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Reactive Glutamines and a Binding Site Region Contribute to FXIII Substrate Specificity for Fibrinogen αC

Mohammed Hindi
University of Louisville

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Mohammed M. Hindi

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Department of Chemistry

University of Louisville

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Abstract

The reaction between transglutaminase factor XIII (FXIII) and fibrin is a key process in the final stages of blood coagulation. The αC region of fibrin contains three reactive glutamines (Q237, Q328, and Q366) which are crosslinked by FXIII, as well as a FXIII anchoring site (E396). Previous work has helped elucidate the FXIII-αC interaction, but much is still unknown about the key residues in the αC region.

Four crosslinking assays were carried out to characterize the contribution of the αC anchoring site and reactive glutamines in crosslinking by FXIII. The effects of individual E396A, Q366N, and Q328P (Fibrinogen Seoul II mutant) mutations in Fbg αC were monitored through a GEE crosslinking/ MALDI-TOF MS strategy. These studies revealed that, at physiological conditions, the E396 anchoring site plays the largest role in crosslinking of the most reactive glutamine, Q237, while the other two glutamines had minimal impacts. In addition, the two less reactive glutamines, Q328 and Q366, appear to compete with each other as substrates for FXIII.

Introduction

Of all the body's homeostatic functions, perhaps the most crucial is the ability to stop or slow bleeding, referred to as hemostasis. This function involves blood coagulation, which is a highly time-sensitive process that must be closely regulated. The circulatory system is responsible for maintaining a delicate balance such that exsanguination is prevented, while at the same time avoiding potentially fatal thrombosis [1].

Coagulation is a complex process involving several protein factors and a few non-protein cofactors including Ca^{2+} and phospholipid. The coagulation cascade proceeds through two main pathways, the intrinsic (contact) and extrinsic (tissue factor) pathways (Figure 1) [2]. The intrinsic pathway seems more involved in inflammation and immune function, whereas the extrinsic pathway plays a larger part in responding to bleeding caused by trauma [3,4].

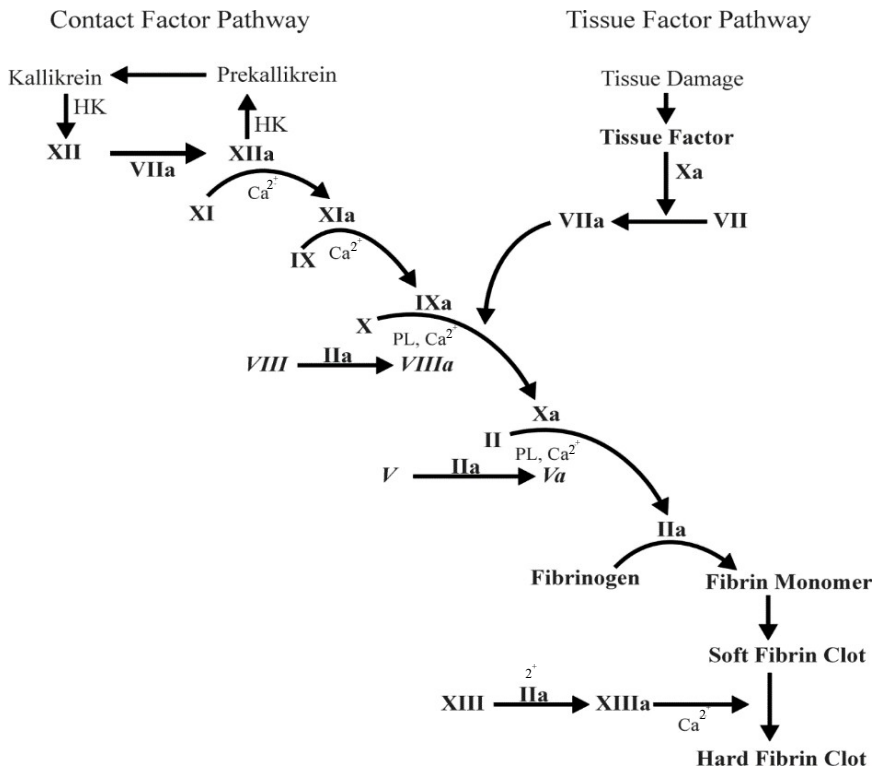


Figure 1. Overview of the coagulation cascade, with cofactors calcium (Ca^{2+}), phospholipid (PL).

With stroke and cardiovascular disease being among the leading causes of death in the United States, the importance of proper regulation of coagulation cannot be overstated [5]. Inhibition of certain factors can lead to inadequate levels of clotting, such as in hemophilia [6]. In contrast, overly heightened levels of clotting can also lead to several serious conditions, such as deep vein thrombosis (DVT) [7]. It should also be noted that post-surgical coagulopathies are common, with the possibility for both excessive bleeding and excessive clotting [8, 9]. This phenomenon is due to a variety of factors, such as hemodilution and temperature.

Many drugs are available which target various stages of the coagulation cascade. One early example is warfarin, which inhibits the production of vitamin K, thus inhibiting many key vitamin K-dependent coagulation factors (II, VII, IX, X) [10]. Other drugs, such as heparin, and more recently apixaban and those similar to it, work by inhibiting a specific factor involved in the coagulation cascade (Factors II and X, respectively) [11, 12].

As shown in Figure 1, the final steps of the coagulation cascade are shared between the intrinsic and extrinsic pathways. The two meet at the point of activation of Factor X, after which there is one common pathway. This common pathway utilizes three active clotting factors, fibrin (factor Ia), thrombin (factor IIa), and transglutaminase factor XIIIA (FXIIIA) to form a hard clot resistant to fibrinolysis [2].

Fibrin is derived from Fibrinogen (Fbg), a 340-kDa dimer of trimers containing Aα, Bβ, and γ chains (Figure 2) [13]. Overall, it is composed of a central E domain and two terminal D domains. Fibrinogen is converted to active fibrin by the serine protease thrombin. Thrombin cleaves fibrinopeptides A (FpA) and B (FpB) from the fibrinogen Aα and Bβ chains, respectively. Upon release of FpA, fibrin monomers polymerize linearly and form protofibrils. Following the cleavage of FpB, the protofibrils then aggregate to form a dense mesh of fibers

composed of fibrin monomers. This forms a soft clot, which is not resistant to degradation (fibrinolysis).

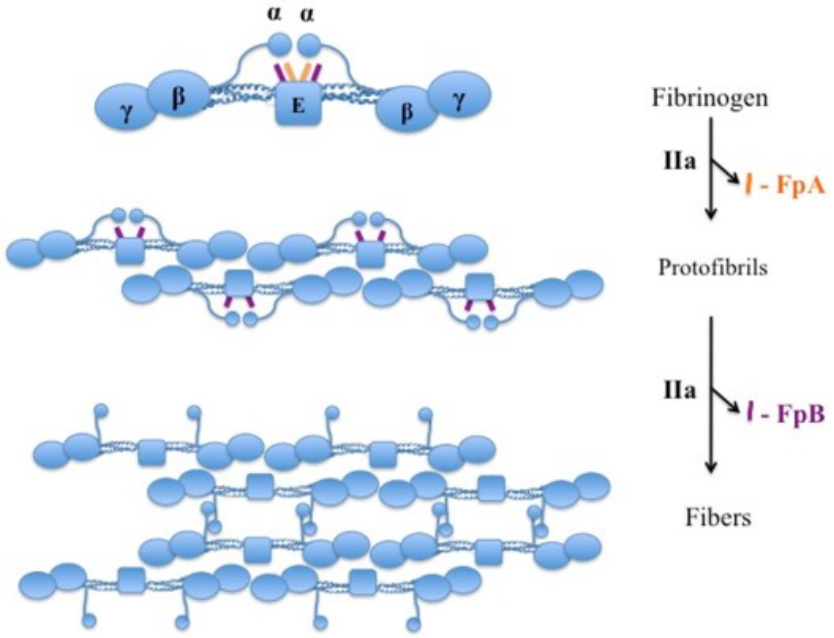


Figure 2. Crosslinking of human fibrinogen by thrombin. Adapted by Marina Malovichko from Undas, A, and Ariens, R.A. (2011) Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromoboembolic diseases. Arteriosclerosis, thrombosis, and vascular biology 31, e88-99.

Active thrombin is derived from prothrombin, a 72-kDa protein containing an A domain, a C-terminal serine protease domain (B domain), and an N-terminal propeptide containing a Gla domain and two kringle domains [14]. Cleavage of prothrombin to yield active thrombin (IIa) is carried out by Factor Xa, along with Factor V, Ca^{2+}, and phospholipid as cofactors [2].

In addition to cleaving fibrinogen to form fibrin, thrombin also activates FXIII to form active FXIIIA*. FXIII is found in both a platelet and plasma form [15]. The platelet form of FXIII has an A_2 conformation, while the plasma form has an A_2B_2 conformation. The A subunits are responsible for the catalytic activity of FXIII (Figure 3). The plasma concentration of an individual A subunit ranges from 60 to 180 nM [16]. In both platelet and plasma FXIII, the A subunits contain an N-terminal activation peptide, β sandwich domain, catalytic core, β-barrel 1, and β-barrel 2 domains [17, 18].

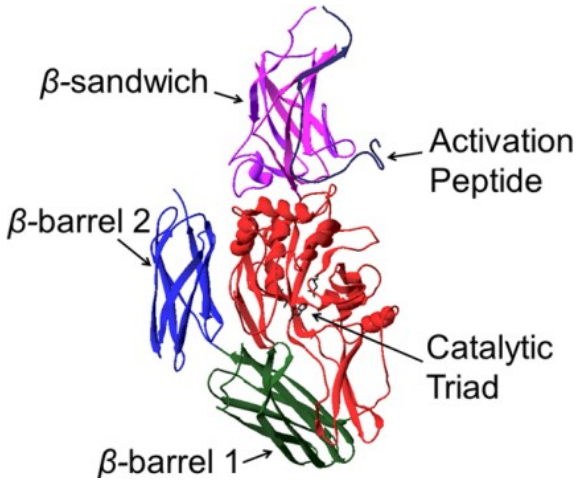


Figure 3. An individual A subunit of FXIII. PDB Code: 1FIE

The B subunits of plasma FXIII act as carriers of the A subunits, and the resultant A_2B_2 binds to fibrinogen with high affinity. The active site of the A subunit contains a conserved Cys314, His373, and Asp396 catalytic triad. In its inactive form, the activation peptide of FXIII blocks the catalytic cysteine residue, so cleavage by thrombin is required for activation. Once activated, the catalytic triad carries out a mechanism similar to a reverse proteolysis reaction of cysteine proteases such as those found in papain. However, rather than form peptide bonds in the backbone of its substrates, it instead forms crosslinks between side chain glutamine and lysine residues [15].

Although the platelet and plasma form of FXIIIA share the same structure, the mechanism of activation differs slightly between the two. Both involve thrombin hydrolyzing the activation peptide of FXIII at the R37-G38 peptide bond, with low concentration Ca^{2+} (1-10 mM) acting as a cofactor. In platelet FXIII, thrombin initially cleaves the activation peptide of FXIII to form intermediate FXIIIA′, which is then converted to active FXIIIA* by Ca^{2+}. However, since plasma FXIII has the addition of the B subunits, Ca^{2+} is also used to facilitate the

dissociation of A' and B subunits, as well as to yield active FXIIIA* (Figure 4). Activation of FXIII also causes the A subunits to dissociate from each other, resulting in individual A* monomers [19].

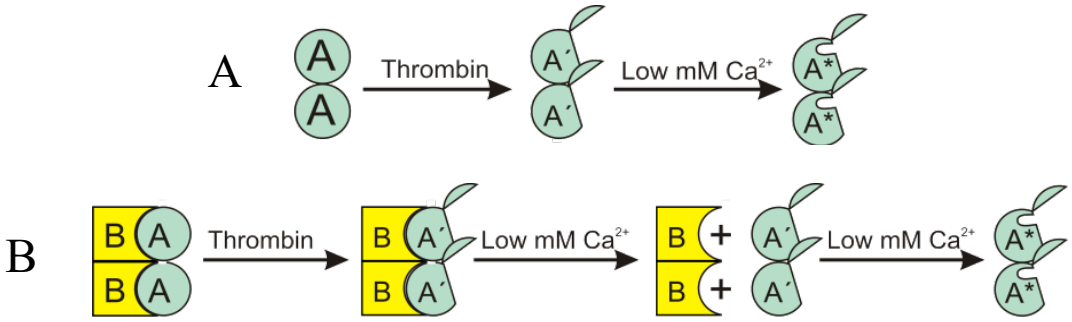
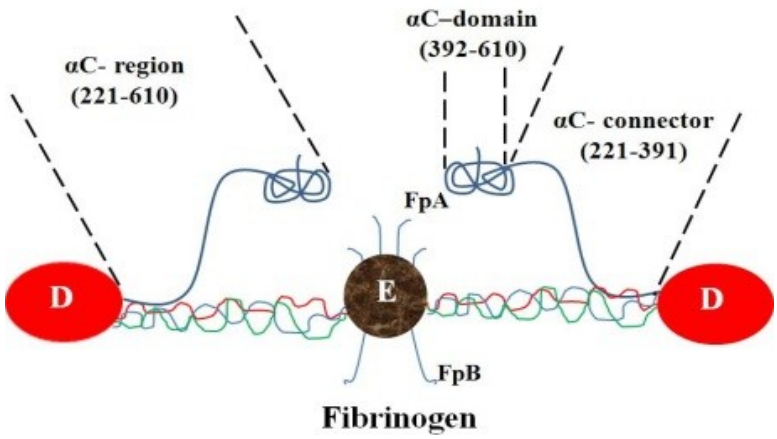


Figure 4. Activation of platelet FXIII (A) compared to plasma FXIII (B).

Upon activation by IIa, the active A subunits (A*) catalyze γ-glutamyl-ε-lysyl crosslinks between fibrin units to form a hard clot. FXIIIA* forms both α-α and γ- γ crosslinks between fibrin monomers. A clot with glutamine (Q)-lysine (K) crosslinks is more resistant to fibrinolysis than a soft clot [15].

Located at the carboxy terminal end of the Aα chains of Fibrinogen (Fbg) is the αC region. The αC region contains reactive glutamines and a binding site (389-403) for FXIII, which allows for crosslinking of fibrin units [20]. This region is heavily targeted by FXIII during crosslinking, with FXIII A_2B_2 having a K_D of 7 nM for Fbg αC [21]. In inactive fibrinogen, the αC regions are bound to the central E domain, and upon conversion to fibrin by thrombin, they are released and act as "free-swimming appendages" attached to the D domains (Figure 5) [22]. Due to this flexible conformation, the exact structure of the αC region has not been elucidated by X- ray crystallography [23].



T D M P ^{237}**Q** M R M E L E R P G G N E I T R G G S T S Y G T G S E
T E S P R N P S S A G S W N S G S S G P G S T G N R N P G S S G T
G G T A T W K P G S S G P G S A G S W N S G S S G T G S T G N
^{328}**Q** N P G S P R P G S T G T W N P G S S E R G S A G H W T S E S
S V S G S T G ^{366}**Q** W H S E S G S F R P D S P G S G N A R P N N P D W G T F E
^{396}**E** V S G N V S P G T R R E Y H T E K L V T S K G D K E L R T

Figure 5. Figure 5. Structure of fibrin with highlighted αC domains (top) and primary sequence of Fbg αC (233-425) (bottom). Reactive glutamine residues are bolded and binding site is underlined.

Factor XIII forms α-α crosslinks between the reactive glutamines and lysine residues of adjacent fibrin monomers. Formation of these crosslinks allows for clots with thicker, stiffer fibers, as well as greater red blood cell retention [24]. The rate of crosslinking by FXIII is an important measure, since it is crucial in maintaining hemostasis. Previous studies have shown that, of the three αC glutamines (Q237, Q328, and Q366), Q237 is by far the most reactive, with Q328 and Q366 having low and comparable reactivities [25]. In addition to these glutamines, the αC (233-425) region contains E396, part of a key anchoring site (P389-P403) for FXIII in plasma (Figure 5) [26]. FXIIIA has been shown to interact with αC, potentially affecting its activity [21,22]. The E396A binding site and three reactive glutamines have been thought to contribute to this interaction [27].

Previous studies have investigated the effects of modification of the three reactive glutamines or E396 binding site. Work with a Q237N mutant revealed that the crosslinking of the remaining two glutamines was somewhat hindered, and work with the αC E396A mutant showed that its Q237 was crosslinked to a lysine mimic at a somewhat slower rate than that of WT αC, likely due to weaker binding of FXIII [27]. However, those studies were performed at FXIII concentrations far above those found in plasma. Such conditions allowed for investigation of the slower-reacting Q328 and Q366, but the resulting rate of reaction for Q327 was too rapid for quantitative analysis. Loss of the E396 binding site in the E396A mutation has also been determined to create weaker binding to FXIII [21]. In addition, the naturally occurring fibrinogen Seoul II mutation (Q328P) has been shown to hinder polymerization of fibrin monomers, and was discovered in a patient with recurrent myocardial infarcts [28]. This decrease in reactivity may be due to the structural effects of a bulky proline (P) group, but the loss of a reactive glutamine may also play a role. Prior studies also revealed that a Q328P, Q366P double mutant had impaired thrombin-catalyzed polymerization compared to Fbg αC WT, but that other glutamines in the Fbg α chain were capable of maintaining normal levels of FXIIIA*-catalyzed crosslinking [29]. No previous crosslinking study has been performed for the Fbg αC Q366N mutation.

The current study aimed to further explore the interactions of FXIII and Fbg αC(233-425). Many previous studies have investigated the γ region of fibrinogen, which is also involved in crosslinking and contains a FXIII binding site [30]. In plasma, FXIII is thought to circulate bound to the Fbg γ (390-396) binding site, and loss of this binding site ($\gamma^{390\text{-}396A}$) has been shown to hinder the activation of FXIII [28]. However, less is known about the αC region and its binding site.

The methods employed in this study are based on MALDI-TOF mass spectrometry (MS) techniques developed by Doiphode *et al.* and Shimba *et al.* [32,33]. Crosslinking studies for the E396A mutant were performed at a lower, more physiological concentration of FXIII. This allowed for a more direct physiological connection, as well as a more precise measurement of the extent of reaction, due to the slower rate of crosslinking. Crosslinking studies were also performed with the αC Q366N mutant with results compared to previous studies by Mouapi *et al* and Park *et al,* which employed Fbg αC Q237N and Q366P mutants, respectively [27, 29]. Finally, studies were also performed for αC Q328P, with the aim of elucidating the effect of this mutant on the crosslinking of Fbg αC.

Specific Aims

The fibrinogen αC region (Fbg αC) region contains three glutamine residues that are crosslinked by FXIII. These include Q237, Q366, and Q328. The αC region also contains E396, which is thought to be a key binding site for FXIII. Studies at higher non-physiological concentrations of FXIII demonstrated that αC E396A produces comparable crosslinking rates to WT αC but Q237 is modestly hindered [27]. These studies also revealed that Q328 and Q366 become somewhat less reactive with inactive Q237N, since crosslinking of Q237 may promote targeting of Q residues to the FXIIIA active site. A similar study investigating a Q366N mutation had not been done prior to the current one. Given the previous Q237N results, determining the effect of Q366N mutation allowed for a useful comparison. Finally, the naturally occurring Q328P mutation (Seoul II) has been shown to cause decreased fibrin crosslinking, though the mechanism behind this is unknown. By further investigating the three glutamines and their effects on each other, a more complete understanding of the relationship between αC residues could be developed. The following work proposed four studies employing three αC mutations (E396A, Q366N, Q328P) to expand upon previous work with αC.

Primary Hypothesis:

The Fibrinogen αC region (spanning amino acids 233-425) contains an enzyme interaction site and distinct reactive glutamine environments which play specific roles in controlling the ability of αC to serve as a substrate for FXIII. Loss of this interaction site or reactive glutamines may hinder interaction with transglutaminase FXIII.

Aim 1:

The E396A mutation was previously shown to decrease the reactivity of Q237 at high concentrations of FXIII. Observing the effect at lower concentrations allowed for a more

direct physiological connection, as well as a more quantitative analysis due to the decreased rate of crosslinking. A mixture containing FXIII, thrombin, and calcium was used to yield fully activated FXIIIA*. Thrombin was inhibited with D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK). The activation mix was then added into a reaction solution of αC (WT or E396A), glycine ethyl ester (GEE, lysine mimic), and calcium. Crosslinking of GEE to Q237 by FXIII A* was quantitated by MALDI-TOF mass spectrometry (MS) following a GluC digest, as described by previous studies [25,32].

Aim 2:

Previous work with Q237N revealed decreased reactivity of the remaining two glutamines. Introduction of a Q366N mutation was thought to similarly hinder crosslinking of Q237 and Q328. A low FXIII concentration assay and a high FXIII concentration assay were carried out to monitor Q237 and Q328, respectively. A GluC digest was used to obtain fragments containing Q237, and a chymotrypsin digest was employed to yield fragments containing Q328. The levels of crosslinking for Q237 and Q328 were analyzed by MALDI-TOF MS.

Aim 3:

The fibrinogen Seoul II mutation (Q328P) in Fbg αC (233-425) hinders fibrin crosslinking. This experiment further explored the effect of that mutation. A crosslinking assay was carried out for the αC Q328P mutant, with the level of Q237 crosslinking being analyzed by MALDI-TOF MS.

This study also aimed to investigate the extent of Q366 crosslinking in Q328P, as well as employ a ^{1}H-^{15}N 2D HSQC NMR study for the Q328P mutant. However, due to a combination of previous difficulty with viewing Q366 fragments, time constraints, and issues with the 700 MHz NMR, these aims were not met.

Materials and Methods

Proteins and Chemicals:

FXIII A_2 was kindly supplied by the late Dr. Paul Bishop (ZymoGenetics). In-house mutagenesis performed by the undergraduate researcher was used to obtain Fbg αC (233-425) Q328P. Cell stocks of Fbg αC WT, E396A, and Q366N each were prepared prior to the current study through the use of *E. coli* expression systems.

Site –Directed Mutagenesis of αC (233-425) to generate Q328P Substitution:

The pGEX plasmid encoding GST-tagged Fbg αC (233-425) was used to introduce a single Q to P point mutation. The substitution was performed using a Quick-Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Forward and reverse primers were designed manually and are shown below, with the proline codons in bold:

Fbg αC Q328P Coding Strand Primer (Forward Primer):

5’ – ctggaactggaagtactggaaac**CCG**aaccctgggagccctagac – 3’

Fbg αC Q328P Template Strand Primer (Reverse Primer):

5’– gtctagggctcccagggtt**CGG**gtttccagtacttccagttccag – 3’

The primers shown are each 45 bases in length, which is longer than the kit’s recommended length of 20-25 bases. However, prior attempts at designing a shorter Q328P primer led to improper annealing.

PCR Amplification:

Primers were used to initiate a PCR reaction containing 1 µL 10x reaction buffer, 20 ng Fbg αC dsDNA, 1 µL dNTP mix, 240 µg coding primer, 170 µg template primer, and 41µL distilled H_2O to reach a total volume of 50 µL. Since some PCR components were proprietary, exact final concentrations were unknown. The reaction was transferred to a thermal cycler.

After an initial heating phase up to 95°C, the reaction underwent 16 heating/cooling cycles (95 °C and 55 °C, respectively), as recommended for a single amino acid change.

Cell Transformation and DNA Extraction

After the PCR reaction, the mixture was cooled to 4°C. To digest parent DNA which had not undergone mutagenesis, 1 µL of 10 U/µL Dpn I, a restriction enzyme which cleaves methylated and hemimethylated DNA, was added. After incubation at 37°C for one hour, 2 µL of the resulting PCR product was transformed into 50 µL XL-1 Blue Supercompetent cells in a Falcon tube. Cells were placed in a shaker at 37°C for 1 hour and then plated on ZYP 0.8g agar plates with 100 µg/mL ampicillin for incubation.

The QIAgen QIAprep spin Miniprep kit was used for DNA extraction and purification. The purified DNA concentration was measured at 260 nm with a NanoDrop spectrophotometer. Substitutions were confirmed with DNA sequencing at the University of Louisville CGeMM DNA Facility Core.

Expression*:*

Acella (EdgeBio) cell stocks of Fbg αC E396A and Fbg αC Q366N were already available, whereas Q328P αC was produced as described above. The following methods for obtaining pure αC variant protein were common to all three mutants.

Variants of αC were expressed from Acella cell lines (EdgeBio) using ZYP 5052 media, which is used for auto-induction. This method utilizes a glucose-rich non-inducing medium (starter) for early cell growth, followed by lactose-rich auto-inducing medium for protein expression [34]. A 1 L stock of ZY was made containing 10g Tryptone, 5 g yeast, and 925 mL H_2O. A 300 mL stock of 5052 (0.5% glycerol, 0.05% g glucose, 0.2% lactose) and a 1 L stock of 20x NPS (50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 50 mM Na_2HPO_4) were also prepared.

Starter cultures for expression were made containing 2.32 mL ZY, 100 µg/mL ampicillin, 0.4% glucose, 0.5x NPS, and 0.5 mM $MgSO_4$. Cultures of transformed cells were used to inoculate the starter culture, which then shook at 180 rpm and 37 °C for 15-16 hours.

A 500 mL auto-induction ZY medium containing 1 mM $MgSO_4$, 2% 5052, and 25 mL 0.5x NPS was made and inoculated with starter culture. The medium shook at 180 rpm for 1 hour at 37°C, then for 24 hours at room temperature. Cell growth was monitored at the 1 hour and 24 hour time points using a Cary 50 Bio UV-Visible spectrophotometer. For each mutant, absorbance at 600 nm was 0.4 – 0.7 at 1 hour. At 24 hours, a 1:41 dilution was made prior to scanning since the mixture became too turbid for accurate readings. Absorbances for the diluted variants at 24 hours was 0.1 – 0.2.

Harvesting*:*

After 24 hours, the medium was spun down in a Sorvall RC 6 Plus centrifuge with SLC 4000 rotor (Thermo Scientific) for 25 minutes at 4680 RCF and 4 °C. After this initial centrifugation, the supernatant was discarded, and the pellet was resuspended in *E. coli* wash buffer (1 M Tris, 5 M NaCl). After stirring, the suspension was spread between four 50-mL VWR conical tubes. These tubes were spun down in an Allegra R-20 swinging-bucket rotor centrifuge (Beckman-Coulter) for 30 minutes at 5000 RCF and 4 °C. The resulting supernatant was discarded.

Extraction*:*

For a 500 mL culture, four pellets were resuspended in a total of 60 mL *E. coli* lysis buffer (1 M Tris, 5 M NaCl, 1.1 mg/mL EDTA) divided across the tubes. Suspensions were combined in a 100-mL beaker. The final volume of the suspension was roughly 80 mL. Protease inhibitors (35 mg benzamidine 50 µL 2 mg/mL aprotinin, 50 µL 1 mM pepstatin, and 130 µL 4 M leupetptin) were added. Cells were lysed with 50 mg lysozyme, 25 mg sodium deoxycholate (SDC), and 2.25 mL 20% Triton X- 100. After adding 250 µL 1 M MgSO4, 25 µL 10 mg/mL

DNAse 1 and 25 µL 10 mg/mL RNAse A were added. This mixture stirred at 4 °C for 2 hours, and NaCl concentration was adjusted by adding 1500 uL of 5M NaCl. In addition, 50 µL of 1 M DTT was added to the mixture. Exact final concentrations were unknown due to the final volume of the resuspension being unknown.

After stirring, the lysate was returned to room temperature and divided between two round bottom Beckman tubes. The tubes were balanced and centrifuged at 22,000 RCF in a Beckman centrifuge with JA-20 fixed angle rotor at 4 °C for 20 minutes. The clarified lysate was filtered through a 0.2 µM filter. In the case that filtration proceeded too slowly, the lysate was clarified with streptomycin to precipitate any remaining nucleic acids.

Purification*:*

The GST-tagged proteins were loaded onto GE healthcare GSTrap 4B 1 mL columns using a GE ÄKTAprime liquid chromatography unit, then treated with PreScission Protease to remove the GST tag. This cleavage left Fbg αC free in the column, with the cleaved GST tag bound to the resin. The loaded column incubated for 18 hours at 4 °C.

Proteins were eluted from the column in two fractions, with the first being a lower concentration 500 µL fraction, and the second being a higher concentration 1 mL fraction. This elution strategy was done by monitoring UV absorbance and collecting fractions at certain absorbance levels. The low concentration fraction was collected once absorbance began to increase slightly, and the high collection fraction was collected during the peak of UV absorbance. After αC elution, a fraction containing GST was eluted using GST elution buffer (0.6 mg/mL GST, 1 mM dithiothreitol in Tris buffer). Finally, 5 M urea was run through the column to collect a fraction containing all remaining protein. The GST and Urea fractions were used to assess the efficiency of the PreScission protease and purification in general.

Polyacrylamide Gel Analysis of Purification:

SDS-PAGE Gels (15%) were cast and run on a Bio-Rad gel apparatus. Stock solutions included 30% acrylamide, 10% SDS, 0.5 M pH 6.8 Tris, 1.5 M pH 8.8 Tris, 10% ammonium persulfate (made fresh), and TEMED. To monitor purification progress, samples of lysate (prior to loading), load waste, and two dilute fractions of eluted Fbg αC protein were each run in individual lanes in the gel. Samples were run under reducing conditions.

GEE/MALDI-TOF Assay:

Glycine ethyl ester (GEE) is a lysine mimic which can be crosslinked to reactive glutamines by activated FXIIIA*. Since the αC (233-425) region does not contain any reactive lysine residues of its own, GEE acts as a convenient reactant for monitoring the level of crosslinking of αC by FXIII. This assay follows the extent of Q-GEE crosslinking by comparing the relative amounts of crosslinked and uncrosslinked glutamines at various timepoints in the reaction. This reaction was monitored using MALDI-TOF MS, since the addition of GEE adds 86 *m/z* to the molecular weight of the reactive glutamine. With this assay design, distinct "reactant" and "product" peaks in the spectra can be followed and quantitated (Figure 6).

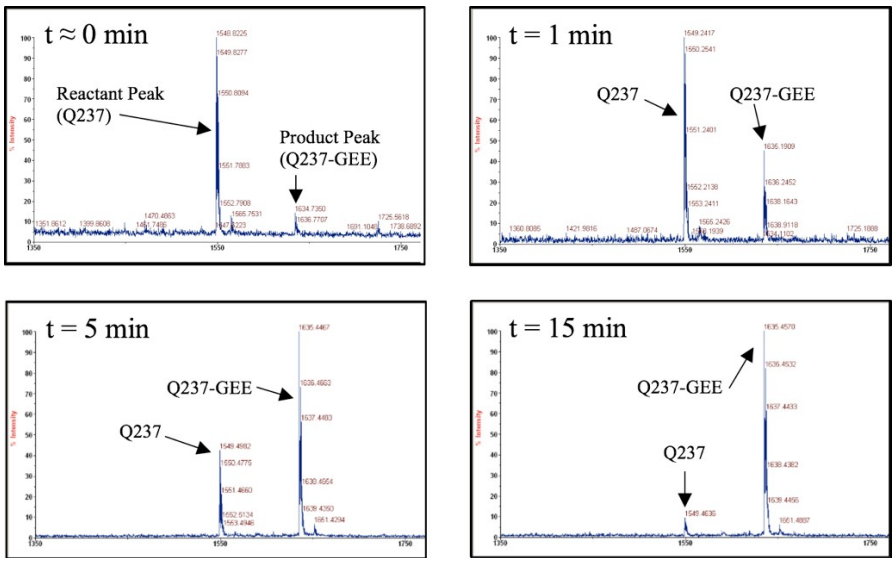


Figure 6. Representative spectra of reaction progression as observed in MALDI-TOF MS.

Two versions of the GEE crosslinking assay were performed, with one being a low concentration assay (50 nM FXIIIA) and the other being a high concentration assay (2μM FXIIIA). The low concentration assay was needed to more closely monitor crosslinking of Q237, since it reacts too quickly at higher concentrations of FXIIIA*. The high concentration assay was used to monitor Q328, which reacts much more slowly, and thus needs a higher concentration of FXIII to achieve appreciable levels of crosslinking in a reasonable time frame. Both were run in Tris assay buffer (100 mM Tris-acetate, 150 mM NaCl, 0.1% polyethylene glycol, pH = 7.40).

The low concentration assay contained a final FXIIIA concentration of 50 nM, and was carried out across two separate mixtures. The first mixture (pre-mix) was used for activation of FXIII, and the second (reaction mix) was used for dilution, as well as the actual reaction. This method also allowed for identical activations of FXIII across all assays. To obtain fully activated FXIIIA*, a pre-mix containing 1 μM FXIIIA, 9.3 U/mL bovine thrombin (bIIa), and 4 mM Ca^{2+} in a total volume of 200 μL was made. The pre-mix was incubated for 30 minutes at 37 °C, then quenched with 2μL 190 μM D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK), a thrombin inhibitor. The pre-mix was diluted 10-fold in reaction buffer, then again 2-fold in the reaction mixture to obtain a final concentration of 50 nM FXIIIA*. The final reaction mixture thus contained 50nM FXIIIA*, 17 mM GEE, and 4 mM Ca^{2+}, and the crosslinking reaction was initiated with the addition of the desired αC variant of (WT, E396A, Q328P, or Q366N). The final concentration of αC was 13.6 μM. The overall reaction volume was 200 μL. The reaction was run at 37 °C in a Thermo Scientific water bath, and then quenched with EDTA , which sequesters Ca^{2+} ions, at time points up to 30 minutes.

Unlike the low concentration assay, crosslinking assays with 2 μM FXIII did not utilize separate pre-mixes and reaction mixes. Instead, the assay took place in one mixture, since a dilution was not needed. Activation of FXIIIA, quenching by 2 μM PPACK, and the crosslinking

reaction took place in the same tube. This allowed for a more direct comparison with earlier studies of the same type [27]. Reaction mixtures contained 2 μM FXIIIA, 4 mM Ca^{2+}, 17 mM GEE, 8.4 U/mL bIIa, and were initiated with the addition of the desired variant of αC such that the final concentration of αC was 13.6 μM. Unlike in the previous assays, 8.4 U/mL bIIa was used instead of 9.3 U/mL, also to allow for a more direct comparison with assays from previous studies. The overall reaction volume was 200 μL. The reaction was run at 37 °C in a Thermo Scientific water bath and was quenched with EDTA at time points up to 60 minutes.

Depending on the desired αC fragments, either a proteolytic GluC or chymotrypsin digest was used (Figure 7). Endoproteinase GluC selectively cleaves glutamic acid C-terminal peptide bonds, and was used to obtain fragments containing Q237 and Q366. For these samples, a GluC digest (0.075 μg GluC/sample) was performed for 2 hours at 25 °C after combining 6 μL of reaction sample with 6 μL of GluC digest buffer (25 mM NH_4HCO_3, pH = 7.8). Chymotrypsin cleaves aromatic C-terminal peptide bonds, and was used to obtain fragments containing Q328. For these samples, a chymotrypsin digest (1.5 μg chymotrypsin/sample) was performed for 1 hour at 25 °C by combining 6 μL of reaction sample with 6 μL of chymotrypsin digest buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH = 7.4). Both digests were run in a VWR Standard heat block.

GluC Fragment Containing Q237	**GluC Fragment Containing Q366:**
m/z = 1549	*m/z* = 1348
^{229}GPLGSTMP^{237}**Q**MRM^{241}E	^{358}SSVSGSTG^{366}**Q**WHS^{370}E
Chymotrypsin Digest Containing Q328:	
m/z = 2448	
^{316}NSGSSGTGSTGN^{328}**Q**NPGSPRPGSTGT^{341}W	

Figure 7. Fragments containing reactive glutamines of αC.

The resulting digested samples were worked up with a ZipTip preparation (Millipore) to remove salts which would form Na^+ adducts. Pipet tips containing a C_{18} resin were rinsed three times with 6 µL of acetonitrile and equilibrated three times with 6 µL 0.1% trifluoroacetic acid (TFA). Digested reaction samples were taken up in the tips, and salts were removed using four rinses of 0.1% TFA. The samples were finally eluted into 6 µL of matrix. For GluC reactions, the matrix employed was 7.0 mg/mL ferulic acid in a 1:1 mixture of acetonitrile and 0.1% TFA. For chymotrypsin reactions, the matrix used was 10 mg/mL α-Cyano-4-hydroxycinnamic acid (αCHCA) in a 1:1:1 mixture of acetonitrile, 0.1% TFA, and ethanol.

Each timepoint was spotted three times on a 100-well MALDI-TOF plate (Applied Biosystems) for each assay and analyzed in a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) with an N_2 UV laser. Laser intensity ranged from 2000-2200 arbitrary units. Each assay was repeated in triplicate. The concentration of uncrosslinked reactant glutamine remaining was determined using the following calculation:

$$\frac{Reactant\ Peak\ Height}{Reactant\ Peak\ Height + Product\ Peak\ Height} * Peptide\ Concentration$$

Results

Expression and Purification of Fbg αC (233-435) E36A, Q328P, and Q366N:

Following expression, affinity purification was carried out to obtain Fbg αC protein. For each mutant, two fractions were collected, with one being low concentration and the other being high concentration. These fractions typically had concentrations of 10-30 μM and 100-120 μM, respectively. The low concentration fraction of Fbg αC E396A had an abnormally low concentration (<1 μM). All experiments in this study used the high concentration fractions of Fbg αC. Progress during expression and purification of αC mutants was monitored with 15% SDS PAGE gels stained with Coomassie Brilliant Blue dye. Bands representing αC fractions represent 1:10 dilutions of collected protein sample collected. Dilution was carried out to prevent excessive smearing. Purity was assessed by the absence of any other significant bands in the lanes with αC fractions (Figure 8).

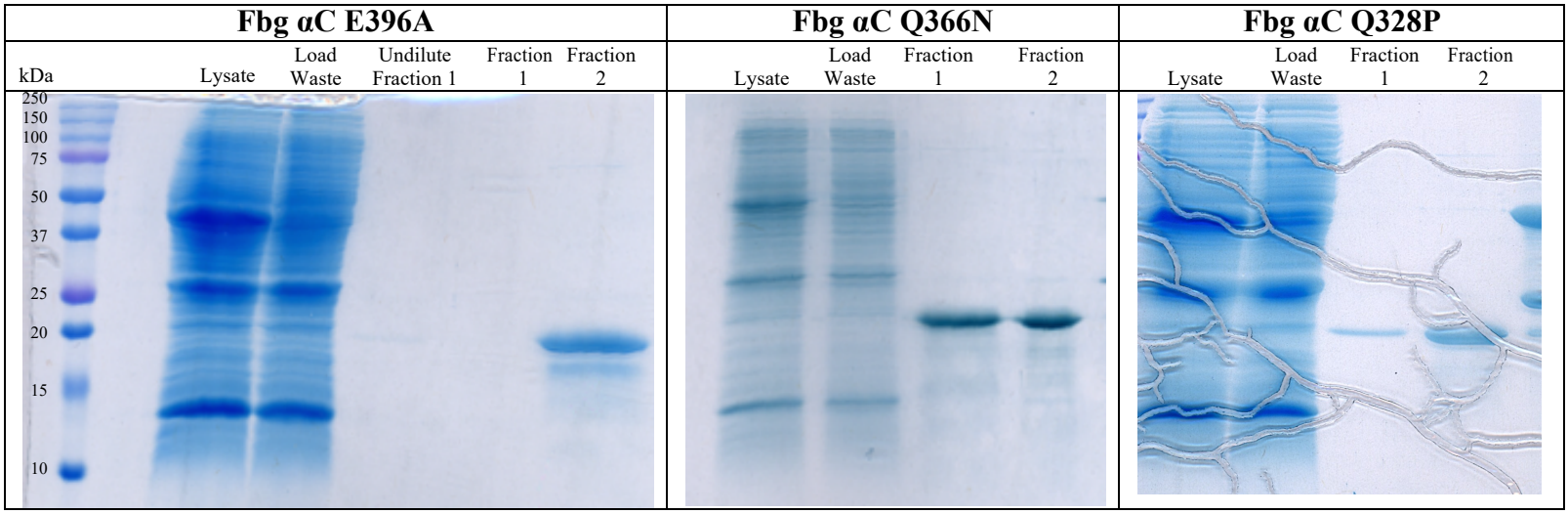


Figure 8. SDS PAGE gels monitoring expression and purification of Fbg αC mutants. Note: The concentration of fraction 1 of E396A was unusually low (<1 μM), so an undiluted sample was run in addition to the normal diluted fraction. Even when undiluted, the band is hardly visible. In addition, there was some cracking during the drying process of the αC Q328P gel.

MALDI- TOF MS Spectra to Confirm Q328P Mutation:

In addition to DNA sequencing, MALDI-TOF MS was also used to confirm the Q328P point mutation. Using a chymotrypsin digest, fragments of αC WT and Q328P were compared, with the difference in *m/z* values being 31 (Figures 9, 10). This represents a successful point mutation of glutamine (146.14 Da) to proline (115.13 Da). The other variants of αC employed in this study were confirmed previously with gene sequencing and MALDI-TOF MS [35,36].

Chymotrypsin Fragments of Interest:

WT *m/z* = 2448	Mutant *m/z* = 2416
^{316}NSGSSGTGSTGN^{328}**Q**NPGSPRPGSTGT^{341}W	^{316}NSGSSGTGSTGN^{328}**P**NPGSPRPGSTGT^{341}W

Figure 9. Fragments of αC containing Q328 (WT) and P328 (mutant).

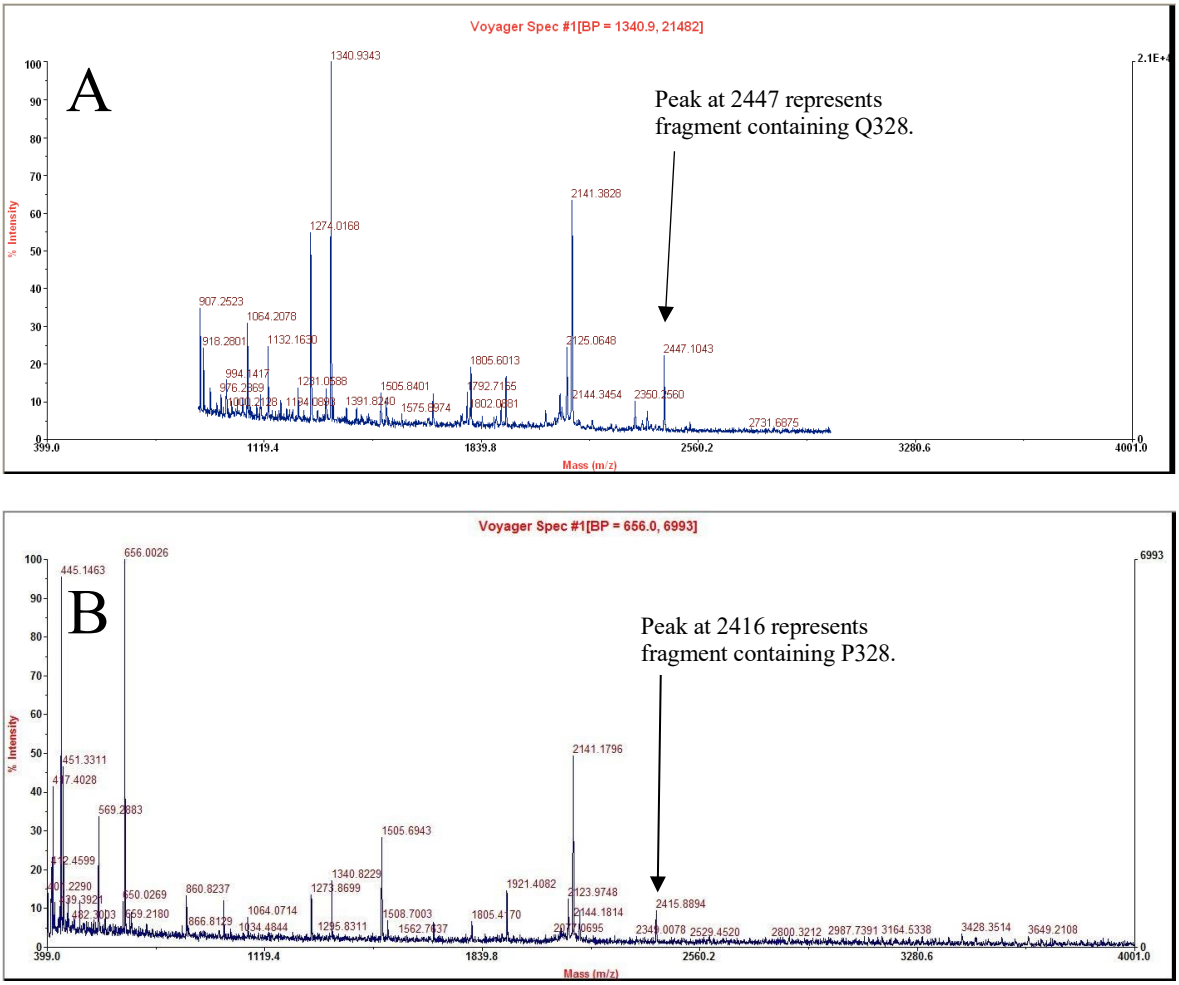


Figure 10. MALDI-MS spectra of the chymotrypsin digest of WT (A) and Q328P (B) αC. In WT αC, the peak at 2448 represents the fragment containing Q328. The presence of a peak at 2417 confirms the mutation to P238.

Aim 1: Comparison of Q237 Reactivity in Fbg αC WT and E396A, 50 nM FXIIIA*:

Upon removal of the E396A anchoring site, Q237 exhibited decreased reactivity. The low concentration GEE/MALDI-TOF assay was applied to monitor the extent of crosslinking of Q237 by 50 nM FXIIIA* in αC E396A, as compared to WT. The concentration of Q237 remaining was plotted against time (Figure 11). At a physiological concentration of FXIIIA (50 nM), Q237 was crosslinked more slowly in Fbg αC E396A compared to WT. Previous work with the αC E396A mutant at higher FXIIIA* concentrations showed no effect on Q328 or Q366, but a moderate hindrance of Q237 crosslinking [27]. The current study strengthens those findings, since the effect is also present at physiological concentrations of FXIIIA*.

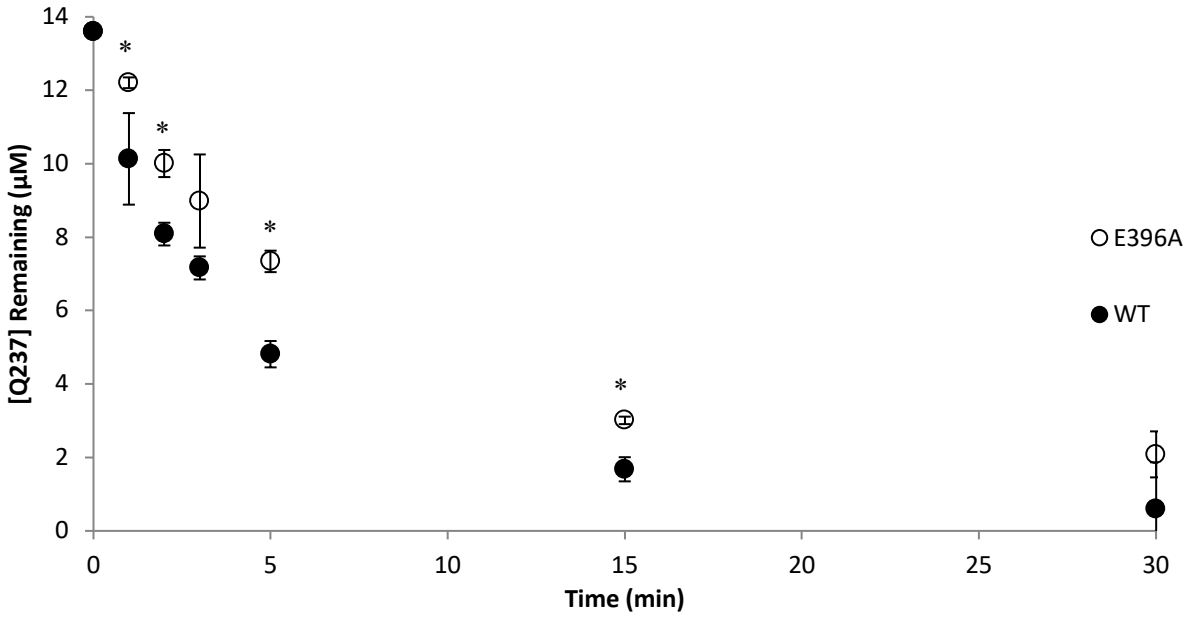


Figure 11. Comparative plot of reactivity of Q237 in WT (black circles) and E396A (white circles) αC. Error bars show 1 SD (n = 3). Stars represent a significant difference ($p<0.05$) between data points based on Student's t-test.

Aim 2: Comparison of Reactivities in Fbg αC WT and Q366N:

The low concentration GEE/MALDI-TOF assay was applied to monitor the reactivity of Q237 by 50 nM FXIIIA* in αC Q366N, as compared to WT. The concentration of Q237 remaining was plotted against time (Figure 12). At physiological concentrations of FXIIIA* (50 nM), Q237 was crosslinked to a comparable extent in Fbg αC Q366N and WT.

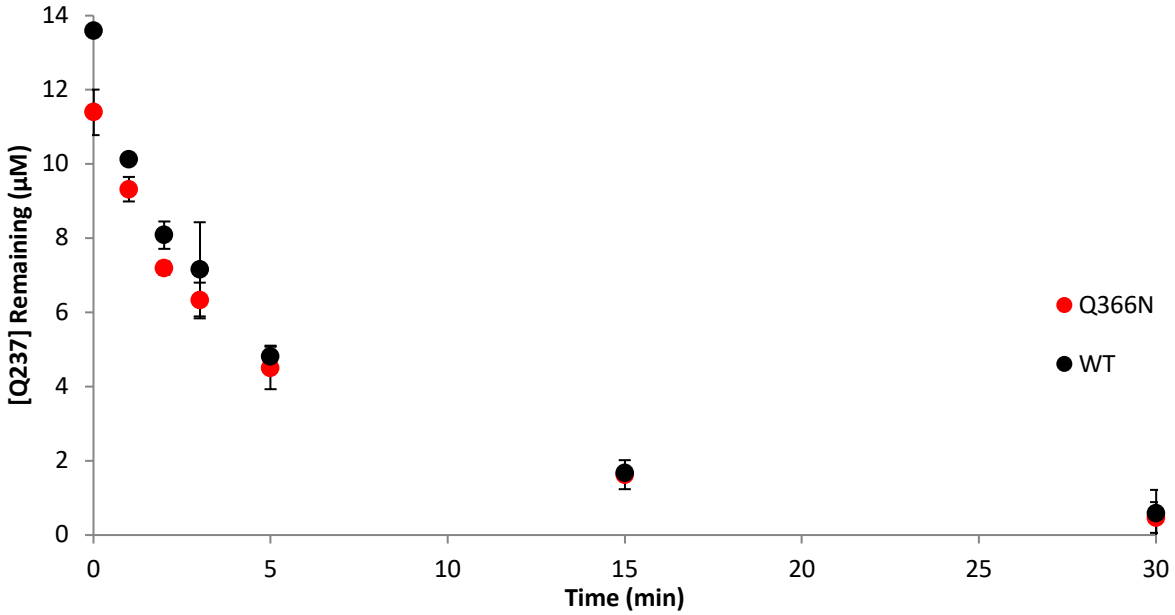


Figure 12. Comparative plot of reactivity of Q237 in WT (black circles) and Q366N (red circles) αC. Error bars show ± SD (n = 3).

In addition, the high concentration GEE/MALDI-TOF assay was applied to monitor the reactivity of Q328 by 2 μM FXIIIA* in αC Q366N, as compared to WT. This higher FXIIIA* concentration was required because Q328 is not crosslinked at an appreciable rate with 50 nM FXIIIA*. The concentration of Q328 remaining was plotted against time (Figure 13). At high concentrations of FXIII (2 μM), Q328 was crosslinked more rapidly in Fbg αC Q366N compared to Fbg αC WT.

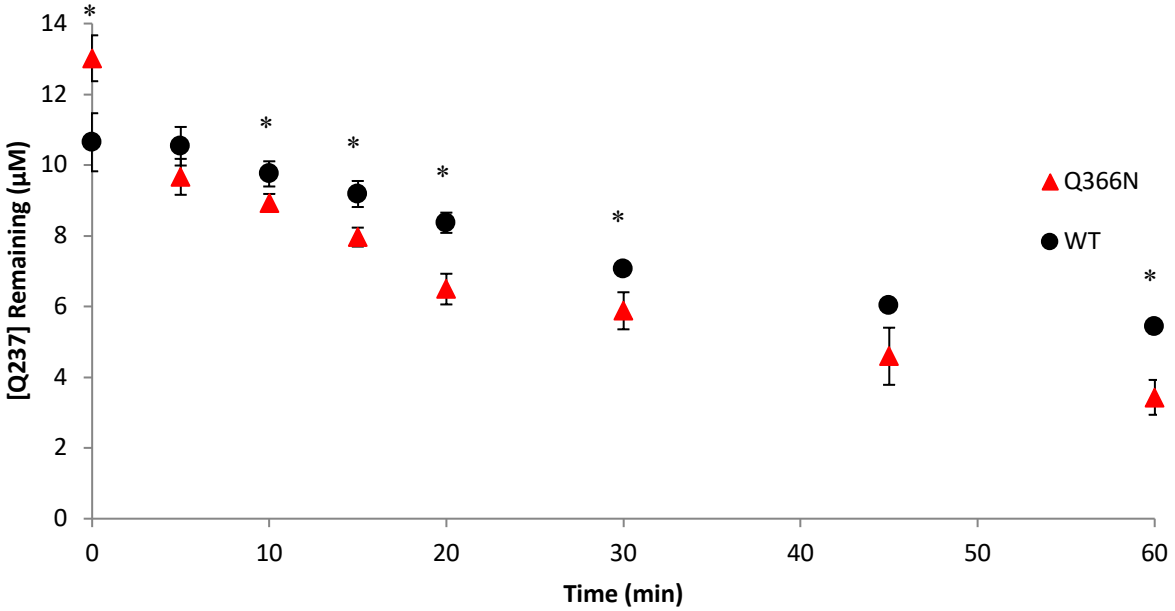


Figure 13. Comparative plot of reactivity of Q328 in WT (black circles) and Q366N (red triangles) αC. Error bars show ± SD (n = 3). Stars represent a significant difference ($p<0.05$) between data points based on Student's t-test.

Aim 3: Comparison of Q237 Reactivity in Fbg αC WT and Q328P:

The low concentration GEE/MALDI-TOF assay was applied to monitor the reactivity of Q237 by 50 nM FXIIIA* in αC Q328P, as compared to WT. The concentration of Q237 remaining was plotted against time (Figure 14). At a physiological concentration of FXIIIA (50 nM), Q237 was crosslinked to a similar extent in Fbg αC Q328P and WT. There does not appear to be an effect similar to that of the αC E396A mutation.

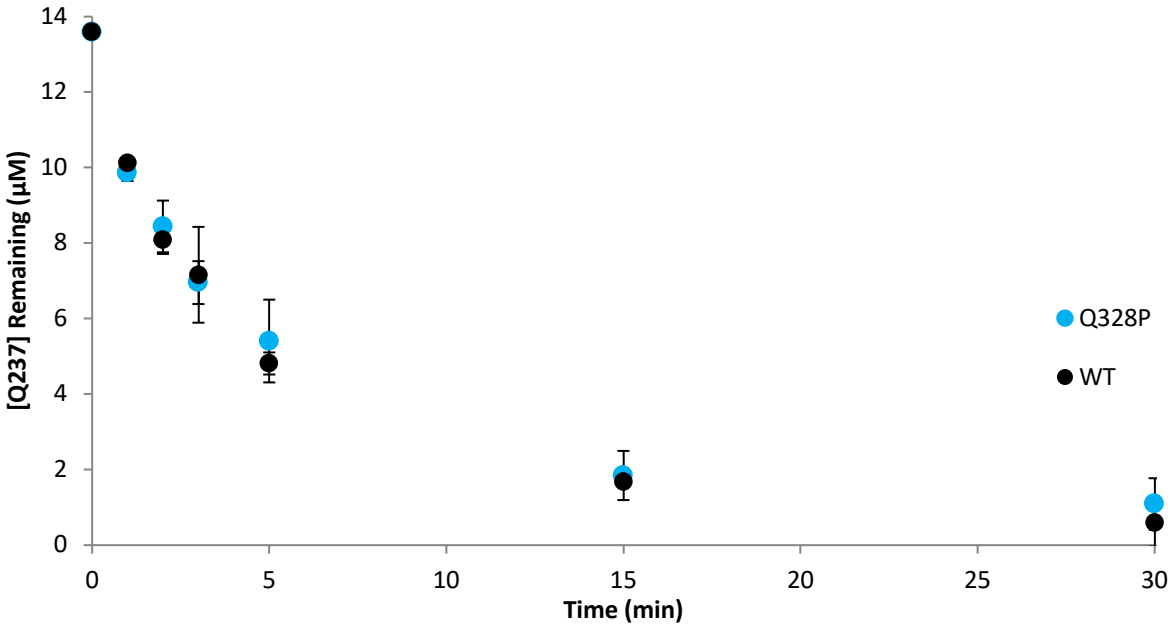


Figure 14. Comparative plot of reactivity of Q237 in WT (black circles) and Q328P (blue circles) αC. Error bars show ± 1 SD (n = 3)

Discussion

The interaction between FXIIIA* and fibrinogen is a vital process in blood coagulation and homeostasis as a whole. Anomalies in these proteins may have serious health consequences, and the mechanisms behind such issues are largely unknown. Historically, the focus was placed on the Fbg γ region, which is crosslinked more quickly than the Fbg αC region by FXIIIA*, and contains a known FXIII binding site (390-396). However, recent studies, such as those by Duval *et al.,* revealed that both α-α and γ-γ crosslinking played crucial and complementary roles in fibrin fiber thickness, density, and resistance to fibrinolysis [37]. Thus, a greater understanding of the contribution of key Fbg αC residues in crosslinking by FXIII is needed in order to elucidate key parts of the coagulation cascade. The four crosslinking assays in the current study demonstrate varying levels of influence of these residues in Fbg αC (233-425).

Work by Mouapi *et al.* demonstrated slightly decreased reactivity of Q237 in the Fbg αC E396A mutant at high, nonphysiological concentrations of FXIIIA* [24]. Results from the Fbg αC E396A assay of the current study (Figure 11) indicate that, at physiological concentrations of FXIIIA, the loss of the E396 binding site decreased the reactivity of the most reactive glutamine, Q237. These findings affirm earlier work with Fbg αC E396A, and confirms that the effect is present at both nonphysiological and physiological concentrations of FXIIIA*.

Work by Park *et al.* revealed that the Seoul II mutation (Q328P) decreased clot thickness and impaired α-chain crosslinking of fibrin units [28]. They also demonstrated that a Fbg αC Q366P, Q328P double mutant had impaired polymerization compared to Fbg αC WT, but that other glutamines were capable of maintaining FXIIIA*-catalyzed crosslinking [29]. Results of the current study indicate that neither the loss of Q366 nor Q328 (Q366N and Q328P mutants, respectively) had an impact on the crosslinking of Q237 (Figures 12, 14). These results support

the finding that, in the absence of Q328 and Q366, other glutamines are capable of maintaining normal levels of FXIIIA*-catalyzed crosslinking.

The combined results of the E396A, Q366N, and Q328P assays indicate that the ^{389}P-^{403}P anchoring site containing E396 is a more significant contributor to the specificity of αC as a substrate for FXIIIA*, compared to the less reactive glutamines. However, despite Q237 being the most reactive of the three glutamines, an unaffected level of Q237 crosslinking does not necessarily indicate an unaffected level of α-chain crosslinking, overall. Given that previous studies demonstrated the life-threatening effects of the Fbg αC Q328P mutant, there must be another factor beside Q237 which is affected by the Seoul II mutant [28,29]. There are other glutamine residues located outside the αC (233-425) region which are also crosslinked by FXIIIA*, and have had their lysine partners identified [38]. If one or more of these glutamines is also highly reactive, their reactivity may be impaired in the presence of P328.

In addition to work with Fbg αC E396A, work with a Q237N mutant was also performed by Mouapi *et al*. [27]. Their studies found that the loss of Q237 impaired crosslinking of Q328 and Q366. This effect was hypothesized to be caused by a conformational change in αC following Q237 crosslinking which facilitates crosslinking of the remaining glutamines. Prior work also revealed that crosslinking of the three glutamines by FXIII* occurs independently [25]. No single glutamine is required for the other two to be crosslinked.

In the context of these previous findings, the results of Q366N crosslinking assay are interesting (Figures 12, 13). The Q237N mutation was previously found to hinder crosslinking of the other two glutamines [27]. In the current study, the Q366N mutation had no effect on Q237 crosslinking, and actually aided in Q328 crosslinking. These findings lead to two conclusions. The first is that there must be some inherent difference between Q237 and Q366 which allows

Q237 to exert some force on Q366, but not vice versa. One possible explanation is the relative locations of the residues, since Q237 is found far upstream of the other two glutamines and E396 anchoring site. Others have used this observation to form hypotheses regarding crosslinking interactions in Fgb αC, but the causes behind these effects are still unknown [27]. The second conclusion from this experiment can be considered as a revision to previously made conclusions, which stated that no one glutamine is required for the crosslinking of the others [25]. The results of this study expand upon those observations, and reveal that, rather than simply being independent of each other, the two less reactive glutamines (Q328 and Q366) may actually be in competition with each other for crosslinking by FXIIIA*. It appears that the presence of one hinders the reaction of another. Monitoring the reactivity of Q366 in the Fbg αC Q328P mutant would have helped to further strengthen this conclusion, since currently, only one side of the interaction between the two glutamines has been studied.

In summary, these four crosslinking assays suggest that, at physiological conditions, only the loss of the E396 binding site has an impact on the reactivity of the most reactive glutamine, Q237. Loss of the less reactive glutamines (Q328, Q366) neither hinders nor aids the crosslinking of Q237. At higher FXIIIA* concentrations, loss of one of the less reactive glutamines appears to aid in the crosslinking of the other, suggesting the two are in competition with each other, but cannot outcompete Q237.

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Appendix

p-Values for Figures 11 and 13:

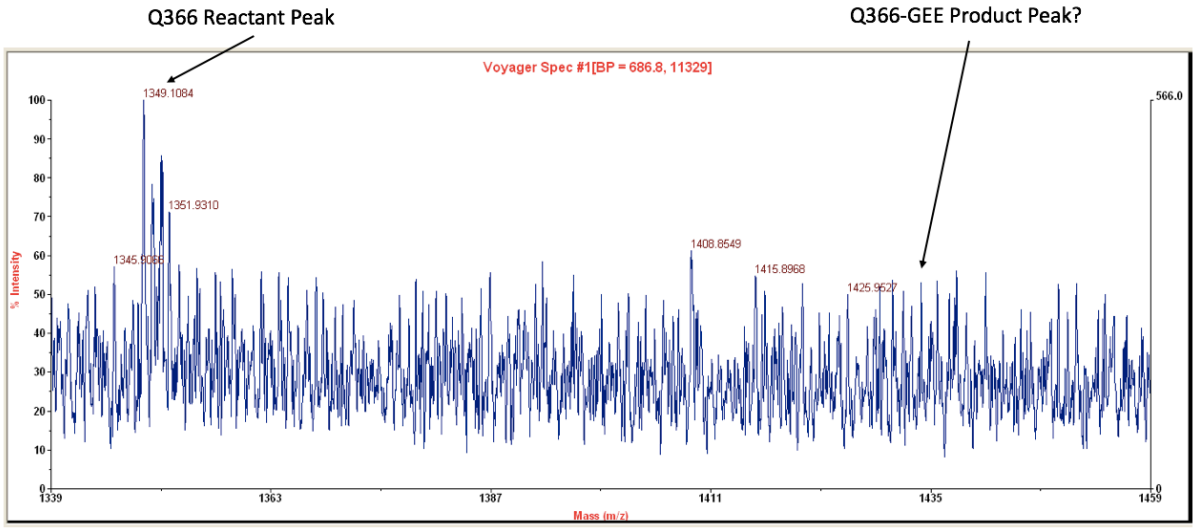
Figure 11:

Time (min)	*p*-Value
0	N/A (identical)
1	0.0459
2	0.0023
3	0.0734
5	0.0007
15	0.0026
30	0.1432

Figure 13:

Time (min)	*p*-Value
0	0.0171
5	0.1146
10	0.0313
15	0.0097
20	0.0033
30	0.0205
45	0.0995
60	0.0135

Previous Difficulty with Viewing Q366 Crosslinking:



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