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Ranking Reactive Glutamines in the Fibrinogen αC Region that are Targeted by Blood Coagulant Factor XIII

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Key Findings:

1. FXIIIa exhibits a preference for Q237 in crosslinking reactions within fibrinogen αC (233-425) followed by Q366 and Q328.
2. None of the reactive glutamines in αC 233-425 (Q237, Q328, Q366) are required to react first before the others can crosslink.

Key words: αC region, Fibrinogen, Factor XIII, MALDI –TOF mass spectrometry, NMR

Abstract

Factor XIIIa (FXIIIa) introduces covalent γ-glutamyl-ε-lysyl crosslinks into the blood clot network. These crosslinks involve both the γ and α chains of fibrin. The C-terminal portion of the fibrin α chain extends into the αC region (210-610). Crosslinks within this region help generate a stiffer clot which is more resistant to fibrinolysis. Fibrinogen αC (233-425) contains a binding site for FXIIIa and three glutamines Q237, Q328, and Q366 that each participate in physiological cross-linking reactions. Although these glutamines were previously identified, their reactivities towards FXIIIa have not been ranked. MALDI-TOF mass spectrometry and NMR methods were thus used to directly characterize these three glutamines and probe for sources of FXIIIa substrate specificity. Glycine ethyl ester (GEE) and ammonium chloride served as replacements for lysine. Mass spectrometry and 2D HSQC NMR revealed that Q237 is rapidly crosslinked first by FXIIIa followed by Q366 and Q328. Both $^{15}NH_4Cl$ and ^{15}N-GEE could be crosslinked to the three glutamines in αC (233-425) with a similar order of reactivity as observed with the MALDI-TOF mass spectrometry assay. NMR studies using the single αC mutants Q237N, Q328N, and Q366N demonstrated that no glutamine is dependent on another to react first in the series. Moreover, the remaining two glutamines of each mutant were both still reactive. Q237, Q328, and Q366 are located in a fibrinogen region susceptible to physiological truncations and mutation. The current results suggest that these glutamines play distinct roles in fibrin crosslinking and clot architecture.

Introduction

Abnormal fibrinogen levels and fibrin clot structure have been associated with several pathological conditions including cardiovascular disease, arteriosclerosis, and bleeding disorders.[1, 2] The N-termini of the fibrinogen $(A\alpha B\beta\gamma)_2$ chains form the central E region, which extends via a coil-coiled region to two terminal D regions. A flexible portion of the Aα chain—the αC region—extends from the ends of the D regions and is tethered to the central E region.[3, 4] To initiate fibrin polymerization, thrombin cleaves fibrinopeptides A and B from the Aα and Bβ chains of fibrinogen to form fibrin monomers $(\alpha\beta\gamma)_2$.[5, 6] These cleavages lead to protofibril formation among the monomers and also releases the αC region.[4, 7, 8]

At the final stage of blood coagulation, Factor XIIIa (FXIIIa) introduces γ-glutamyl-ε-lysyl isopeptide bonds between selective glutamines (Q) and lysines (K) within fibrin to form a clot with enhanced elastic properties.[6, 9-13] Moreover, the overall fibrin network has decreased fiber diameter, increased fiber density, reports of thinner fibers, and increased clot stiffness.[11, 14-16] FXIIIa first crosslinks fibrin γQ398 or γQ399 to fibrin γK406 thereby forming γ-γ dimers. Crosslinks involving the fibrin α chain appears later and involves the generation of α-α dimers, γ-α hybrids, and higher α-α polymers.[17-20] Five known reactive glutamines in the αC region include Q221 (and/or 223), Q237, Q328, and Q366.[16, 21-23] FXIIIa also crosslinks α_2-antiplasmin (α_2AP), plasmin activator inhibitor 2 (PAI-2), and fibronectin into the fibrin clot network.[24-27] FXIII(a) and fibrinogen play additional supporting roles in the presence of red blood cells (RBC). FXIII binding to fibrinogen γ390-396 and subsequent crosslinking of the alpha chain helps mediate RBC retention in venous thrombi.[28, 29]

The αC region of fibrinogen contributes its own critical roles in the assembly and properties of clot structure.[8, 30-33] This region has also been directly correlated with adhesion events.[34-36] Fibrinogen αC region (221-610) is composed of a flexible αC connector (221-391) and a more

structured αC domain (392-610).[4, 7] During fibrin polymerization, crosslinking in the αC region has been shown to promote lateral aggregation and protofibril staggering.[8, 30, 37] Studies with native fibrinogen versus the mutant (γQ398N, γQ399N, αK406R) have revealed independent contributions coming from α-α crosslinks.[16, 38] Such crosslinks play major roles in promoting clot stiffness, fiber straightening, and hindering fibrinolysis.

Truncations to the αC region have been shown to alter the nature of the clot formed.[32] With Fibrinogen Otago, the Aα chain ends at P270 resulting in severe hypofibrinogenemia. Further analysis revealed that loss of (271-610) leads to impaired fibrin polymerization and the production of much thicker fibers. [32, 33, 39] Fibrinogen Seoul II (Q328P mutation) exhibits impaired fibrin polymerization and a reduced extent of α-α crosslinking due in part to loss of a reactive glutamine within the αC region.[40]

Several studies have identified specific crosslinking sites in the αC region responsible for lateral aggregation but little is known about the individual reactive glutamines and the roles played by the surrounding residues.[17, 18, 20, 22, 23, 41, 42] Antibody studies by Procyk et al., identified a FXIIIa binding site within αC (242-424) and then narrowed the site more specifically to αC (389-402).[43] Using recombinant αC (233-425), Smith et al demonstrated that the key contact region for FXIII A_2' (thrombin-activated) and A_2B_2 involves αC (371-425) with E396 playing an important role.[44, 45] In close proximity to this FXIII binding region, αC (233-425) contains three reactive glutamines (Q237, Q328, and Q366) that each become crosslinked to a selective set of lysines within the fibrin clot network.[17, 23]

The aim of the current study was to characterize the glutamines within the fibrinogen αC region (233-425) that are known to be crosslinked by FXIIIa under physiological conditions. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry and 2D

Heteronuclear Single Quantum Coherence (HSQC) NMR assays were used to directly monitor FXIIIa-catalyzed reactions between the substrate glutamines in αC (233-425) and a set of lysine mimics.[46] The results demonstrate that Q237 is the most reactive glutamine of fibrinogen αC (233-425) followed by Q366 and Q328. Moreover, no glutamine is dependent on another to react first in the series. New knowledge gained about these three glutamines is considered relative to the conformational features of fibrinogen and to the mutants Fibrinogen Otago and Fibrinogen Seoul II.

Materials and methods

Proteins and chemicals

Human cellular FXIII was a generous gift from the late Dr. Paul Bishop. Stock aliquots of FXIII were prepared in 18 MΩ deionized water and the concentration measured on a Cary 100 Bio UV-Visible spectrophotometer using an extinction coefficient of 1.49 (ml mg^{-1} cm^{-1}) and stored at -70 ºC. Thrombin and glycine ethyl ester (GEE) were obtained from Sigma Aldrich (St. Louis, MO). $^{15}NH_4Cl$ and ^{15}N-GEE were obtained from Cambridge Isotope Laboratories (Andover, MA).

Site-directed mutagenesis of αC (233-425) to generate Q to N substitutions

The PGEX-6P-1 plasmid vector contained cDNA for GST-tagged αC fragment (233-425). This αC sequence was derived from the full-length fibrinogen Aα chain cDNA.[44] Individual glutamine substitutions, Q237N, Q328N, and Q366N, were introduced into αC (233-425) using the QuikChangeII Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The resulting substitutions were confirmed with DNA sequencing. DNAs coding for the different αC (233-425) species were transformed into BL21 Gold DE3 E. coli cells.

Fibrinogen αC (233-425) expression

GST-αC (233-425) and its mutants were expressed as described previously by Smith et al.[44] with few modifications. Briefly, GST-αC expressing cells were incubated in Terrific Broth (1.2% typtone, 2.4% yeast extract, 0.4% glycerol, 742 mM K_2HPO_4, 171 mM KH_2PO_4, and 0.1 mg/ml ampicillin) at 37°C on a rotary shaker until the optical density at 600 nm reached 0.9. αC expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 30°C for 16 h. Cells were harvested by centrifugation at 5000 g at 4°C. Biomass was resuspended

in Wash Buffer (20 mM Tris Base pH 8.0, 50 mM NaCl) and centrifuged at 5000 g at 4°C. Resulting pellets were stored at -20°C.

Frozen pellets were resuspended in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and incubated with 1 mM dithiothreitol and 1 mg/ml lysozyme, followed by the addition of 2 µg/ml aprotinin, 1 µM pepstatin A, 10 µM leupeptin, 4 mM benzamidine hydrochloride, 0.5 mM phenylmethansulphonylfluoride (PMSF), 0.05% sodium deoxycholate, 0.02% triton X-100, 5 µg/ml Dnase I, and 5 mM MgCl. The Dnase I reaction was quenched with 6 mM EDTA, and the lysate was centrifuged at 22,000 g at 4°C. Supernatant was supplemented with 1% (w/v) streptomycin sulfate and centrifuged at 22,000 g at 4°C. The resulting soluble fraction was passed through a 0.2 µm membrane filter and loaded on a GST-affinity column using an AKTAprime™ system (Amersham Biosciences, Piscataway, NJ).

An on-column procedure employing PreScission™ protease was used to cleave αC (233-425) from the GST-tag. The released αC (233-425) was eluted with PBS buffer. Complete cleavage of the GST tag was confirmed by SDS–PAGE and Western blot analysis (Amersham™ anti-GST-HRP conjugate antibody). The resultant αC (233-425) was concentrated in a 5,000 MWCO Vivaspin 2 concentrator (Sartorius, Goettingen). Protein concentrations were determined using an extinction coefficient of 41480 $M^{-1}cm^{-1}$ calculated from ExPASy (www.expasy.org).

MALDI-TOF mass spectrometry kinetic assay: Monitoring crosslinking reaction of glutamines in αC (233-425)

The ability of FXIIIa to crosslink each reactive glutamine in αC (233-425) to the lysine mimic GEE was monitored using our previously optimized MALDI-TOF mass spectrometry kinetic assay.[47] Final concentrations of 500 nM FXIII, 17 mM GEE, 4 mM $CaCl_2$, and MALDI assay buffer [100 mM Tris-acetate, 150 mM NaCl and 0.1% PEG_{8000} pH 7.4] were incubated at 37°C in a 1.5ml reaction tube. The FXIII was activated with bovine thrombin (8.4 U/ml final) for 10 min and then 200 nM PPACK (D- phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) was added to inhibit the thrombin. The crosslinking reaction between αC (233-425) and GEE was initiated by adding a final concentration of 13.6 μM αC (233-425) to a total assay volume of 250 μL. At different time points, a 25 μL aliquot of this reaction mixture was removed and quenched in 1.6 μL of 160 mM EDTA (10 mM final). Control experiments were performed in the absence of FXIII.

All samples were subjected to proteolytic digestion with chymotrypsin and GluC (Roche, Indianapolis, IN). For the digests, 6 μL of either the control or time point sample were combined with 6 μL chymotrypsin buffer (100 mM Tris-HCl, 10 mM $CaCl_2$ pH 7.4) and 1.5 μL chymotrypsin (1 μg/μL) and incubated for 1 hr at 25°C. This digest was quenched with 2 μL 5% TFA. GluC digest was performed in a similar manner with 6 μL of the sample, 6 μL GluC buffer (25 mM NH_4HCO_3 pH 7.8) and 1.5 μL GluC (0.05 μg/μL) incubated for 2hrs at 25°C and quenched with 5% TFA. All digested samples were zip-tipped and analyzed using a MALDI-TOF mass spectrometer (Applied Biosystems Voyager DE-PRO).[47] α-Cyano-4-hydroxycinnamic acid (αCHCA) matrix was employed for chymotrypsin digests and ferulic acid matrix for GluC. This assay was done in triplicate using αC (233-425) from three independent expression trials. The peak-height ratio method

was utilized to determine the extent of crosslinking of GEE to each reactive glutamine with time. The amount of reactant left at each time was calculated as follows:

$$\frac{\sum \text{Reactant Peak Height}}{\sum \text{Reactant Peak Height} + \sum \text{Product Peak Height}} * \text{Peptide concentration}$$

^{15}N-HSQC NMR spectroscopy: Validating αC (233-425) crosslinking with ^{15}N-labeled substrates

FXIIIa-catalyzed crosslinking of ^{15}N-labeled NH_4Cl or ^{15}N-labeled GEE to reactive glutamines in αC (233-425) was monitored using ^{15}N- heteronuclear single quantum coherence (HSQC) NMR spectroscopy.[46] All assays were carried out in a total volume of 400 μL with 10% D_2O added for the NMR deuterium lock. Final concentrations are presented. For crosslinking reactions with ^{15}N-GEE, 800 nM FXIII was proteolytically activated with 21 U/ml bovine thrombin in the presence of 5 mM $CaCl_2$ and 20 mM Borate buffer. Thrombin activity was quenched with 460 nM PPACK. 10 mM ^{15}N-GEE and 35 μM αC (233-425) were added and incubated for 1hr at 37°C. For the $^{15}NH_4Cl$ exchange reactions, 400 nM FXIII was non-proteolytically activated in the presence of excess calcium (50 mM) and 20 mM borate buffer (pH 8) for 10 mins at 37°C. Then, 100 mM $^{15}NH_4Cl$ and 40 μM αC (233-425) were added and the mixture was incubated for 1hr at 37°C. 10% D_2O was added and the mixtures transferred into Shigemi NMR tubes. All reactions were analyzed at 25°C using a single z-axis gradient triple resonance (HCN) cryoprobe run on a 700 MHz Varian Inova NMR spectrometer. The parameters for the 1D HSQC were number of transients (nt) =512, number of increments (ni) = 1, number of points (np) =2048, and sweep width (sw) = 7022.5. For the 2D HSQC, the parameters used were nt =64, ni = 64, np = 2048 and sw =7022.5. The spectra were processed using NMRPipe and nmrDraw.[48]

Results

Preparation and characterization of recombinant αC (233-425)

Recombinant αC (233-425) containing an N-terminal GST-tag and a PreScission™ protease cleavage site was expressed in E.coli and purified. SDS-PAGE results and Western Blot analysis using an anti-GST antibody confirmed that there was no GST-αC or free GST protein detected in the αC samples. The amino acid sequence of αC (233-425) can be found in Figure 1A.

MALDI-TOF mass spectral analysis of separate chymotrypsin and GluC digests of αC (233-425) showed peptide fragments containing all three reactive glutamines. Theoretical protease digests (Protein Prospector, UCSF) and MS/MS analyses were used to identify the αC fragments. The chymotrypsin digest exhibited a peak containing Q328 (2448 m/z) whereas the GluC digest showed peaks for Q366 (1349 m/z) and for Q237 (1550 m/z) (Figure 1B, 1C and Supplemental Table 1).

Monitoring crosslinking of Q237, Q328, and Q366 with GEE

Our MALDI-TOF mass spectrometry kinetic assay was used to monitor FXIIIa-catalyzed crosslinking of each reactive αC (233-425) glutamine to the lysine mimic GEE. The crosslinking reaction was quenched at distinct time points and then digested separately with appropriate protease. A representative set of mass spectral data for a chymotrypsin (Figure 2A) and a protease GluC digest (Figure 2B) show the reactant peak containing the Q of interest and the subsequent product peak (+ 86 m/z for the new Q-GEE). No reactions products were observed in the absence of FXIIIa.

The amount of reactant left after each time point was calculated using the peak height ratio method. As shown in Figures 2 and 3A, the peptide containing Q237 was rapidly crosslinked by 20 seconds and completely depleted by 5 minutes of reaction with GEE. FXIIIa was able to very effectively crosslink the lysine mimic GEE to the Q237 side chain amine. Q328 and Q366 could

also undergo FXIII-catalyzed crosslinking with GEE but at a slower rate than Q237. A plot displaying all three αC glutamines indicates that the order of reactivity toward GEE crosslinking was Q237 >> Q366 ≈ Q328. Reactions with Q366 and Q328 could be brought closer to completion by increasing the FXIIIa concentration by 3-fold and the incubation time to 120 minutes (Figure 3B). When the original FXIIIa concentration was reduced 15-fold, the Q237 reaction rate became much slower with a 50% loss of reactant occurring by 5 minutes (data not shown). There have been previous reports on the α-chain glutamine residues that can be crosslinked by FXIIIa and the lysine partners involved.[23, 42] Using the current assay strategy, the individual reactivities of each glutamine could, for the first time, be directly monitored.

2D ^{15}N HSQC NMR confirms that ^{15}N labeled substrates crosslink to reactive glutamines

^{15}N-labeled substrates have been used to follow reactions and conformational changes in proteins. Here, 2D HSQC NMR was used to further examine the crosslinking of ^{15}N-labeled substrates (GEE and $^{15}NH_4Cl$) to the reactive glutamines on αC (233-425). With the ^{15}N-GEE reaction, the magnetically silent $^{14}NH_2$ on the αC glutamine side chains was replaced with NMR active ^{15}NH amide product that is part of the FXIIIa-catalyzed GEE dependent reaction (Figure 4A). The 2D HSQC spectrum of the FXIIIa-catalyzed reaction shows three crosspeaks with each peak corresponding to an amide ^{1}H (8.4-8.1 ppm) directly attached to a ^{15}N (114 ppm range) (Figure 4B). The observed HSQC peaks revealed that FXIIIa had successfully crosslinked ^{15}N-GEE into the three reactive glutamines within αC (233-425). One crosspeak clearly had an intensity greater than the other two (Figure 4B). Additional HSQC studies demonstrated that $^{15}NH_4Cl$ could also be utilized as a lysine replacement (Supplemental Figure 1).

HSQC experiments with ^{15}N-GEE were then carried out using αC (233-425) mutants in which a single Q was replaced each time with a non-reactive N. As shown in Figure 4C, each mutant displayed two as opposed to the three crosspeaks seen in wild-type αC (233-425). A comparison of chemical shift positions and the mutant employed revealed that the strong intensity peak at (114 ppm, 8.35ppm) corresponds to the Q237. Q328 occurs at (113.8 ppm, 8.20 ppm) and Q366 at (113.9 ppm, 8.16 ppm) (Figure 4B and 4C). The high intensity peak for Q237 matches well with the highest reactivity observed with the MALDI-TOF mass spectrometry kinetics assay and followed by Q366 and Q328.

DISCUSSION

Within a blood clot, FXIIIa introduces γ-glutamyl-ε-lysyl crosslinks between specific reactive glutamines and lysines located in the fibrin γ and α chains.[12, 13, 17, 41] Little is known about the individual contributions of each reactive glutamine and how FXIII selects one glutamine over another. We characterized and ranked three reactive glutamines in fibrinogen αC (233-425) for their abilities to be crosslinked by FXIIIa to the lysine mimic GEE. MALDI-TOF mass spectrometry and NMR strategies were employed that would allow us to directly measure individual reactivities.

Several studies have demonstrated that FXIIIa first introduces γ-γ crosslinks into the blood clot followed by α-α crosslinks.[15, 19] Putative binding sites for FXIIIa have been identified within α and γ fibrinogen chains, and such sites are located either upstream or downstream from the crosslinking glutamines.[28, 44] γ-γ crosslinking plays key roles in fiber appearance time and the generation of fiber density whereas α-α crosslinking is a major determinant in fibrin clot stiffness and hindering fibrinolysis ability.[16, 49] Interestingly, there are far more covalent Q-K pairs involving the α chains than the γ.[23] FXIIIa crosslinks fibrin γQ398 or γQ399 to γK406.[19] By contrast, the αC region contains multiple reactive glutamines [Q221 (and/or Q223), Q23, Q328, and Q366] and multiple reactive lysines [K418, K448, K508, K539, K556, K563, K580, and K601].[22, 23, 41]

Curiously, there is no obvious consensus sequence for the Q-containing substrates of FXIIIa.[50-52] The reactive glutamines are often found in flexible regions of the substrates. It is important to point out that not all freely available glutamines are good FXIII substrates. Fibronectin contains three glutamines toward its N-terminus but only Q3 is highly reactive, minor crosslinking involves Q7 and Q9.[27] α_2-Antiplasmin (N1) contains two reactive glutamines near the N-terminus but only Q2 is utilized. Reactive glutamines of α_2-antiplasmin and PAI-2 become crosslinked to reactive lysines (K230, K303, and K413) within αC (233-425).[25] α_2-Antiplasmin is one of the few FXIIIa

substrates that can be studied employing short peptides. Sequences based on α_2- antiplasmin (N1, 1-15) have been used to characterize individual glutamines, evaluate roles of surrounding residues, and assess effects of peptide length.[53-55] By contrast, similar sized peptides based on the fibrinogen Aα and γ chains make poor Q-substrates for FXIIIa.[56, 57] These observations suggest that fibrinogen chains require a larger, more protein-like environment to promote substrate specificity.

αC (233-425), containing close to 200 amino acids, has been shown to be a highly promising system for probing the FXIIIa substrate specificity toward the Aα chain.[44, 45] αC (233-425) has three reactive glutamines and a putative FXIIIa binding site. GEE serves successfully as a lysine mimic and targets physiological, reactive glutamines.[18, 55, 57] Unlike spectrophotometric assays that indirectly monitor crosslinking activity or SDS PAGE gels that provide more general conclusions about crosslinking, our mass spectrometry kinetic assay directly follows each reactive glutamine. By focusing on αC (233-425), the challenges of working with multiple, competing glutamines and lysines both on fibrinogen Aα and γ chains were also avoided.

Using the MALDI-TOF mass spectrometry assay, we were able, for the first time, to rank the individual glutamine players in fibrinogen αC (233-425). Results revealed that Q237 was the most reactive followed by Q366 and Q328. Our complementary NMR techniques validated these findings. Interestingly, the αC region is proposed to be mostly disordered and no X-ray crystallographic information is available. Despite this disordered nature, all three glutamines in αC (233-425) could still serve as FXIIIa substrates. Other FXIIIa substrates may take advantage of even more defined structural features. Fibronectin, α_2-antiplasmin, and PAI-2 each contain a flexible region for the reactive glutamine(s) that is further supported by a folded protein architecture. Unlike Aα based peptides, we propose that the larger αC (233-425) exhibits some localized conformational environment that allows or promotes interactions with FXIIIa.

The αC reactivity ranking Q237 >> Q366 ≈ Q328 provides new information to help interpret crosslinking pairs reported recently for full length fibrinogen.[23] For that study, the FXIIIa catalyzed crosslinking reactions were followed by trypsin digests and mass spectral analysis. Wang demonstrated that Q237 could be crosslinked to more lysine pairs (αK418, αK508, αK539 αK556 and αK601) than all other reactive glutamines that were probed.[23] The high reactivity we documented for Q237 suggests that this amino acid is well positioned to crosslink a variety of reactive lysines. Unlike Q237, Wang reported that αC Q366 only crosslinks to K539. It should be noted that Q328 was not identified in Wang's study. Previous investigations, however, have shown that Q328 is an important reactive glutamine.[22, 40] In our assay, we were able to characterize Q328 following a chymotrypsin digest. Our ability to monitor all three reactive glutamines simultaneously in αC (233-425) is enhanced by digesting each sample time point separately with chymotrypsin and Glu C (Figure 1).

Using an NMR assay, we were able to confirm that FXIIIa crosslinks both ^{15}N-GEE and ^{15}N-NH_4Cl to reactive glutamines (Figure 4 and supplemental Figure 1) in a similar order as seen in the MALDI-TOF kinetic assay. Furthermore, FXIIIa still crosslinks Q237 fastest following Q328N or Q366N mutations (Figure 4). Also in the absence of the most reactive glutamine Q237N, both Q328 and Q366 are shown to be crosslinked to GEE by FXIIIa (Figure 4). Thus, FXIIIa does not need to crosslink the fast acting αC Q237 before moving on to the other reactive glutamines. Further mutant combinations reveal that if any of the three Qs is replaced with a nonreactive N, the other two Qs can still participate in the crosslinking reactions (Figure 4). The data collected thus far suggest there is no obvious sequentiality in FXIIIa crosslinking.

Our current results can also be considered relative to disease-associated fibrinogen truncations and mutants. In Fibrinogen Otago, a major portion of the αC region (271-610) is absent and in

Fibrinogen Keokuk, a single point mutation changes Q328 to a stop codon generating a larger truncation.[40, 58] In both cases, Q221, Q223, and Q237 are still available for crosslinking by FXIIIa but Q328, Q366, and multiple reactive lysines are missing. Our MS assay suggests that Q237 is highly reactive and the Wang studies revealed that this glutamine interacts with several lysines. FXIIIa may catalyze a heterodimeric reaction between fibrin αC Q237 and fibrin γK406. In normal fibrinogen, the α-γ heterodimer has been reported to make up 2% of the fibrin crosslink contributions, but the reactive Q and K partners have not been identified.[59] αC Q237 (and/or Q221, Q223) might therefore help support the dominant fibrin γ-γ crosslinks observed in SDS-PAGE gels of Fibrinogen Otago treated with FXIIIa and calcium.

In Fibrinogen Seoul II, a single point mutation results in αC Q328 being replaced with proline (Q328P). With this mutation, fibrin γ-γ crosslinking occurs but α-α is impaired.[40] Considering our data, Q237 and Q366 would still be available in the Seoul II patients along with potential K residues in the more C-terminal portion of αC. Multiple crosslinks involving highly reactive Q237 may, however, not be sufficient to overcome the introduction of Q328P.

Recombinant variants that model Fibrinogen Seoul II revealed that Q328P/Q366P exhibited greater impairment of fibrin polymerization than the single mutants Q328P or Q366P.[60] The current work with αC (233-425) suggests Q328 and Q366 exhibit similar reactivities. With each single P substitution, the pyrrole group may induce a conformational change that hinders fibrin architecture and subsequent crosslinking. A greater disruption occurs with the double mutant. Fibrin α-α crosslinking, however, was still observed and likely involved Q237, Q221, and/or Q223. Unlike working with Q328P and Q366P, our Q to N substitutions within αC (233-425) provide a more conservative strategy for understanding the contributions of the different reactive Q residues. Moreover, any glutamines still present within αC mutants can be individually monitored and

kinetically ranked. Q237 always remained the most reactive glutamine but no obvious sequentiality in FXIIIa crosslinking was observed. Park and coworkers propose that conformational problems resulting from P328 help explain the consequences of Seoul II. Another possibility to consider is that crosslinks containing Q328 (and/or Q366) play structural roles within the fibrin clot that differ from those of Q237, Q221, and Q233. These issues can be further probed in the future using full chain fibrinogens.

In summary, we have characterized and ranked three known reactive glutamines Q237, Q328, and Q366 in fibrinogen αC (233-425) using a MALDI-TOF mass spectrometry kinetics assay and a complementary 2D HSQC NMR approach. αC Q237 was shown to be the most reactive glutamine followed by Q366 and Q328. Moreover, FXIIIa can independently crosslink these reactive glutamines. Additional studies will help better understand the individual cross-linking contributions from each reactive Q and their unique effects on clot properties. Continuing investigations on the αC region are anticipated to provide new clues about sources of FXIII substrate specificity and their applications in future drug or assay substrate designs.

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Authorship Section

Contribution: K.N.M and M.C.M designed the research, analyzed the data, and wrote the manuscript; K.N.M and J.C.B performed the research; M.C.M supervised the research; K.A.S, R.A.S.A. and H.P contributed vital samples and critically reviewed the research design and manuscript.

Conflict of Interest: The authors declare no competing financial interests.

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References:

[1] Ariëns, R. A. S. (2013) Fibrin(ogen) and thrombotic disease, *Journal of Thrombosis and Haemostasis 11*, 294-305.

[2] Standeven, K. F., Ariëns, R. A. S., and Grant, P. J. (2005) The molecular physiology and pathology of fibrin structure/function, *Blood Reviews 19*, 275-288.

[3] Weisel, J. W., and Litvinov, R. I. (2013) Mechanisms of fibrin polymerization and clinical implications, *Blood 121*, 1712-1719.

[4] Medved, L., Weisel, J. W., On Behalf Of, F., Factor Xiii Subcommittee Of The Scientific Standardization Committee Of The International Society On, T., and Haemostasis. (2009) Recommendations for nomenclature on fibrinogen and fibrin, *Journal of Thrombosis and Haemostasis 7*, 355-359.

[5] Yang, Z., Mochalkin, I., and Doolittle, R. F. (2000) A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides, *Proceedings of the National Academy of Sciences 97*, 14156-14161.

[6] Mosesson, M. W. (2005) Fibrinogen and fibrin structure and functions, *Journal of Thrombosis and Haemostasis 3*, 1894-1904.

[7] Tsurupa, G., Pechik, I., Litvinov, R. I., Hantgan, R. R., Tjandra, N., Weisel, J. W., and Medved, L. (2012) On the Mechanism of αC Polymer Formation in Fibrin, *Biochemistry 51*, 2526-2538.

[8] Weisel, J. W., and Medved, L. (2001) The Structure and Function of the αC Domains of Fibrinogen, *Annals of the New York Academy of Sciences 936*, 312-327.

[9] Lorand, L., and Graham, R. M. (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions, *Nat Rev Mol Cell Biol 4*, 140-156.

[10] Bagoly, Z., Koncz, Z., Hársfalvi, J., and Muszbek, L. (2012) Factor XIII, clot structure, thrombosis, *Thrombosis Research 129*, 382-387.

[11] Hethershaw, E. L., Cilia La Corte, A. L., Duval, C., Ali, M., Grant, P. J., Ariëns, R. A. S., and Philippou, H. (2014) The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis, *Journal of Thrombosis and Haemostasis 12*, 197-205.

[12] Richardson, V. R., Cordell, P., Standeven, K. F., and Carter, A. M. (2013) Substrates of Factor XIII-A: roles in thrombosis and wound healing, *Clinical science (London, England : 1979) 124*, 123-137.

[13] Nikolajsen, C. L., Dyrlund, T. F., Poulsen, E. T., Enghild, J. J., and Scavenius, C. (2014) Coagulation Factor XIIIa Substrates in Human Plasma: Indentification and incorporation into the clot, *Journal of Biological Chemistry 289*, 6526-6534.

[14] Kurniawan, N. A., Grimbergen, J., Koopman, J., and Koenderink, G. H. (2014) Factor XIII stiffens fibrin clots by causing fiber compaction, *Journal of thrombosis and haemostasis : JTH 12*, 1687-1696.

[15] Ryan, E. A., Mockros, L. F., Stern, A. M., and Lorand, L. (1999) Influence of a Natural and a Synthetic Inhibitor of Factor XIIIa on Fibrin Clot Rheology, *Biophysical Journal 77*, 2827-2836.

[16] Duval, C., Allan, P., Connell, S. D. A., Ridger, V. C., Philippou, H., Ariëns, R. A. S. (2014) Roles of fibrin α- and γ-chain specific cross-linking by FXIIIa in fibrin structure and function, *Thrombosis and haemostasis 111*, 842.
[17] Cottrell, B. A., Strong, D. D., Watt, K. W. K., and Doolittle, R. F. (1979) Amino acid sequence studies on the .alpha. chain of human fibrinogen. Exact location of crosslinking acceptor sites, *Biochemistry 18*, 5405-5410.
[18] Chen, R., and Doolittle, R. F. (1969) Identificatioin of the polypeptide chains involved in the cross-linking of fibrin *Proceedings of the National Academy of Sciences 63*, 420-427.
[19] Chen, R., and Doolittle, R. F. (1971) γ-γ Cross-linking sites in human and bovine fibrin, *Biochemistry 10*, 4486-4491.
[20] Doolittle, R. F., Cassman, K. G., Cottrell, B. A., and Friezner, S. J. (1977) Amino acid sequence studies on the α chain of human fibrinogen. Isolation and characterization of two linked α-chain cyanogen bromide fragments from fully cross-linked fibrin, *Biochemistry 16*, 1715-1719.
[21] Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) Human Factor XIII from Plasma and Platelets: Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin *Journal of Biological Chemistry 248*, 1395-1407.
[22] Matsuka, Y. V., Medved, L. V., Migliorini, M. M., and Ingham, K. C. (1996) Factor XIIIa-Catalyzed Cross-Linking of Recombinant αC Fragments of Human Fibrinogen†, *Biochemistry 35*, 5810-5816.
[23] Wang, W. (2011) Identification of Respective Lysine Donor and Glutamine Acceptor Sites Involved in Factor XIIIa-catalyzed Fibrin α Chain Cross-linking, *Journal of Biological Chemistry 286*, 44952-44964.
[24] Sakata, Y., and Aoki, N. (1980) Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor, *The Journal of Clinical Investigation 65*, 290-297.
[25] Ritchie, H., Lawrie, L. C., Mosesson, M. W., and Booth, N. A. (2001) Characterization of Crosslinking Sites in Fibrinogen for Plasminogen Activator Inhibitor 2 (PAI-2), *Annals of the New York Academy of Sciences 936*, 215-218.
[26] Barry, E. L., and Mosher, D. F. (1989) Factor XIIIa-mediated cross-linking of fibronectin in fibroblast cell layers. Cross-linking of cellular and plasma fibronectin and of amino-terminal fibronectin fragments, *Journal of Biological Chemistry 264*, 4179-4185.
[27] Hoffmann, B. R., Annis, D. S., and Mosher, D. F. (2011) Reactivity of the N-terminal Region of Fibronectin Protein to Transglutaminase 2 and Factor XIIIA, *Journal of Biological Chemistry 286*, 32220-32230.
[28] Aleman, M. M., Byrnes, J. R., Wang, J.-G., Tran, R., Lam, W. A., Di Paola, J., Mackman, N., Degen, J. L., Flick, M. J., and Wolberg, A. S. (2014) Factor XIII activity mediates red blood cell retention in venous thrombi, *The Journal of Clinical Investigation 124*, 3590-3600.
[29] Byrnes, J. R., Duval, C., Wang, Y., Hansen, C. E., Ahn, B., Mooberry, M. J., Clark, M. A., Johnsen, J. M., Lord, S. T., Lam, W., Meijers, J. C., Ni, H., Ariens, R. A., and

Wolberg, A. S. (2015) Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking, *Blood In Press*.
[30] Protopopova, A. D., Barinov, N. A., Zavyalova, E. G., Kopylov, A. M., Sergienko, V. I., and Klinov, D. V. (2015) Visualization of fibrinogen αC regions and their arrangement during fibrin network formation by high-resolution AFM, *Journal of Thrombosis and Haemostasis 13*, 570-579.
[31] Belkin, A. M., Tsurupa, G., Zemskov, E., Veklich, Y., Weisel, J. W., and Medved, L. (2005) Transglutaminase-mediated oligomerization of the fibrin(ogen) αC domains promotes integrin-dependent cell adhesion and signaling, *Blood 105*, 3561-3568.
[32] Collet, J.-P., Moen, J. L., Veklich, Y. I., Gorkun, O. V., Lord, S. T., Montalescot, G., and Weisel, J. W. (2005) The αC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis, *Blood 106*, 3824-3830.
[33] Gorkun, O. V., Henschen-Edman, A. H., Ping, L. F., and Lord, S. T. (1998) Analysis of Aα251 Fibrinogen: The αC Domain Has a Role in Polymerization, Albeit More Subtle Than Anticipated from the Analogous Proteolytic Fragment X, *Biochemistry 37*, 15434-15441.
[34] Yermolenko, I. S., Gorkun, O. V., Fuhrmann, A., Podolnikova, N. P., Lishko, V. K., Oshkadyerov, S. P., Lord, S. T., Ros, R., and Ugarova, T. P. (2012) The Assembly of Nonadhesive Fibrinogen Matrices Depends on the αC Regions of the Fibrinogen Molecule, *Journal of Biological Chemistry 287*, 41979-41990.
[35] Yakovlev, S., Mikhailenko, I., Cao, C., Zhang, L., Strickland, D. K., and Medved, L. (2012) Identification of VLDLR as a novel endothelial cell receptor for fibrin that modulates fibrin-dependent transendothelial migration of leukocytes, *Blood 119*, 637-644.
[36] Safiullin, R., Christenson, W., Owaynat, H., Yermolenko, I. S., Kadirov, M. K., Ros, R., and Ugarova, T. P. (2015) Fibrinogen matrix deposited on the surface of biomaterials acts as a natural anti-adhesive coating, *Biomaterials 67*, 151-159.
[37] Litvinov, R. I., Yakovlev, S., Tsurupa, G., Gorkun, O. V., Medved, L., and Weisel, J. W. (2007) Direct Evidence for Specific Interactions of the Fibrinogen αC-Domains with the Central E Region and with Each Other, *Biochemistry 46*, 9133-9142.
[38] Helms, Christine C., Ariëns, Robert A., Uitte de Willige, S., Standeven, Kristina F., and Guthold, M. (2012) α−α Cross-Links Increase Fibrin Fiber Elasticity and Stiffness, *Biophysical Journal 102*, 168-175.
[39] Ridgway, H. J., Brennan, S. O., Faed, J. M., and George, P. M. (1997) Fibrinogen Otago: a major α chain truncation associated with severe hypofibrinogenaemia and recurrent miscarriage, *British Journal of Haematology 98*, 632-639.
[40] Park, R., Doh, H.-J., An, S.-S. A., Choi, J.-R., Chung, K.-H., and Song, K.-S. (2006) A novel fibrinogen variant (fibrinogen Seoul II; AαGln328Pro) characterized by impaired fibrin α-chain cross-linking, *Blood 108*, 1919-1924.

[41] Sobel, J. H., and Gawinowicz, M. A. (1996) Identification of the α Chain Lysine Donor Sites Involved in Factor XIIIa Fibrin Cross-linking, *Journal of Biological Chemistry 271*, 19288-19297.
[42] Murthy, S. N. P., Wilson, J. H., Lukas, T. J., Veklich, Y., Weisel, J. W., and Lorand, L. (2000) Transglutaminase-catalyzed crosslinking of the Aα and γ constituent chains in fibrinogen, *Proceedings of the National Academy of Sciences 97*, 44-48.
[43] Procyk, R., Bishop, P. D., and Kudryk, B. (1993) Fibrin - recombinant human factor XIII a-subunit association, *Thrombosis Research 71*, 127-138.
[44] Smith, K. A., Adamson, P. J., Pease, R. J., Brown, J. M., Balmforth, A. J., Cordell, P. A., Ariëns, R. A. S., Philippou, H., and Grant, P. J. (2011) Interactions between factor XIII and the αC region of fibrinogen, *Blood 117*, 3460-3468.
[45] Smith, K. A., Pease, R. J., Avery, C. A., Brown, J. M., Adamson, P. J., Cooke, E. J., Neergaard-Petersen, S., Cordell, P. A., Ariëns, R. A. S., Fishwick, C. W. G., Philippou, H., and Grant, P. J. (2013) The activation peptide cleft exposed by thrombin cleavage of FXIII-A2 contains a recognition site for the fibrinogen α chain, *Blood 121*, 2117-2126.
[46] Shimba, N., Yokoyama, K.-i., and Suzuki, E.-i. (2002) NMR-Based Screening Method for Transglutaminases: Rapid Analysis of Their Substrate Specificities and Reaction Rates, *Journal of Agricultural and Food Chemistry 50*, 1330-1334.
[47] Doiphode, P. G., Malovichko, M. V., Mouapi, K. N., and Maurer, M. C. (2014) Evaluating factor XIII specificity for glutamine-containing substrates using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay, *Analytical Biochemistry 457*, 74-84.
[48] Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes, *Journal of biomolecular NMR 6*, 277-293.
[49] Standeven, K. F., Carter, A. M., Grant, P. J., Weisel, J. W., Chernysh, I., Masova, L., Lord, S. T., and Ariëns, R. A. S. (2007) Functional analysis of fibrin γ-chain cross-linking by activated factor XIII: determination of a cross-linking pattern that maximizes clot stiffness, *Blood 110*, 902-907.
[50] Coussons, P. J., Price, N. C., Kelly, S. M., Smith, B., and Sawyer, L. (1992) Transglutaminase catalyses the modification of glutamine side chains in the C-terminal region of bovine beta-lactoglobulin, *Biochemical Journal 283*, 803-806.
[51] Sugimura, Y., Hosono, M., Wada, F., Yoshimura, T., Maki, M., and Hitomi, K. (2006) Screening for the Preferred Substrate Sequence of Transglutaminase Using a Phage-displayed Peptide Library: Identification of Peptide Substraes for TGASE 2 and Factor XIIIA, *Journal of Biological Chemistry 281*, 17699-17706.
[52] Severina, E., Nunez, L., Baker, S., and Matsuka, Y. V. (2006) Factor XIIIa Mediated Attachment of S. aureus Fibronectin-Binding Protein A (FnbA) to Fibrin: Identification of Gln103 as a Major Cross-Linking Site, *Biochemistry 45*, 1870-1880.

[53] Cleary, D. B., and Maurer, M. C. (2006) Characterizing the specificity of activated Factor XIII for glutamine-containing substrate peptides, *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1764*, 1207-1217.
[54] Cleary, D. B., Doiphode, P. G., Sabo, T. M., and Maurer, M. C. (2009) A non-reactive glutamine residue of α2-antiplasmin promotes interactions with the factor XIII active site region, *Journal of Thrombosis and Haemostasis 7*, 1947-1949.
[55] PÉNzes, K., KÖVÉR, K. E., Fazakas, F., Haramura, G., and Muszbek, L. (2009) Molecular mechanism of the interaction between activated factor XIII and its glutamine donor peptide substrate, *Journal of Thrombosis and Haemostasis 7*, 627-633.
[56] Gorman, J. J., and Folk, J. E. (1980) Structural features of glutamine substrates for human plasma factor XIIIa (activated blood coagulation factor XIII), *Journal of Biological Chemistry 255*, 419-427.
[57] Fickenscher, K., Aab, A., and Stuber, W. (1991) A photometric assay for blood coagulation factor XIII, *Thrombosis and haemostasis 65*, 535-540.
[58] Lefebvre, P., Velasco, P. T., Dear, A., Lounes, K. C., Lord, S. T., Brennan, S. O., Green, D., and Lorand, L. (2004) Severe hypodysfibrinogenemia in compound heterozygotes of the fibrinogen AalphaIVS4 + 1G>T mutation and an AalphaGln328 truncation (fibrinogen Keokuk), *Blood 103*, 2571-2576.
[59] Siebenlist, K. R., and Mosesson, M. W. (1996) Evidence for Intramolecular Cross-Linked Aα·γ Chain Heterodimers in Plasma Fibrinogen, *Biochemistry 35*, 5817-5821.
[60] R. Park, L. P., J. Song, J-Y. Seo, T.-Y. Choi, J.-R. Choi, O.V. Gorkun, S.T. Lord. (2013) An engineered fibrinogen variant AαQ238, 366P does not polymerise normally but retains the ability to form α crooslinks, *Thrombosis and haemostasis 109*, 199-206.

Figure Legends

Figure 1. Proteolytic digests of fibrinogen αC (233-425) show three reactive glutamines in a MALDI-TOF MS

(A) αC (233-425) sequence showing position of reactive glutamine in bold (B) MALDI- TOF MS spectrum of αC (233-425) digested with chymotrypsin contained a fragment with one reactive glutamine Q328 at 2448.7 m/z (C) GluC digest of αC (233-425) showed two peaks (1550.5 m/z and 1349.3 m/z) containing Q366 and Q237, respectively in the MALDI-TOF MS

Figure 2. Crosslinking of reactive glutamines in fibrinogen αC (233-425) with lysine mimic glycine ethyl ester (GEE)

Representative MALDI-TOF spectra showing crosslinking reaction between reactive glutamine in αC (233-425) and the lysine mimic GEE catalyzed by FXIIIa (A) Chymotrypsin digest at time points 0, 5, 10 and 15 mins shows that the fragment peak containing reactive glutamine Q328 (2448.5 m/z) was crosslinked to GEE by FXIIIa to form a product peak Q328-GEE (2534.5 m/z). Each spectrum was obtained from the same starting assay and represents a quenched time point followed by a chymotrypsin digest analyzed using MALDI-TOF MS (B) A similar plot with GluC digest at time points 0, 0.4, 5, and 10 mins shows reactant and product peaks containing Q237 and Q366 and their respective GEE-crosslinked products (1636.5 m/z and 1434.3 m/z). Q237 is mostly crosslinked to product by 20 seconds as compared to Q366, which was only crosslinked to product by 10 mins.

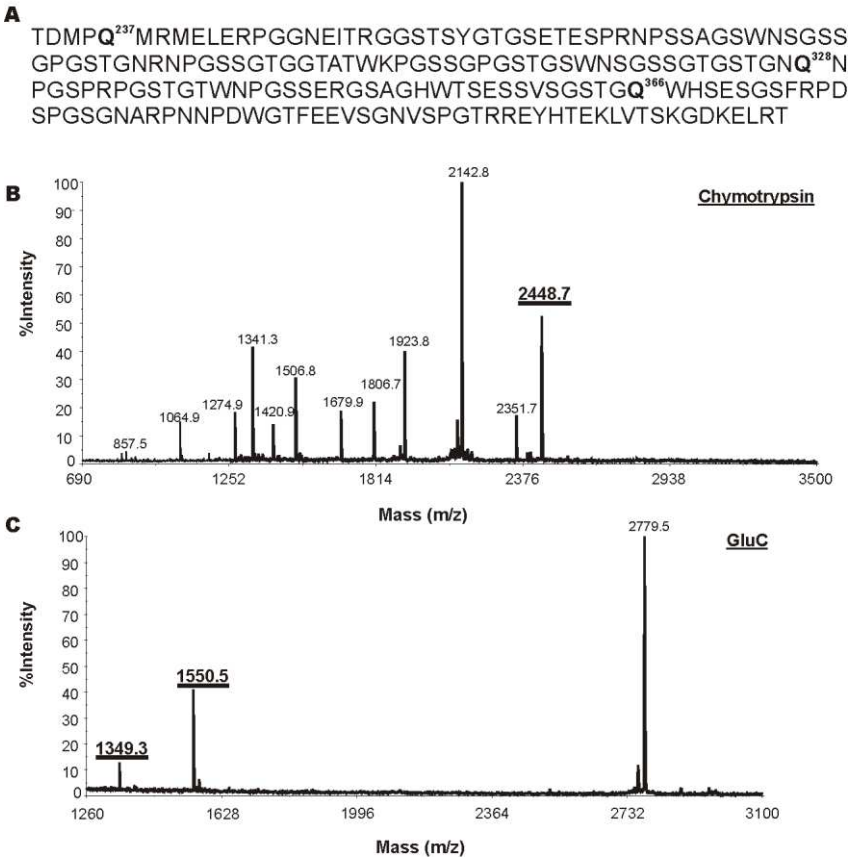
Figure 3. Plot showing combined reactivities of Q237, Q328 and Q366 in fibrinogen αC (233-425)

(A) A combined graph showing the rate of consumption of all three reactive glutamines Q237 (diamonds ♦), Q328 (squares ■), and Q366 (triangles ▲). The peak height ratio method was used to calculate the amount of reactant left in triplicate experiments and plotted as mean ± STD. These reactive glutamines were ranked as Q237 > > Q366 ≈ Q328. (B) An increase in FXIIIa concentration by 3-fold shows that Q366 reacts comparably with Q328 even at higher time points.

Figure 4. Reactive glutamines in αC (233-425) are crosslinked by FXIIIa in wild-type αC and following single Q to N substitutions

A) Reaction scheme for FXIIIa-catalyzed crosslinking reaction of ^{15}N-GEE with reactive glutamines in αC (233-425) as monitored by NMR spectroscopy. B) 2D ^{1}H-^{15}N HSQC NMR spectrum for the reactions between αC (233-425) and ^{15}N-GEE. The three NMR peaks observed correspond to successful crosslinking reactions with the three glutamines Q237, Q328, and Q366. C) FXIIIa-catalyzed reactions between ^{15}N-GEE and the individual αC mutants Q237N, Q328N, and Q366N as monitored by 2D ^{1}H-^{15}N HSQC NMR. In αC Q237N, peaks corresponding to Q328 and Q366 were seen (top). Q237 reacted fastest following either Q328N or Q366N substitutions (middle and bottom panels).

Figure 1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PGSPRPGSTGTWNPGSSERGSAGHWTSESSVSGSTGQ366WHSESGSFRPD
SPGSGNARPNNPDWGTFEEVSGNVSPGTRREYHTEKLVTSKGDKELRT
B
Chymotrypsin
%Intensity
2142.8
2448.7
1341.3
1923.8
1506.8
1806.7
1274.9
1679.9
1064.9
1420.9
2351.7
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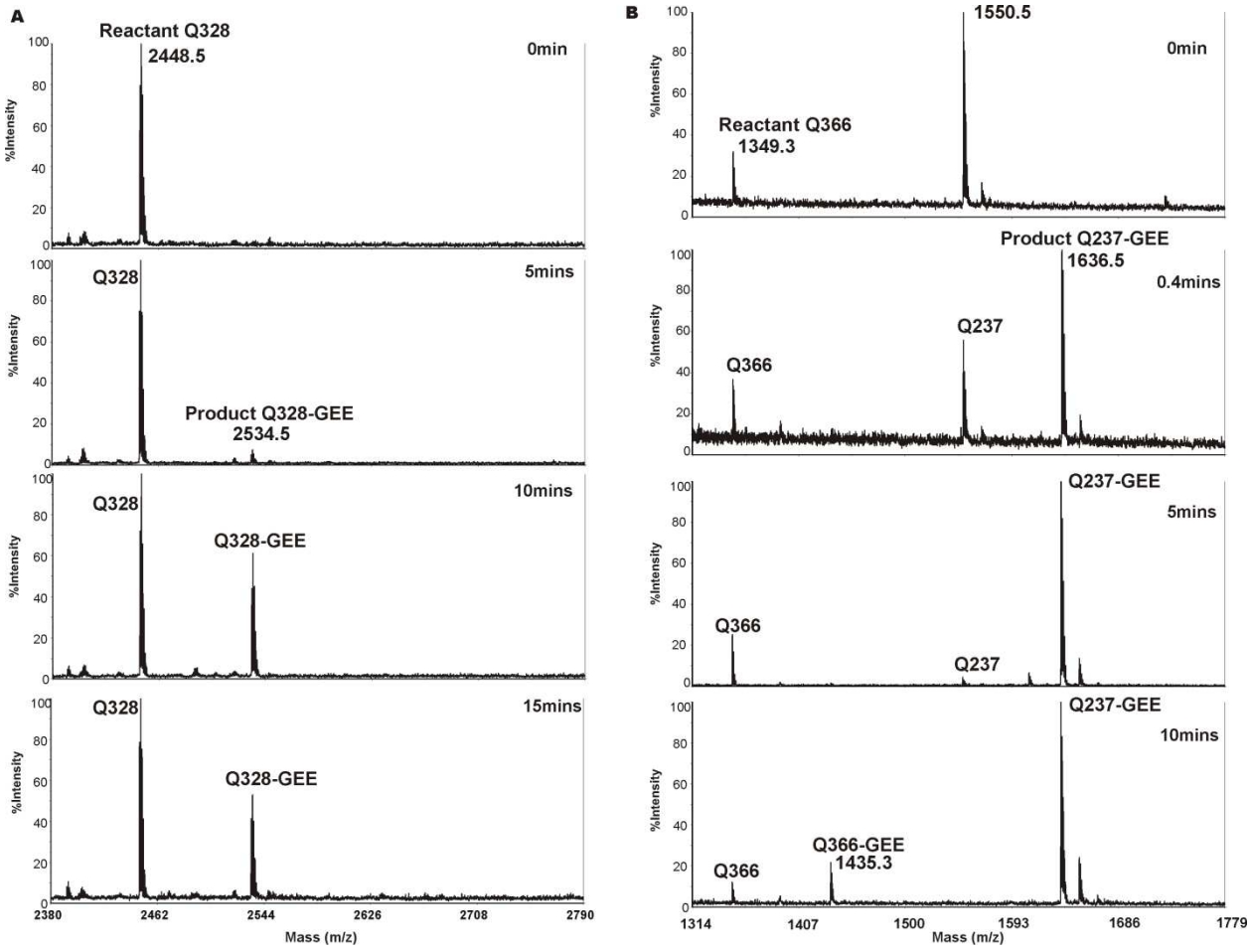
Mass (m/z)
C
GluC
%Intensity
2779.5
1550.5
1349.3
Mass (m/z)

Figure 2


A
Reactant Q328
2448.5
0min
Q328
5mins
Product Q328-GEE
2534.5
Q328
10mins
Q328-GEE
Q328
15mins
Q328-GEE
%Intensity
Mass (m/z)
2380
2462
2544
2626
2708
2790
B
1550.5
0min
Reactant Q366
1349.3
Product Q237-GEE
1636.5
0.4mins
Q237
Q366
Q237-GEE
5mins
Q366
Q237
Q237-GEE
10mins
Q366-GEE
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Q366
1314
1407
1500
1593
1686
1779

Figure 3

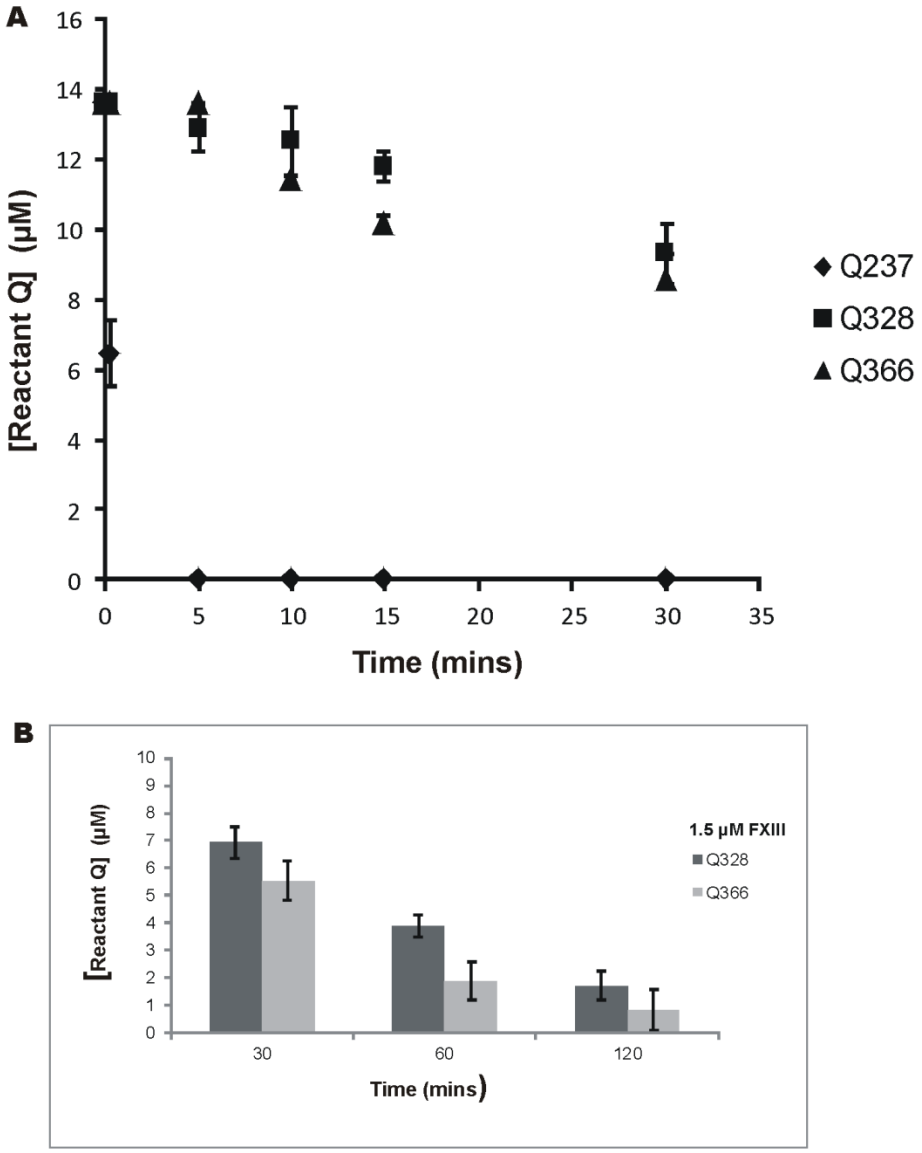
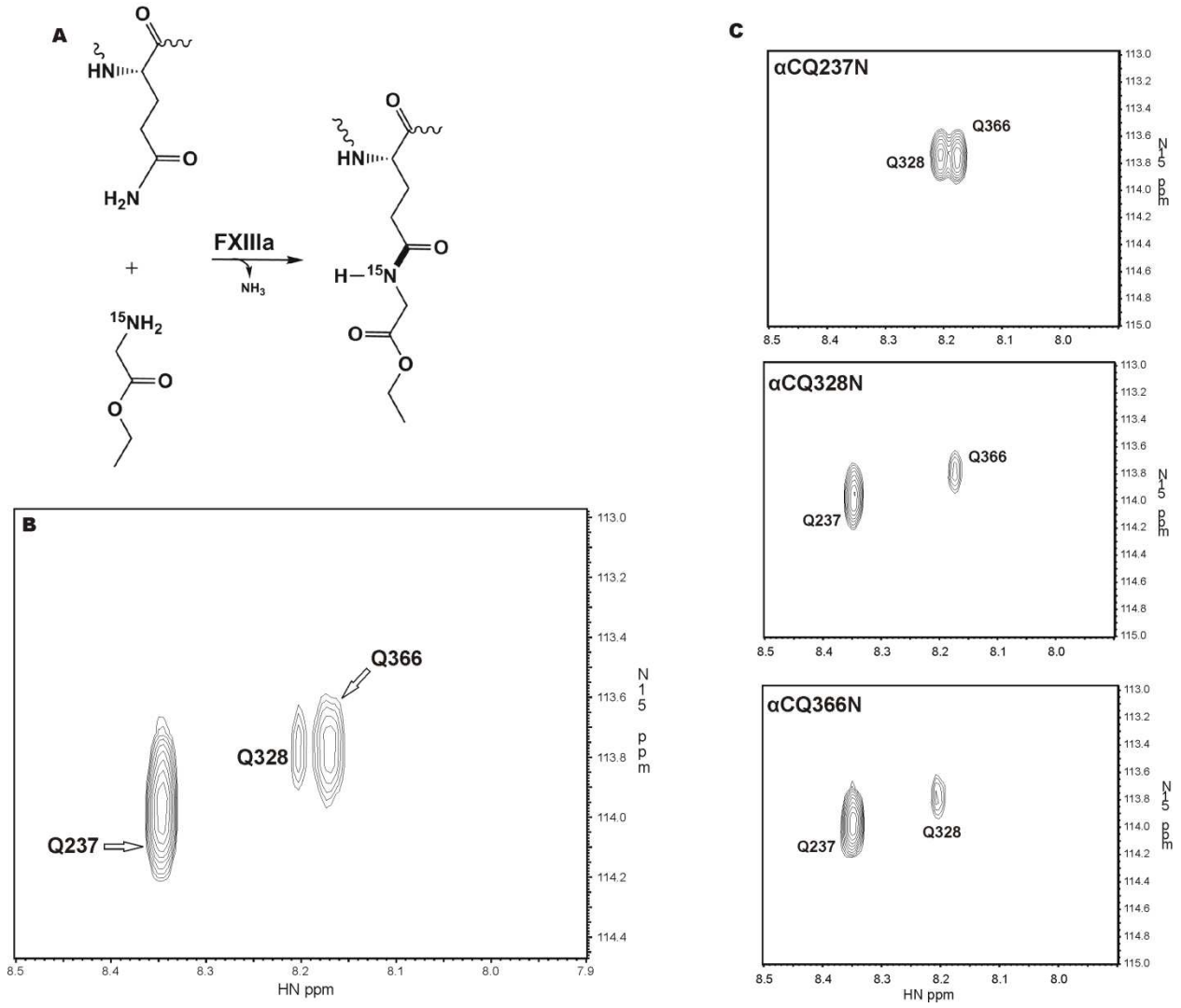

A
[Reactant Q] (μM)
Time (mins)
Q237
Q328
Q366
B
[Reactant Q] (μM)
1.5 μM FXIII
Q328
Q366
30
60
120
Time (mins)

Figure 4


A
FXIIIa
NH3
B
Q366
Q328
Q237
HN ppm
N15 ppm
C
αCQ237N
αCQ328N
αCQ366N
Q366
Q328
Q237
HN ppm
N15 ppm

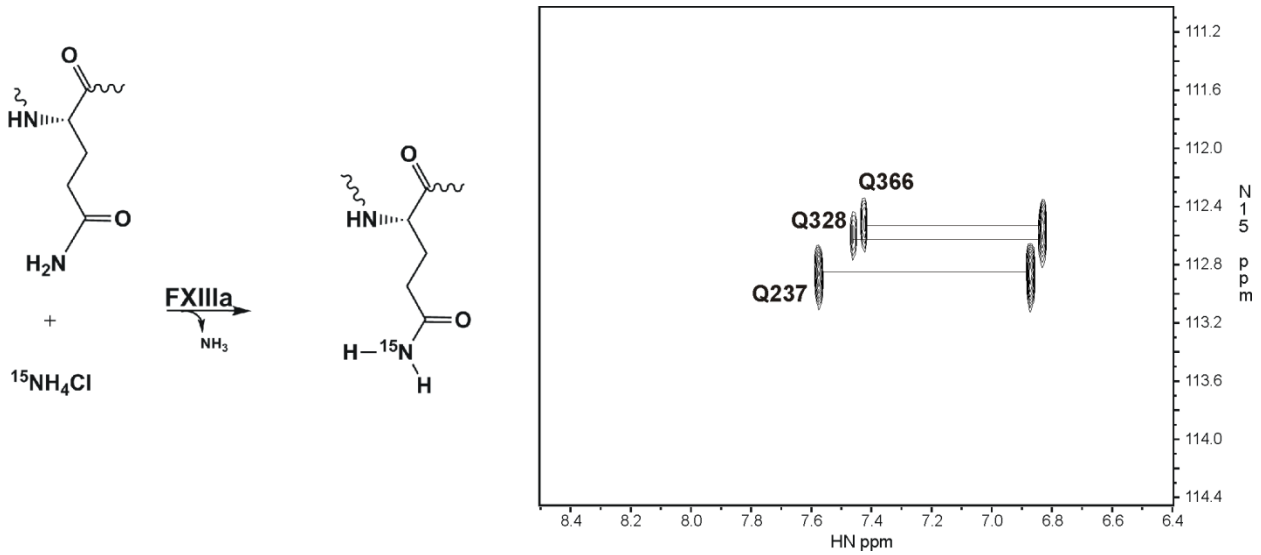
Supplemental Data

Table 1. Sequence and mass of proteolytically derived peptides containing αC Q237, Q328, and Q366

Reactive Glutamine	Amino Acid Sequence	Theoretical m/z	Experimental m/z	Digest
Q237*	GPLGSTMP**Q**MRME	1549.7	1550	GluC
Q328	NSGSSGTGSTGN**Q**NPGSPRP GSTGTWNPGSSERGSAGHW	2448.1	2448	Chymotrypsin
Q366	SSVSGSTG**Q**WHSE	1348.6	1349	GluC

*MS/MS analysis on the peptide containing Q237 revealed that amino acids GPLGS are located just N-terminal to the αC 233-425. These amino acids remain following PreScission protease cleavage of the GST-αC (233-425). The sequences containing Q328 and Q366 were readily identified using a theoretical protease digest (Protein Prospector UCSF).

Supplemental Figure 1. Reaction scheme and a representative spectrum for FXIIIa-catalyzed crosslinking of reactive glutamines in αC (233-425) with $^{15}NH_4Cl$ as monitored by 2D ^{15}N-^{1}H HSQC NMR



FXIIIa substitutes the magnetically silent $^{14}NH_2$ on the αC glutamine side chains with NMR active $^{15}NH_2$ from isotopically labeled $^{15}NH_4Cl$. NMR peaks are observed in the 2D ^{1}H-^{15}N HSQC spectrum confirming that Ca^{2+} activated FXIII successfully catalyzed reactions between glutamines in αC (233-425) and $^{15}NH_4Cl$. Each peak corresponds to a ^{1}H directly attached to a ^{15}N. The ^{15}N for each glutamine was in the 112 ppm range. Two sets of peaks were observed for each glutamine side chain amine since the two protons on the $^{15}NH_2$ group are not equivalent. One proton (^{1}H) has a chemical shift in the 7.5 ppm range whereas the other appears in the 6.8 ppm range. A review of the peak patterns reveals, unexpectedly, two overlapping peaks at 6.84 ppm suggesting similar chemical environments for these two protons.