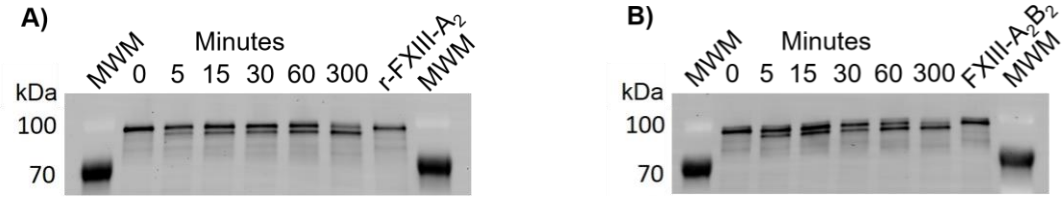


**Figure 5. Solution-phase activation of FXIII-$A_2B_2$ and r-FXIII-$A_2$.** Solution-phase activation rates of r-FXIII-$A_2$ (A) and FXIII-$A_2B_2$ (B) were measured by incubating FXIII in the presence of thrombin (5 nM) and $CaCl_2$ (5 mM). Timed samples were quenched and analyzed by SDS-PAGE and Western blotting with anti-human FXIII-A antibody. Control lanes marked FXIII-$A_2B_2$ and r-FXIII-$A_2$ contained the indicated FXIII and lacked thrombin.

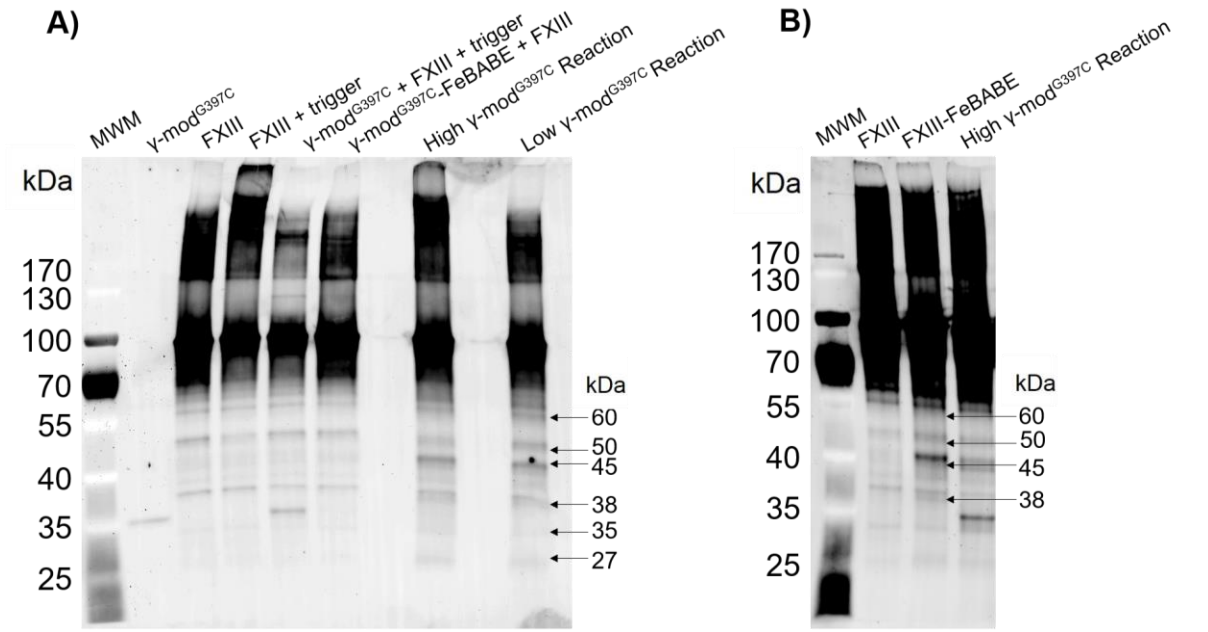


**Figure 6. Tethered cleavage chemistry of γ-mod$^{G397C}$.** In the "Reaction" lanes**,** γ-mod$^{G397C}$ was treated with FeBABE reagent and subsequently incubated with FXIII-$A_2B_2$. "High Reaction" contained a higher concentration of γ-mod$^{G397C}$ compared to the "Low Reaction". "Reactions" and lanes termed "+ trigger" received the ascorbic acid and hydrogen peroxide trigger and were reacted for 30 seconds before being quenched with sample buffer. Controls contained γ-mod$^{G397C}$ alone, FXIII alone, FXIII plus trigger, γ-mod$^{G397C}$ plus FXIII plus trigger, and FeBABE-γ-mod$^{G397C}$ conjugate plus FXIII (not triggered). Samples were analyzed by SDS-PAGE and Western blotting with anti-human FXIII-A antibody. Reaction lanes displayed unique cleavage fragments at approximately 27, 35, 38, 45, 50, and 57 kDa (A). The presence of these same bands in the FXIII-FeBABE control lane (B) suggests that these fragments result from direct conjugation of FeBABE to free cysteines on FXIII-A in the "Reactions".