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<http://doi.org/10.18297/honors/123>

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Evaluating the role that a putative FXIII binding site plays in the reactivity of three glutamines
within the coagulation substrate Fibrinogen A α (233-425)

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Submitted in partial fulfillment of the requirements for graduation *summa cum laude*

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March, 2016

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Abstract

Formation of a stable thrombus *in vivo* depends on the interaction between fibrinogen and the transglutaminase factor XIII (FXIII). Characteristics of blood plasma, such as concentration of Ca^{2+} , are well known to regulate the activation and subsequent reactivity of FXIII. Despite this knowledge, the role fibrin(ogen) plays in modulating FXIII activation and reactivity is not entirely delineated. Currently, the coagulation substrate fibrinogen A α is proposed to contain a binding site for the active factor XIII with fibrinogen A α E396 serving a major role.

A series of mass spectrometry and NMR spectroscopy studies was conducted to assess whether putative interactions between residues 389-403 within Fbg A α and FXIII influence the ability of FXIII A $_2^*$ to crosslink reactive residues within recombinant Fbg α A 233-425. Results indicate the electrostatic interaction of Fbg α A E396 with FXIII does not appreciably influence either the activation of FXIII A $_2$ to FXIII A $_2^*$ or the ability of FXIII A $_2^*$ to recognize and bind to the substrate Fbg α A 233-425 and catalyze the crosslinking reaction.

Introduction

The circulatory system possesses the capability to self-heal. Proper and timely thrombosis prevents continued blood loss and maintains hemostasis within the human body. Abnormal blood clotting may result from one or more variations in the biochemical mechanism of coagulation. Disorders of coagulation can be acquired or hereditary. Most inheritable coagulopathies result from either autosomal or X-linked mutations that encode deficiency in or nonfunctional versions of the proteins involved in coagulation. The most common coagulation disorders inherited in humans include von Willebrand disease and the hemophilias¹ [1]. Prevalent etiologies include deficiencies in the cofactors essential to proper coagulation, such as vitamins K and C [2, 3]. Even the smallest amount of improper coagulation can pose a major risk to health.

Coagulation is intimately linked to cardiovascular health. Cardiovascular diseases, such as coronary heart disease, stroke, and deep vein thrombosis (DVT), constitute the leading cause of hospitalization and death in the United States [4]. Certain genetic variations in clotting proteins have been clinically shown to affect an individual's susceptibility to cardiovascular disease [5]. In addition to changes in lifestyle and diet, the therapeutic modulation of coagulation could serve as a method in both the prevention and intervention against certain cardiovascular diseases. Advances in the coagulation field may also improve current medical testing and certain surgical practices [6]. Research into the protein-to-protein interactions of coagulation provides insight into a biochemical process that continues to affect the livelihood of millions.

Although multiple, interrelated signals initiate thrombosis, the coagulation cascade can be generally divided into two mechanisms: the contact factor and tissue factor pathways. The tissue factor pathway predominates *in vivo*. Tissue factor (TF) while absent in the endothelium, is an

¹ The Centers for Disease Control and Prevention estimate von Willebrand disease affects 1% of the population. The hemophilias affect 1 in 5,000 male births. Approximately 400 children are born with hemophilia each year.

integral membrane glycoprotein present in tissues of the brain, lung, blood vessel wall, and placenta. Extrinsic activation of coagulation occurs when injury allows plasma to contact cells expressing TF [7]. The pathway then proceeds with the combinatory interactions of twenty different coagulation factors to form a hard clot. Notably, monocytes and damaged endothelium within atherosclerotic plaques synthesize TF in the presence of multiple pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin1- β [8]. TF exposure and the subsequent activation of coagulation may cause rapid thrombotic blockage of blood vessels in the event of plaque rupture. Ergo, TF activation of thrombosis is consequently implicated in many cases of myocardial infarction, stroke and DVT [9]. The less critical contact factor pathway involves factor XII activation and couples with signals from the tissue factor pathway. Both pathways converge at the multistep conversion of prothrombin (II) to α -thrombin (II_a).

Figure 1: Coagulation consists of the contact factor and tissue factor pathways, which converge to form a prothrombinase complex (FV_a-FX_a). FV_a-FX_a generates II_a from prothrombin. Various steps are dependent on Ca²⁺, phospholipid (PL), and high-molecular-weight kininogen (HK).

As demonstrated by Figure 1, the coagulation cascade proceeds with the interactions of multiple coagulation factors. With the exception of fibrinogen and factor XIII (FXIII), the coagulation factors consist of multiple serine proteases. Sequence homology suggests most of the coagulation proteases may have arisen from a series of gene duplications [10]. Additionally, clotting proteases demonstrate active site homology with many pancreatic serine proteases involved in digestion, such as chymotrypsin, trypsin, and elastin. The activation of both digestive and clotting proteases requires the cleavage of a N-terminal propeptide, which further supports an evolutionary relationship between the two pools of serine proteases. While similarities exist between digestive proteases and many coagulation factors, both groups of enzymes appear to be regulated differently. Differential forms of regulation allow both groups of proteases to operate appropriately within a specific physiological environment.

Proper coagulation culminates in the timely formation of an ample blood clot; therefore, the cascade involves multiple levels of regulation to prevent inappropriate thrombosis. Unlike the serine proteases involved in digestion, many of the clotting factors are fully activated only in the presence of Ca^{2+} and an anionic phospholipid surface. [11]. Anionic phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), are nearly exclusive to the cytofacial leaflet of the plasma membrane [12]. Exposure of an anionic phospholipid surface occurs predominately during injury. Consequently, anionic phospholipid can attract plasma Ca^{2+} . Multiple clotting factors contain one or more γ -carboxyglutamate (Gla) binding domains that then chelate Ca^{2+} ions with high affinity [2, 3].² In this way, the anionic surface serves as an electrostatic docking station to collect multiple clotting factors and orients coagulation at a site of

² A vitamin K dependent posttranslational modification converts glutamate residues into γ -carboxyglutamate (Gla) residues within Gla domains of a proenzyme. Gla domains chelate Ca^{2+} .

injury. Additionally, concise modulation of Ca^{2+} levels in plasma can further control the extent of activation and propagation of the cascade.

The formation of a blood clot is immediately dependent on the activation and interaction of three coagulation factors: prothrombin, FXIII, and fibrinogen. In the first of two steps to form a thrombus, activated thrombin (IIa) cleaves two anionic N-terminal fibrinopeptides (A and B) from fibrinogen to form fibrin monomers [13]. Noncovalent agglomeration of multiple monomers forms a fragile clot of protofibrils. Secondly, activated FXIII mediates the covalent crosslinking of fibrin monomers to effectively convert the soft clot into a hard thrombus. The integrity of the resulting thrombus depends on multiple factors. Rate of crosslinking and incorporation of anticoagulants, such as α_2 -antiplasmin, have been experimentally shown to affect clot structure [14]. Given the intricate activation and interaction of these three critical factors, regulation must be equally as efficient to ensure immediate and proper thrombosis.

Prothrombin is a 579-residue zymogen present in the blood at 100 $\mu\text{g/mL}$ (1.388 μM). Human prothrombin consists of three general domains: a large N-terminal propeptide, a 36-residue A chain, and a 259-residue catalytic B chain. The N-terminal propeptide, released upon prothrombin activation, is further composed of a Gla domain and two kringle domains in tandem. A single disulfide bond persistently links the A and B chains together throughout a multistep activation process [15]. Prothrombin activation *in vivo* begins with cleavage of the Arg 271 – Thr272 and Arg 320 – Ile 321 peptide bonds by the $\text{FV}_a\text{-FX}_a$ prothrombinase complex in the presence of Ca^{2+} and anionic phospholipid. Prothrombinase cleavage facilitates the release of the propeptide and dissociation of activated thrombin from the phospholipid surface. Formed thrombin then autocleaves to trim 13 residues from the enzyme's N-terminus to generate α -thrombin (IIa) [16]. IIa proceeds to serve both coagulant and anticoagulant roles.

An important role of IIa is to convert fibrinogen to fibrin. Human fibrinogen is a 340-kDa plasma glycoprotein that circulates within the blood at 2.0-4.0 mg/mL. Structurally, fibrinogen is a dimer of trimers consisting of two 610-residue A α chains, two 461-residue B β chains, and two 411-residue γ chains. X-ray crystallography suggests the center regions of the α , β , and γ chains associate into a symmetric set of α -helical coiled-coils. Globular D domains hang at both ends of the molecule and a globular E domain sits between the coiled-coils [17]. Anionic fibrinopeptides (FpA and FpB) hang from the central E domain and prevent premature aggregation of the protein in circulation. Upon IIa cleavage of FpA and FpB, multiple non-covalent interactions between the D and E domains promote the agglomeration of the newly formed fibrin monomers [13].

Fibrin formation also permits the release of the α C region of A α fibrinogen (Fbg α C) from the E domain. Fbg α C contains specific residues used by activated FXIII to crosslink a reactive glutamine of one fibrin monomer with a reactive lysine of another fibrin monomer [18]. The γ chain is also crosslinked by FXIII, with γ - γ crosslinks forming faster than α - α crosslinks. FXIII activity also affects other characteristics of a thrombus *in vivo*, such as the retention of red blood cells within the linked fibrin network [19, 20].³ Therefore, FXIII activation and interaction with fibrinogen are critical areas of study to better understand thrombogenesis *in vivo*.

Figure 2: Fbg α C are released upon IIa cleavage as fibrin agglomerates to form a soft clot.

³ Trapped red blood cell may account for up to half the weight of a thrombus *in vivo*.

The transglutaminase factor XIII (FXIII) presents *in vivo* as one of two zymogens. Platelets, monocytes, and multiple cells of the placenta express FXIII as a homodimer of two 731-residue A chains (FXIII A₂). Inactive plasma FXIII circulates as a tetramer composed of the two aforementioned A chains in addition to two 641-residue B chains (FXIII A₂B₂). Both A subunits are composed of an activation peptide, a β -sandwich domain, two β -barrel domains, and a catalytic center. FXIII B subunits bind FXIII A subunits with a K_d of 1.0×10^{-10} M and 1% of FXIII A₂ exists in the free form [21]. The association of the A and B subunits appears to increase the half-life of FXIII A₂B₂ in circulation. While FXIII activity serves multiple roles, the enzyme is best known to crosslink fibrin into a thrombus [22].

Figure 3: (Left) An individual FXIII A subunit is composed of multiple domains. (Right) Two FXIII A subunits dimerize as indicated: activation peptides in purple, β -sandwiches in blue, β -barrels 1 in green, β -barrels 2 in yellow, and catalytic core in red.

In plasma, IIa cleavage removes a 37-residue portion of the activation peptide to convert FXIII A₂B₂ to an FXIII A₂'B₂ intermediate. Experiments in X-ray crystallography show a subsequent Ca²⁺-dependent conformational change pushes both β -barrel domains away to expose and set up the FXIII active site [23]. Ca²⁺ also contributes to the release of each A subunit from the corresponding B subunit (FXIII A₂^{*}). FXIII A₂^{*} immediately proceeds to catalyze formation of ϵ -amino(γ -glutamyl)lysine isopeptide bonds between two fibrin monomers [24].

Platelet FXIII A₂Plasma FXIII A₂B₂

Figure 4: (*Left*) With platelet FXIII, IIa cleaves the activation peptide each monomer of FXIII A₂. Ca²⁺ ion converts FXIII A₂' to functional FXIII A₂*. (*Right*) In plasma, after IIa cleavage, Ca²⁺ mediates the dissociation of FXIII A₂' from FXIII B₂ and setup of the FXIII A₂* active site.

FXIII-A₂* operates via a catalytic triad similar to those of the cysteine proteases [25].

The transglutaminase mechanism of FXIII A₂* involves attack by the catalytic cysteine thiolate on the amide group of a reactive glutamine to form a thioester linkage. Ammonia is concurrently released. Subsequently, the thioester intermediate transfers the glutamyl unit to an amine to form an amide linkage. Implicit to this mechanism is the prediction that the protein presenting the reactive glutamine localizes preferentially within FXIII A₂* through some binding interaction [26]. Putative binding sites of FXIII have been restricted to the γ 390-396 and Aα 389-403 segments of human fibrinogen [27].

Figure 5: FXIII A₂* uses a cysteine protease-like catalytic triad to link macromolecules. The catalytic C314 thiolate is stabilized by an electrostatic interaction with the imidazolium ion of H373. D369 is suspected to accept a hydrogen bond from H373. The reactive thiolate attacks a reactive Gln to form the thioester intermediate as shown to the right.

The proposed FXIII binding site on Fbg Aα may facilitate interaction between Fbg αC and FXIIIa A₂* and/or enhance activation of FXIII. A binding study by Smith *et. al* suggests a segment of the Fbg αC₂₄₂₋₄₂₄ truncation could play a regulatory role in either the activation of FXIII and/or the binding of a recombinant FXIII A₂* (rFXIII A₂*). Fbg αC₂₄₂₋₄₂₄ wild type has

been shown to bind FXIII A₂B₂ with a K_d of approximately 10 nM and to the rFXIII-A₂^{*} with a K_d of $2.35 \pm 0.09 \mu\text{M}$ [28], [26]. Studies indicate E396A mutagenesis decreased Fbg αC 242-424 binding to a rFXIII-A₂^{*}. Further *in silico* calculations by Smith *et al.* predict salt bridge formation between Fbg αC E396 and R158 within the activation cleft of FXIII A₂^{*} facilitates enzyme docking. Association of Fbg αC 242-424 E396 with FXIII is also predicted to cause downstream stabilization within Fbg αC that could further influence protein interaction.

No known study has been conducted to assess whether a mutation at Fbg αC 233-425 E396 affects the FXIII A₂^{*} crosslinking of the reactive glutamines within the Fbg αC 233-425 truncation. Fbg αC 233-425 is expected to contain the same FXIII binding site within Fbg αC 389-403 previously investigated by Smith *et al* [26]. The current work entails three reactivity studies used to compare an expressed Fbg αC 233-425 E396A mutant with the Fbg αC 233-425 wild type. Strategies utilizing matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and ¹H-¹⁵N heteronuclear spin quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy were employed to investigate effects of mutagenesis at Fbg αC 233-425 E396. With this study, the role of Fbg A α E396 can be further understood.

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GPLGSTDMP237QMRMELERPGGNEITRGGSTSYGTGSETESPRNPSSAGSWNSGSSGPGSTGN
RNPSSGTGGTATWKPSSGPGSTGSWNSGSSGTGSTGN328QNPSPRPGSTGTWNPSSERG
SAGHWTSESSVSGSTG366QWHSESGSFRPDSPGSGNARPNNPDWGTFE396EVSGNVSPGTRRE
YHTEKLVTSKGDKELRT

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Figure 6: The primary sequence of Fbg αC 233-425 is shown above. The three reactive glutamines are depicted in red, while the glutamate proposed as critical to formation of the putative FXIII A₂^{*} binding site (underlined) is shown in green.

Specific Aims

The α C region of human fibrinogen (Fbg α C) contains three reactive glutamine residues (Q237, Q328 and Q366) involved in FXIII A₂^{*} crosslinking of the α -chains of fibrin [24]. Studies suggest, in addition to reactive glutamines, Fbg α C may contain certain amino acids that play a role in either FXIII enzyme-substrate interaction or activation. Previous experiments demonstrate that FXIII interacts with truncations of human fibrinogen α C, with Fbg α C E396 potentially facilitating enzyme docking. The glutamyl residue at this position has been proposed to aid in electrostatic stabilization between Fbg α C and FXIII. Additionally, structural effects afforded by the residue are suggested to enhance FXIII activation and reactivity. To address these research issues, the following work entailed three reactivity studies to compare an expressed Fbg α C 233-425 E396A mutant against the Fbg α C 233-425 wild type.

Primary Hypothesis: Fbg α C 233-425 contains a binding site within Fbg α C 389-402 that may modulate the ability of FXIII to crosslink α C Q237, Q328 and Q366.

AIM I: The putative Fbg α C binding site Fbg α C 389-402 may facilitate enzyme-substrate interaction. Pre-activated FXIII-A₂^{*} would crosslink reactive glutamines of Fbg α C 233-425 E396A with glycine ethyl ester slower than those of the Fbg α C 233-425 wild type.

FXIII A₂ was activated by IIa in the presence of Ca²⁺. IIa was inhibited with PPACK before the introduction of GEE and Fbg α C 233-425 (WT or E396A).

Transamidation of each reactive glutamine of Fbg α C 233-425 (Q237, Q328 and Q366) by pre-activated FXIII A₂^{*} was quantitated by a previously designed MALDI-TOF mass spectrometry strategy [24].

AIM II: A complementary 2D ¹H-¹⁵N HSQC NMR strategy was used to assess FXIII A₂^{*} crosslinking of Fbg α C 233-425 E396A with ¹⁵N-labeled glycine ethyl ester.

Results from the ¹H-¹⁵N HSQC NMR spectroscopy experiment were compared to findings for the Fbg α C 233-425 E396A mutant from the MALDI-TOF mass spectrometry studies.

AIM III: Fbg α C 389-403 may contribute to FXIII activation. FXIII A₂ is proposed to take longer to activate in the presence of Fbg α C 233-425 E396A than in presence of the wild type. The rate of crosslinking of Q237 within the Fbg α C 233-425 E396A mutant with glycine ethyl ester is proposed to be slower than the rate observed for the wild type.

Ila was introduced to a solution of FXIII A₂, Fbg α C 233-425 (WT or E396A), glycine ethyl ester and Ca²⁺. Crosslinking before Ila inhibition with PPACK was monitored.

Transamidation of Q237 within Fbg α C 233-425 (E396A / WT) by FXIII-A₂^{*} was quantified over time by an identical MALDI-TOF mass spectrometry strategy [\[24\]](#).

Materials and Methods

Site-Directed Mutagenesis

A single point mutation was inserted into a PGEX GST – Fbg α C 233-425 plasmid within XL1-Blue Supercompetent *Escherichia coli* cells (Agilent Technologies) by means of mutagenic DNA primers. DNA primers (Integrated DNA Technologies) were designed in accordance to the QuikChange II Site-Directed Mutagenesis Kit protocol. The amino acid substitution E396A was accomplished by a single nucleotide base change, GAG to GCG, at the codon coding for E396.

Both mutagenic primers utilized are listed below:

Fbg α C 233-425 E396A Coding Strand Primer:

5'- CTGGGGCACATTTGAA**GCG**GTGTCAGGAAATG -3'

Fbg α C 233-425 E396A Template Strand Primer:

5'- CATTCCTGACAC**GCG**TTCAAATGTGCCCCAGTCTGG -3'

	E396A Coding Primer	E396A Template Primer
Nucleotide Length	32 bases	37 bases
Molecular Weight	9969.5 g·mol ⁻¹	11236.3 g·mol ⁻¹
Melting Temperature (T _M)	65.6°C	67.8°C
Mass Received	23.9 nmol	23.2 nmol

PCR Amplification and XL-1 Blue Supercompetent Cell Transformation

A PCR reaction solution of 5.0 μ L 10x PCR reaction buffer, GST- Fbg α C 233-425 construct stock, 20 μ g E396A Coding Primer, 22 μ g E396A Template Primer and a dNTP mix were diluted to 50 μ L with 39.0 μ L of distilled H₂O. The resulting reaction mixture was subjected to a round of PCR amplification in accordance to the QuikChange II Site-Directed Mutagenesis Kit protocol for a 5.646-kBase PGEX plasmid. After amplification, the reaction

was treated with 2.0 μL of 10 U/ μL Dpn1 restriction enzyme to digest parental DNA plasmid. Transformation of XL-1 Blue Supercompetent cells onto ampicillin-infused LB agar plates was accomplished in accordance to the QuikChange II Site-Directed Mutagenesis Kit protocol. Cell colony growth was monitored for 18 hours while incubating plates at 37°C. Select colonies were chosen and liquid cultures were made with ampicillin.

DNA Purification and Sequencing

DNA extraction and purification from XL1-Blue Supercompetent small liquid cultures were conducted in accordance to the Plasmid DNA Purification QIAprep Spin Miniprep Kit protocol using a microcentrifuge. DNA concentration measurements were performed with a NanoDrop spectrophotometer at 260 nm. The obtained DNA samples were submitted for sequencing to the CGeMM DNA Facility Core at the University of Louisville's Health Science Center. DNA confirmed to contain the mutation of interest was stored for future transformation of BL-21 Gold DE3 *E. coli* cells.

BL-21 Gold DE3 Cell Transformation and Glycerol Cell Stocks

The transformation of BL-21 Gold DE3 *E. coli* cells was conducted over ice. Approximately 26 ng of mutated DNA was added to 100 μL of thawed BL-21 Gold DE3 cells. The solution was mixed and allowed to sit over ice for 30 minutes. The solution was subjected to a 45 second heat pulse in a 42°C water bath. The reaction solution was incubated in 0.9 mL aliquot of a super optimal broth with catabolite suppression (SOC broth) for an hour before plating onto LB agar plates with ampicillin. The inoculated plates sat at 37°C for 17 hours. Selected colonies were chosen for small culture incubation. Three small cultures were prepared.

An IPTG test was conducted to assess the prolificacy of each small culture. The fastest growing culture was selected to make glycerol stocks. Cell stocks were made by first aliquoting 100 μ L of cooled, autoclaved 50% glycerol into a 1.5 mL tube. 100 μ L of BL-21 Gold DE3 cell culture was added to each solution of glycerol. Stocks were mixed gently and frozen at -80°C.

GST-Fbg α C₂₃₃₋₄₂₅ E396A Expression, Extraction, and Purification

Media for two starter cultures were prepared with sterile technique by combining 180 mL LB broth, 20 mL phosphate buffer, and 200 μ L 100 mg/mL ampicillin into two 500 mL Erlenmeyer flasks. Media were inoculated with frozen BL21 Gold DE3 glycerol cell stock. The starter cultures were allowed to shake at 225 rpm at 37°C for 17 hours. Two large BL21 Gold DE3 cell cultures were then prepared. A 200 mL amount of autoclaved phosphate buffer was introduced to 1.8 L of terrific broth. 2.0 mL of 100 mg/mL ampicillin was then added. Cultures were shaken by hand. The BL21 Gold DE3 small cultures were introduced to large cultures using sterile technique. The two LB large cultures were incubated at 37°C with shaking at 225 rpm. Using a Cary 50 Bio UV-Visible spectrophotometer, simple scans were used to monitor cell growth as a function of time for both large cultures. Scans at 600 nm were taken in 1 cm colorless cuvettes until each culture measured an optical density between 0.6 and 0.7. Protein expression was then induced with 2.2 mL of 1.0 M IPTG. Induced cultures were allowed to shake at 180 rpm at 30°C for 15 hours. A lower incubation temperature was used to slow protein synthesis and prevent intracellular toxicity.

After incubation, the two large BL21 Gold DE3 cell cultures were divided evenly and balanced within six 500 mL Beckman centrifuge bottles. Cells were spun down in a Beckman JA-10 centrifuge rotor at 3317 g for 10 minutes at 4°C. The supernatants were decanted and

subsequently bleach treated. The resulting pellets were then suspended in 360 mL of an *E. coli* wash buffer. The suspension was divided equally among eight 50 mL VWR centrifuge tubes. Tubes were spun at 5000 g in an Allegra R-20 swinging-bucket centrifuge for 1 hour at 4°C. The supernatants were discarded and bleach treated. A total of eight *E. coli* cell pellets were frozen at -20°C for future protein extraction.

Cell pellets were suspended by transferring pellets with spatula to a 100 mL glass beaker containing 176 mL of PBS. An analytical wash using an additional 176 mL of PBS was done to increase yield. The cell suspension was allowed to spin at a low rpm for 30 minutes at room temperature. 1.76 mL 100 mg/mL lysozyme and 177.6 µL DTT were added via pipette to the spinning solution. The cell suspension was allowed to spin at a low rpm for another 30 minutes at ambient temperature. Protease inhibitors were added: 177.8 µL 2 mg/mL Aprotinin, 177.8 µL 1 mM Pepstatin A, 444 µL 4M Leupeptin, and 0.138 g benzamidine were added into the cell suspension. The cell suspension was stirred at a low rpm at 4°C for 30 minutes. The suspension was returned to room temperature before adding 444 µL 100 mM PMSF in DMSO and 898 µL 10% sodium deoxycholate. The suspension mixed at room temperature for another 30 minutes.

Next, 9.0 mL of 20% Triton X100 was quickly introduced to the solution as the mixing speed was increased. The detergent was allowed to mix for 5-10 minutes at room temperature. 94.5 µL of 10 mg/mL DNase 1 and 945 µL 1 M MgCl₂ were added to the suspension. The suspension was mixed for 10 minutes at a low rpm at 4°C. DNase 1 was quenched by scavenging Mg²⁺ with 2.29 mL of 0.5 M EDTA. The suspension was returned to room temperature.

The final suspension was evenly decanted into six small round-bottom Beckman centrifuge tubes. Using a beam balance, the tubes were balanced to even out the rotor in the centrifuge. Tubes were spun down for 20 minutes at 14275 g in a Beckman JA-20 rotor at a

temperature of 4°C. A sticky, grey-green solution formed containing cell lysate. A streptomycin paste (2.0 g streptomycin sulfate and distilled H₂O) was added to the supernatant to remove any remaining nucleic acids. The streptomycin-infused filtrate was again divided between six small round-bottom Beckman centrifuge tubes, balanced, and spun at 14275 g for 20 minutes at a rotor temperature of 4°C. A small pellet formed. Supernatants from each of the six round-bottom Beckman centrifuge tubes were decanted into a 0.45 µm polyethersulfone (PES) sterile filter (Cole-Parmer). The filtrate was then passed through a smaller 0.20 µm PES sterile filter.

The resulting sample was loaded onto two 5.0 mL GST-HiTrap (GE Healthcare) affinity columns in tandem over seven hours via an AKTApurifier fast protein liquid chromatography (FPLC) instrument. Columns contained the glutathione ligand coupled to Sepharose by reaction of the thiol group with oxirane groups made by epoxy-activation of the Sepharose matrix. The glutathione stationary phase binds to the GST (glutathione S-transferase) tags of fusion proteins with high affinity. Once the solution had successfully loaded onto the column, the fusion protein was cleaved with PreScission Protease⁴. Chromatographic elution after cleavage of the fusion protein by PreScission Protease produced a set of 2.0 mL fractions suspected to contain free Fbg αC 233-425 E396A. SDS PAGE and Western Blotting with an anti-GST HRP conjugate (GE Healthcare) were employed to check for GST contamination. Spectrophotometry at 280 nm with a molar attenuation coefficient of 41480 M⁻¹s⁻¹ estimated a total protein yield of 2.74 mg protein (0.685 mg protein per L culture). All nine fractions found to contain free Fbg αC 233-425 E396A were further concentrated into stock solutions using a VivaSpin centrifugal concentrator with molecular weight cut-off of 30 kDa.

⁴ PreScission Protease is a fusion protein of the human rhinovirus protease and GST that facilitates the specific cleavage between the Gln and Gly residues of the sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro.

AIM I: MALDI-TOF Mass Spectrometry

FXIII A₂ activation was conducted by incubating final concentration of 500 nM FXIII A₂ (ZymoGenetics) with 8.40 U/mL IIa and 4.0 mM CaCl₂ for 10 minutes at 37°C. The activation reaction was subsequently quenched with 0.3 μM PPACK solution. Final assay concentrations of 13.8 mM CaCl₂, 17 mM glycine ethyl ester and 13.6 μM Fbg αC E396A were diluted to a total reaction volume of 350 μL with 39.6 μL of Tris reaction buffer (100 mM Tris-acetate, 150 mM NaCl, 0.1% PEG at pH= 7.40). Note that extra CaCl₂ was added in the listed amount to completely counter the presence of phosphates from the Fbg αC E396A solution (1x PBS ≈ 12 mM PO₄³⁻). Total reaction volume was 350 μL. The FXIII A₂* crosslinking reaction was incubated over two hours at 37°C. Time points were taken as needed and Ca²⁺ scavenged with 10 mM EDTA before freezing at -80°C.

A 6.0 μL aliquot of the reaction solution at each time point was mixed with 6.0 μL of chymotrypsin digest buffer (100 mM Tris-HCl, 10 mM CaCl₂ at pH = 7.40) and GluC digest buffer (25 mM NH₄HCO₃ at pH = 7.80). To initiate the chymotrypsin digest, 1.10 μg chymotrypsin (dissolved in 1.0 mM HCl) was added to the mixture containing chymotrypsin digest buffer. The chymotrypsin digest was incubated at 25°C for 1 hour. To initiate the GluC digest, 0.100 μg GluC was added to the reaction mixture containing GluC digest buffer. The GluC digest was incubated at 25°C for 2 hours. Both digests were quenched with 2.0 μL 5% trifluoroacetic acid (TFA).

Solution cleaning and plating was done according to the ZipTip (Millipore) procedure for MALDI-TOF MS analysis. Pipet tips containing C₁₈ resins were initially rinsed with 4.0 μL acetonitrile thrice and equilibrated with 4.0 μL 0.1% TFA thrice. Digest samples were loaded

onto the resins with vigorous pipetting. Any unbound sample was rinsed off the resins with 5.0 μL of 0.1% TFA four times. Digest samples were eluted into 6.0 μL of matrix with vigorous pipetting. For the chymotrypsin digests, 10 mg/mL α -Cyano-4-hydroxycinnamic acid (αCHCA) in equal parts acetonitrile, ethanol and 0.1% TFA was used. For the GluC digests, 7.0 mg/mL ferulic acid matrix in equal parts acetonitrile and 0.1% TFA was used. Both digests were spotted onto a methanol-washed, 100 well MALDI-TOF MS plate in 1.0 μL aliquots. The plate was loaded into a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a nitrogen laser at 337 nm. Samples were analyzed under a positive peptide reflector program with a manual laser intensity of 1997.

Figure 7: (*Below*) A mathematical representation of how FXIII A₂* crosslinking of a glutamine reactant to the glutamyl-GEE isopeptide product is monitored as a function of time. (*Right*) MALDI-TOF MS can monitor the decrease in intensity of the reactant peak as the intensity of the product peak increases.

Peak Height Ratio Formula

$$\text{Reactant left} = \frac{\Sigma R}{(\Sigma R + \Sigma P)} * [\text{Peptide}]$$

$$\Sigma R = \Sigma \text{ height of reactant peak}$$

$$\Sigma P = \Sigma \text{ height of product peak}$$

FXIII A₂ activation was done by incubating final concentrations of 800 nM FXIII A₂ (ZymoGenetics) with 21.0 U/mL IIa and 5.0 mM CaCl₂ for 10 minutes at 37°C. The activation reaction was subsequently quenched with 0.26 μM PPACK. Reaction components were added to

the existing solution and 140.15 μL of borate buffer (20 mM borate at pH = 7.40) at final concentrations of 13.25 mM CaCl_2 , 10 mM ^{15}N -labeled glycine ethyl ester and 40 μM Fbg αC E396A. Total reaction volume was 400 μL . The FXIII A_2^* crosslinking reaction was incubated for 30 minutes at 37°C before scavenging Ca^{2+} with 0.64 mM EDTA. Then, 40 μL of D_2O was added and the final solution was immediately transferred into a Shigemi NMR tube. Solution pH was estimated to be 6.0 by subjecting a small aliquot to a litmus paper test. The NMR tube was placed in a Varian Inova 700 MHz NMR spectrometer, equipped with a triple-resonance HCN probe and Z-axis gradients, at 25°C. The sample was not spun.

The reaction was subjected to a ^1H - ^{15}N HSQC spectroscopy experiment to detect ^{15}N -labeled amides. A 1D study was set up with the following parameters: $Z_0 = 6512$, phase = 20, power = 45, gain = 45, np = 2048, ss = 2, NT = 512, at = 0.146, sw = 7022.5, tof = -254.3, pw = 7.40 and tpwr = 58. The 2D experiment was employed with the following parameter changes from the 1D experiment: ni = 64 and nt = 64.

AIM III: MALDI-TOF Mass Spectrometry

With AIM I, FXIII A_2 was pre-activated and then the crosslinking reaction of Fbg αC E396A with GEE was monitored. By contrast with AIM 2, the FXIII A_2 was activated in the presence of its substrates. Before activation, a final concentration of 500 nM FXIII A_2 was initially incubated with 13.6 μM Fbg αC E396A and 17 mM GEE in the presence of 13.8 mM Ca^{2+} and 39.6 μL Tris buffer. FXIII activation was then conducted by adding IIa to solution at 8.40 U/mL. Total reaction volume was 350 μL . The FXIII A_2^* crosslinking reaction was incubated at 37°C over 30 minutes. Multiple time points were taken as needed and immediately quenched with 10 mM EDTA before freezing at -80°C. A 6.0 μL aliquot of the reaction solution

at each time point was mixed with 6.0 μL of GluC digest buffer (25 mM NH_4HCO_3 at pH = 7.80). To initiate the GluC digest, 0.100 μg GluC was added to the reaction mixture containing GluC digest buffer. The GluC digest was incubated at 25°C for 2 hours. The digest was quenched with 2.0 μL 5% trifluoroacetic acid (TFA). An identical cleaning and spotting protocol was used to prepare samples for analysis by MALDI-TOF MS.

Results

Site-directed Mutagenesis and BL-21 Gold DE3 *E. coli* Stocks

DNA sequencing confirmed mutagenesis of the PGEX GST – Fbg α C 233-425 plasmid within XL-1 Blue Supercompetent *E. coli* cells. PCR produced a sufficient amount⁵ of mutated plasmid to transform BL-21 Gold DE3 cells. Transformed cells were plated on LB agar with ampicillin. Liquid cultures infused with ampicillin were then inoculated with BL-21 Gold DE3 colonies. An IPTG test assessed the prolificacy of each culture and the fastest-growing culture was used to make cell stocks. BL-21 Gold DE3 cells containing DNA for GST – Fbg α C 233-425 E396A were frozen in glycerol and stored at -80°C for future protein expression.

Expression and Purification of Fbg α C₂₃₃₋₄₂₅ E396A

BL-21 Gold DE3 *E. coli* cells from a frozen glycerol stock were used to inoculate a large 4.0 L culture infused with ampicillin. IPTG was used to induce protein expression. Extraction and affinity chromatography purified the GST – Fbg α C 233-425 E396A fusion protein as described in Materials and Methods. Chromatographic elution after cleavage of the fusion protein by PreScission Protease produced a set of 2.0 mL fractions suspected to contain free Fbg α C₂₃₃₋₄₂₅ E396A. Protein content and purity of each fraction was assessed with SDS PAGE, Western blotting, and spectrophotometry.

SDS PAGE gels stained with Coomassie Brilliant Blue dye revealed bands aligned with the 25-kDa molecular weight marker (MW Fbg α C 233-425 E396A $\approx$ 20 kDa) (Figure 8). Both SDS PAGE and an anti-GST Western blot indicated no contamination by free GST or intact fusion protein in any of the collected Fbg α C 233-425 E396A fraction.

⁵ PCR amplification was assessed with 1% agarose gel electrophoresis. The agarose gel matrix was infused with an ethidium bromide stain for visualization under ultraviolet light.

Analysis of Expression and Purification of Fbg α C₂₃₃₋₄₂₅ E396A

Figure 8: (Top Left and Right) 9 of 17 protein fractions collected reveal bands aligned with the 25 kDa molecular marker on 15% T SDS PAGE gels stained in Coomassie Brilliant Blue R250 solution. Note the MW Fbg α C 233-425 E396A $\approx$ 20 kDa. (Bottom Left) Aliquots of multiple steps throughout protein preparation were subjected to SDS PAGE.

Spectrophotometry at 280 nm with a molar attenuation coefficient of $41480 \text{ M}^{-1}\text{s}^{-1}$ estimated a total protein yield of 2.74 mg protein (0.685 mg protein per L culture). All nine fractions found to contain free Fbg α C 233-425 E396A were concentrated into stock solutions using a VivaSpin centrifugal concentrator with molecular weight cutoff of 30 kDa. The protein concentration of each stock solution was similarly assessed by spectrophotometry. Fbg α C 233-425 E396A stock concentrations ranged from $18.0 \mu\text{M}$ to $88.3 \mu\text{M}$ of protein. A $25.0 \mu\text{M}$ Fbg α C 233-425 E396A substock was made with 1X phosphate buffered saline before each mass spectrometry or NMR spectroscopy assay.

Assessment of Fbg α C 233-425 E396A with MALDI-TOF MS

Both chymotrypsin and GluC digests were performed⁶ on stock Fbg α C 233-425 E396A. MALDI-TOF MS assessed whether the fragments previously monitored to quantitate FXIII A₂^{*} crosslinking of the Fbg α C 233-425 wild type could be detected to similar extents when using the Fbg α C 233-425 E396A mutant. Analysis revealed the same three peptide fragments appear after the GluC and chymotrypsin digests of Fbg α C 233-425 E396A and wild type [24]. An additional peptide that previously contained E396 in the wild type decreased by a mass expected after Gln to Ala mutation (2704.78 in the wild type to 2647.78 Da in Fbg α C 233-425 E396A).

Figure 9: MALDI-TOF MS on chymotrypsin (*Left*) and GluC (*Right*) digests detect individual peaks containing the three reactive glutamine residues under study. A one-hour chymotrypsin digest produces the 2446.55-Da peptide fragment containing Fbg α C 233-425 Q237. GluC digestion for two hours produces the 1347.94 and 1548.82-Da peptide fragments containing Fbg α C 233-425 Q366 and Q328, respectively. Before analysis by MS, digests were mixed with organic matrices: chymotrypsin with α CHCA and GluC with ferulic acid.

Control on Iia Cleavage of Fbg α C 233-425 (E396A / WT)

The integrity of the reactivity experiments presented below relied on intact Fbg α C 233-425. As a protease, Iia cleavage of full fibrinogen is limited to the removal of FpA and FpB. It was not known if Iia would show activity towards the Fbg α C 233-425 fragment. Unexpected cleavage of Fbg α C₂₃₃₋₄₂₅ could reduce or eliminate the ability of Fbg α C 233-425 to interact

⁶ Both chymotrypsin and GluC are C-terminal endopeptidases with pH optimums near 7.80. Chymotrypsin cleaves after phenylalanine, tyrosine, and tryptophan. GluC cleaves after asparagine and glutamate.

with or serve as a substrate for FXIII A₂^{*}. Results (not shown), when compared to a positive control, indicated IIa does not cleave Fbg αC 233-425 E396A to any detectable extent.

AIM I: Fbg αC E396A and Rate of FXIII A₂^{*} Crosslinking of Reactive Glutamines

A MALDI-TOF mass spectrometry assay determined the rate of pre-activated FXIII A₂^{*} crosslinking of reactive glutamines within Fbg αC 233-425 E396A by monitoring the disappearance of un-crosslinked Fbg αC 233-425 E396A as a function of time (Figure 10). Data indicate FXIII A₂^{*} crosslinked the reactive glutamines of Fbg αC 233-425 E396A with glycine ethyl ester. Additionally, FXIII A₂^{*} appears to crosslink Fbg αC 233-425 glutamines at different efficiencies (²³⁷Q > ³⁶⁶Q ~ ³²⁸Q). Pre-activated FXIII A₂^{*} crosslinked the reactive glutamines of Fbg αC 233-425 E396A at rates comparable to the rates seen with the wild type under identical conditions (Figure 11) [24].

Figure 10: Pre-activated FXIII A₂^{*} crosslinks reactive glutamines within Fbg αC₂₃₃₋₄₂₅ E396A despite the substitution of E396 with a smaller, nonpolar alanyl residue. FXIII A₂ was activated to FXIII A₂^{*} by IIa in the presence of Ca²⁺. A PPACK quench preceded introduction of GEE and Fbg αC 233-425 E396A. Reaction aliquots were quenched with EDTA and frozen at -80°C prior to digestion and MALDI-TOF MS to quantitate crosslinking. Error bars show ± 1.0 SD (n = 3)

Figure 11: Pre-activated FXIII A₂* crosslinks each reactive glutamine of the Fbg αC 233-425 E396A mutant with GEE at rates comparable to those seen with Fbg αC 233-425 WT under the same reaction conditions. Error bars indicate ± 1.0 SD (n = 3).

AIM II: ¹H-¹⁵N HSQC NMR to Monitor FXIII A₂* Crosslinking of Reactive Glutamines

Preliminary work with a new ¹H-¹⁵N HSQC NMR assay provided insight into a potential, complementary method to confirm results seen with MALDI-TOF mass spectrometry. Previous studies on the Fbg αC 233-425 wild type show the FXIII A₂* crosslinking of each reactive glutamine with ¹⁵N-labeled glycine ethyl ester produces an individual signal when probed by ¹H-¹⁵N HSQC NMR spectroscopy [24]. Results show FXIII A₂* crosslinks Fbg αC 233-425 E396A with ¹⁵N-labeled glycine ethyl ester under identical conditions (Figure 12). ¹H-¹⁵N HSQC NMR produced three amide signals centered at (8.18 ppm, 113.75 ppm), (8.20, 113.75), and (8.35, 113.95). The resonances correspond to the products of Q366, Q328 and Q237, respectively.

Digests of the ¹H-¹⁵N HSQC NMR solution were subjected to MALDI-TOF MS analysis both 24 hours and 5 days after the initial NMR experiment. Notably, the FXIII A₂* crosslinking of both Fbg αC 233-425 E396A Q328 and Q366 appears to continue (Figure 13). These results potentially question the efficacy of the EDTA quench applied during the original ¹H-¹⁵N HSQC NMR experiment. More work is required to investigate this potential issue.

Figure 12: (Left) FXIII A₂* transamidation between a reactive glutamine and ¹⁵N-labeled glycine ethyl ester. The ¹⁵N isotope remains in the isopeptide product. (Right) The ¹H-¹⁵N HSQC NMR spectrum after pre-activated FXIII A₂* crosslinked Fbg αC 233-425 E396A with ¹⁵N-labeled glycine ethyl ester for 30 minutes.

Figure 13: FXIII A₂* crosslinking of Fbg αC 233-425 E396A with ¹⁵N-labeled glycine ethyl ester appears to proceed over time despite an EDTA quench applied the same day as an original ¹H-¹⁵N HSQC NMR experiment.

AIM III: Fbg αC E396 and the Activation of FXIII A₂*

Modulation of the original MALDI-TOF MS assay permitted further investigation of the Fbg αC 233-425 E396A mutant. FXIII A₂ was incubated with Fbg αC 233-425 (E396A / WT), glycine ethyl ester, and Ca²⁺ before IIa activation. The rates of initial FXIII A₂* crosslinking of Q237 of both Fbg αC 233-425 E396A and the wild type were assessed and compared in a similar fashion as in AIM I (Figure 14). Given FXIII A₂* crosslinks Fbg αC 233-425 Q237 with glycine

ethyl ester faster than both Fbg α C 233-425 Q328 and Q366, monitoring the rate of Q328 and Q366 crosslinking became too difficult at lower time points. Consequently, only Fbg α C 233-425 Q237 has been presented. Results show FXIII A₂, when incubated with its substrates, activates and subsequently crosslinks Q237 of Fbg α C 233-425 E396A and the wild type with glycine ethyl ester with similar efficiency.

Figure 14: FXIII A₂* crosslinks Q237 of both Fbg α C 233-425 E396A and the wild type with similar efficiencies. FXIII A₂ was incubated with Fbg α C 233-425 (E396A / WT) and glycine ethyl ester before IIa activation in the presence of Ca²⁺. Reaction aliquots were quenched with EDTA and freezing at -80°C before digestion and MALDI-TOF MS to quantitate crosslinking. Error bars show ± 1.0 SD (n = 3)

Discussion

Coagulation is the body's first reaction to any damage to blood vessels, such as a wound or an internal rupture of a blood vessel. Given the intricacy of the coagulation cascade, the regulation of the numerous clotting factors must be equally as efficient to ensure immediate and proper thrombosis. In addition to bleeding disorders, such as von Willebrand disease and the hemophilias, the effects of abnormal coagulation contribute to many other disorders affecting the circulatory system. Congenital deficiencies of anticoagulants, such as antithrombin, protein C, and protein S, have been correlated with increased risk of venous thrombosis [29]. Results of epidemiological studies suggest plasma levels of fibrinogen and FVII are predictive of coronary heart disease [30]. Given the growing global burden of cardiovascular diseases, research into the intricacies of coagulation and thrombosis may provide insight for prevention and intervention against disorders of the cardiovascular system.

Thrombogenesis *in vivo* culminates in the interaction of FXIII with fibrin(ogen). While much remains unknown of how these two clotting factors cooperate *in vivo*, published studies on the interaction between FXIII and fibrin(ogen) support FXIII binding onto fibrin(ogen) [28]. The early findings of Greenburg and Shuman demonstrated fibrinogen binds to plasma FXIII A₂B₂ with a K_d of 10 nM. Results also indicated FXIII A₂B₂ remained bound with a similar affinity to the resulting fibrin monomer after IIa cleavage of fibrinogen FpA and FpB [28].

Procyk *et al.* first localized a binding site for FXIII A₂^{*} within fibrin Aα 389-402 using an antibody study. The binding of fibrin to placental FXIII A₂^{*} was assessed in the presence of multiple antibodies, including anti-γ 392-406, anti-Bβ 15-21, and anti-Aα 389-402. The anti-Aα 389-402 antibody substantially decreased FXIII A₂^{*} binding to fibrin. Antibodies against the Bβ and γ chains of fibrin did not affect the binding interaction [31]. Results by Procyk *et al.* were in

agreement with previous findings of Credo *et al*, which indicated fibrin A α 242-424 enhanced the binding of FXIII A $_2^*$ activated from FXIII A $_2$ B $_2$ [32]. While studies proposed a new FXIII A $_2^*$ binding site within the α C region of fibrinogen A α , further investigation into the specific binding interaction between FXIII A $_2^*$ and fibrinogen α C was required.

Smith *et al.* further characterized the interaction between FXIII and fibrin(ogen) α C [26]. Methods in surface plasmon resonance (SPR) using recombinant truncations of Fbg α C 233-425 confirmed the previously described FXIII A $_2^*$ binding site within Fbg α C 389-403. In agreement with the observations of Procyk *et al* [31], Smith *et al.* demonstrated Fbg α C 389-403 bound a cleft of the β -sandwich domain of FXIII A $_2^*$ exposed in the presence of Ca $^{2+}$ after Ila cleavage of the FXIII activation peptide [23]. A multiple sequence alignment (MSA) of fibrin(ogen) α C 371-425 in seven species identified conserved residues within fibrin(ogen) α C 389-403. SPR studies on a series of mutant Fbg α C 389-403 peptides revealed an E396A mutation substantially decreased the binding of FXIII A $_2^*$ to the Fbg α C 389-403 peptide. *In silico* calculations using X-ray crystallography data predicted Fbg α C E396 may participate in an electrostatic interaction with R158 within the activation cleft of FXIII A $_2^*$ [23]. Authors proposed that structural effects caused by a salt bridge between Fbg α C E396 and FXIII A $_2^*$ facilitates the observed binding.

An enzyme-linked immunoabsorbant assay (ELISA) and a FXIII A $_2^*$ 5-(biotinamido) pentylamine incorporation assay conducted by Smith *et al.* investigated the inhibitory effect of a synthetic Fbg α C 389-402 E396A peptide on the interaction between Fbg α C and FXIII A $_2^*$ [26]. Results demonstrated a synthetic Fbg α C 389-402 E396A peptide failed to inhibit binding of FXIII A $_2^*$ to Fbg α C 233-425. Consequently, binding of the peptide to FXIII A $_2^*$ did not interrupt FXIII A $_2^*$ crosslinking of an biotinylated glutamine donor with either poly-L-lysine or the reactive K303 residue of fibrin monomers. The collective work by Smith *et al.* on Fbg α C

233-425 and the Fbg α C E396 residue would predict an E396A mutation within a recombinant Fbg α C segment should cause decreased association and reactivity of FXIII A₂* [23, 26]. The work presented herein assessed the effect of the E396A mutation within Fbg α C 389-402 on the crosslinking of three natural substrates (Fbg α C Q237, Q328, and Q366) by FXIII A₂*.

Results of three reactivity studies indicate mutation at Fbg α C E396 does not affect FXIII A₂* reactivity towards the three reactive glutamines of Fbg α C 233-425. Complementary assays utilizing MALDI-TOF mass spectrometry and ¹H-¹⁵N HSQC NMR spectroscopy confirm FXIII A₂* crosslinked Fbg α C 233-425 E396A with glycine ethyl ester. Additionally, pre-activated FXIII A₂* was shown to crosslink the three reactive glutamines of Fbg α C 233-425 E396A at rates comparable to the rates previously seen with the wild type under identical conditions [24]. These results question the extent to which the interactions proposed by Smith *et al.* between Fbg α C and FXIII influence the reactivity of FXIII A₂* towards Fbg α C Q237, Q328, and Q366.

A second MALDI-TOF mass spectrometry assay revealed FXIII A₂, when incubated with its substrates, activates and subsequently crosslinks Q237 of Fbg α C 233-425 E396A and the wild type with glycine ethyl ester at similar rates. When taken together, the three studies presented indicate the loss of Fbg α C E396 does not influence either activation of FXIII A₂ to FXIII A₂* or the ability of FXIII A₂* to recognize and bind to Fbg α C 233-425. Given the information presented, results could be interpreted two different ways. Firstly, if Fbg α C E396 contributes to a putative FXIII binding site, the electrostatic interaction proposed by Smith *et al.* between Fbg α C E396 and FXIII A₂* R158 must not be required for the proper transglutaminase activity of FXIII A₂* [23]. Alternatively, additional structural details of the natural Fbg α C 233-425 segment must exist to successfully compensate for the loss of Fbg α C E396 to FXIII A₂* R158 interaction and any consequential structural changes.

In addition to documenting interactions of FXIII A₂^{*} and Fbg αC 233-425, Smith *et. al* have identified a high-affinity interaction between FXIII A₂B₂ and Fbg αC 371-425 [26]. FXIII A₂B₂ binding to Fbg αC appears to occur before and after IIa cleavage of the FXIII activation peptides. Future work should assess whether the presence of FXIII B₂ subunits target this putative FXIII binding site of Fbg Aα to modulate either FXIII A₂B₂ activation or reactivity towards Fbg αC Q237, Q328, Q366.

Conclusions

Three reactivity experiments indicate an E396A mutation within Fbg α C 233-425 does not influence either the activation of FXIII A₂ to FXIII A₂^{*} or the ability of FXIII A₂^{*} to recognize and bind Fbg α C 233-425. Although Fbg α C E396 contributes to a putative FXIII binding site, a proposed interaction between Fbg α C E396 and FXIII A₂^{*} R158 is not required for the proper transglutaminase activity of FXIII A₂^{*}. Alternatively, additional structural components of the natural FXIII A₂ substrate, Fbg α C 233-425, could exist to compensate for the loss of Fbg α C E396 to FXIII A₂^{*} R158 interaction and any consequential structural changes.

Acknowledgements

I would like to thank the fellow members of my lab for all the help and guidance they have provided over the last year: Kelly Njine Mouapi, Boris Anokhin, Ramya Billur, and Carmela Riposo. I would also like to thank Jacob Bell for his contributions to the fibrinogen α C project and the MALDI-TOF mass spectrometry assay described herein. A gracious thank you is extended to Kelly Njine Mouapi for her expertise and patience throughout this entire process. Lastly, I would like to thank Dr. Muriel Maurer for her enduring support and attention to detail that has made this project possible. My work with Dr. Maurer has allowed me to grow both as a critical thinker and a lifelong learner. I am forever grateful for the opportunity to work with her, as I will carry the experiences and insights I have gained with me for the rest of my life.

Appendix

A. Modified Transglutaminase Mechanism [25]

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