THE ROLE OF THROMBIN EXOSITES 1 AND 2 IN THE ACTIVATION OF FACTOR XI BY THROMBIN

THE ROLE OF THROMBIN EXOSITES 1 AND 2 IN THE ACTIVATION OF FACTOR XI BY THROMBIN

BY VISHAL PATEL, BSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2018) Hamilton, Ontario (Medical Sciences)

TITLE: The Role of Thrombin Exosites 1 And 2 In The Activation Of Factor XI By Thrombin

AUTHOR: Vishal Patel, BSc (McMaster University)

SUPERVISOR: Dr. Jeffrey I. Weitz

NUMBER OF PAGES: 78

ABSTRACT

Factor XI (FXI) is the zymogen of the coagulation protease factor XIa (FXIa) that contributes to thrombin generation through the intrinsic pathway. FXI is activated by the contact pathway protease, factor XIIa (FXIIa), in a high molecular weight kininogen-dependent manner. It is also known to be activated by thrombin in a positive feedback reaction, however, the mechanism of this activation is not yet completely understood. Therefore, our objectives were to identify the role of polyanions in the thrombin mediated FXI activation, and the role of the thrombin exosites in the activation.

To study this activation, we assessed the activation of FXI by thrombin in the presence and absence of the polyanions, dextran sulfate and polyphosphate (polyP). We utilized surface plasmon resonance to determine whether FXI and thrombin bind to the polyanions, and how the exosites effect thrombin's ability to bind using thrombin exosite 1 and 2 variants. To investigate the role of the exosites in FXI activation, we analyzed the activation of FXI by the thrombin variants. In addition, we explored how inhibiting the thrombin exosites using DNA aptamers affects thrombin's ability to bind to polyanions and activate FXI.

We found that polyanions are required as a cofactor for the activation of FXI by thrombin, and stimulate the activation in a concentration dependent fashion, suggesting a template mechanism. Our findings also show that exosite 1 and 2 are required for thrombin to bind to polyanions, however, exosite 2 plays the predominant role in FXI activation. Our aptamer data showed that either

exosite can be targeted to inhibit FXI activation. These findings enrich our understanding of the mechanism of FXI activation by thrombin and provides further insight on how to attenuate the activation for potential antithrombotic therapies.

ACKNOWLEDGEMENTS

Completion of this thesis would not have been possible without the help and support of many people who contributed their ideas, suggestions, and assistance. First and foremost, I would like to express my deepest thanks to Dr. Jeffrey Weitz for the opportunity to become a part of this project and to develop skills related to basic science and translational research. He has been very patient and supportive throughout this entire journey.

I would also like to thank Dr. Jim Fredenburgh for providing the academic and technical training required to complete this project. Thank you for providing key suggestions to improve this project, answering many of my questions, helping me troubleshoot my experiments and for the countless hours he spent editing my reports, manuscripts and presentations. In addition, I would like to thank Alan Stafford and Beverley Leslie for helping me troubleshoot my experiments, teaching me various lab techniques, and execute technically challenging experiments.

I would like to thank each and every member of my supervisory committee Dr. Patricia Liaw and Dr. Colin Kretz for your ideas, suggestions, and comments, which helped to guide and improve this study. Thank you for your continued guidance sharing your expertise to improve my project.

I also want to thank my colleagues and friends in the Weitz lab: Tammy Truong, Ran Ni, Rida Malik, Natalie Cohen, and Dr. Iqbal Jaffer. Thank you promoting a positive work environment and making the lab very enjoyable.

Lastly, thank you to the Canadian Institutes of Health Research for supporting my work through a Canada Graduate Scholarship – Master's.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AT- Antithrombin

b- Biotinylated

C1-Inh- C1-inhibitor

CTI- Corn trypsin inhibitor

DS- Dextran Sulfate

DNA- Deoxyribonucleic acid

DNase- Deoxyribonuclease

DVT- Deep vein thrombosis

ETP- Endogenous thrombin potential

FV- Factor V

FIX- Factor IX

FXa- Activated FX

FXI- Factor XI

FXIa- Activated FXI

FXII- Factor XII

FXIIa- Activated FXII

HBS- HEPES buffered saline

HK- High molecular weight kininogen

HMW polyP- High molecular weight polyphosphates (>1000 monomer)

Kal- Kallikrein

NETs- Neutrophil extracellular traps

NP- Normal pooled platelet poor plasma

PK- Prekallikrein

polyP- Polyphosphates

polyP70- Polyphosphates (70 monomer polyphosphate)

PPX- Exopolyphosphatase

rMZ- Recombinant prothrombin

rMZa- Recombinant meizothrombin

RNA- Ribonucleic acid

Serpin- Serine protease inhibitor

TBS- Tris buffered saline

TF- Tissue factor

TFPI- Tissue factor pathway inhibitor

VWF- von Willebrand factor

Introduction:

Overview of Hemostasis:

Hemostasis maintains the integrity of blood circulation after blood vessel rupture has occurred, while maintaining fluidity of blood in normal circulation (Furie and Furie, 2005; Wolberg and Campbell, 2008). Hemostasis is attained when the procoagulant, anticoagulant and fibrinolytic processes are balanced. However, after vascular injury, subendothelial components such as collagen in the vessel wall are exposed to the blood and this triggers activation of platelets and the coagulation cascade. Hemostasis can be divided into three stages: primary, secondary, and tertiary.

Primary hemostasis involves the formation of a platelet plug. Following vascular injury, subendothelial collagen is exposed to the blood. Platelets bind to collagen, which triggers several signaling events that leads to platelet activation and aggregation. During this phase, platelets also interact with various adhesive and aggregation proteins such as von Willebrand factor (VWF), and interacts with fibrinogen to form a platelet-rich plug that seals the leaks in the injured vascular wall (Boon, 1993).

Secondary hemostasis, also known as coagulation, serves to produce a fibrin hemostatic plug through activation of coagulation proteins. During coagulation, an ordered series of proteolytic reactions convert zymogens in the blood into active enzymes. These coagulation proteins include procoagulant proteins, such as factors II, VII, IX, XI, and X, and regulatory anticoagulant

proteins such as antithrombin, protein C, protein S, and tissue factor pathway inhibitor. Coagulation culminates in thrombin generation. Thrombin can further activate platelets and also converts insoluble fibrinogen into a fibrin network that reinforces the platelet plug (Chu, 2011).

Tertiary hemostasis refers to the lysis and solubilization of fibrin clots in a process known as fibrinolysis. Fibrinolysis is initiated when plasminogen activators released from the vessel wall convert the zymogen plasminogen to the active serine protease, plasmin. Plasmin binds to and degrades the fibrin mesh to dissolve the clot. Fibrinolysis also involves a variety of regulatory proteins, such as α2-antiplasmin and plasminogen activator inhibitor 1, that prevent abnormal fibrinolysis. Therefore, hemostasis depends on the dynamic balance between clot formation and breakdown. Clots that persist can obstruct blood flow, whereas unstable clots that are rapidly degraded can lead to hemorrhage (Cesarman-Maus and Hajjar, 2005).

Overview of coagulation cascade:

The process of coagulation can be described in 3 phases: initiation, amplification, and propagation. In the classical model of coagulation (Figure 1), the extrinsic and intrinsic pathways of coagulation converge into the common pathway to produce thrombin. Exposure of tissue factor (TF) at sites of vascular injury or release of polyanions into the blood initiates coagulation via the extrinsic

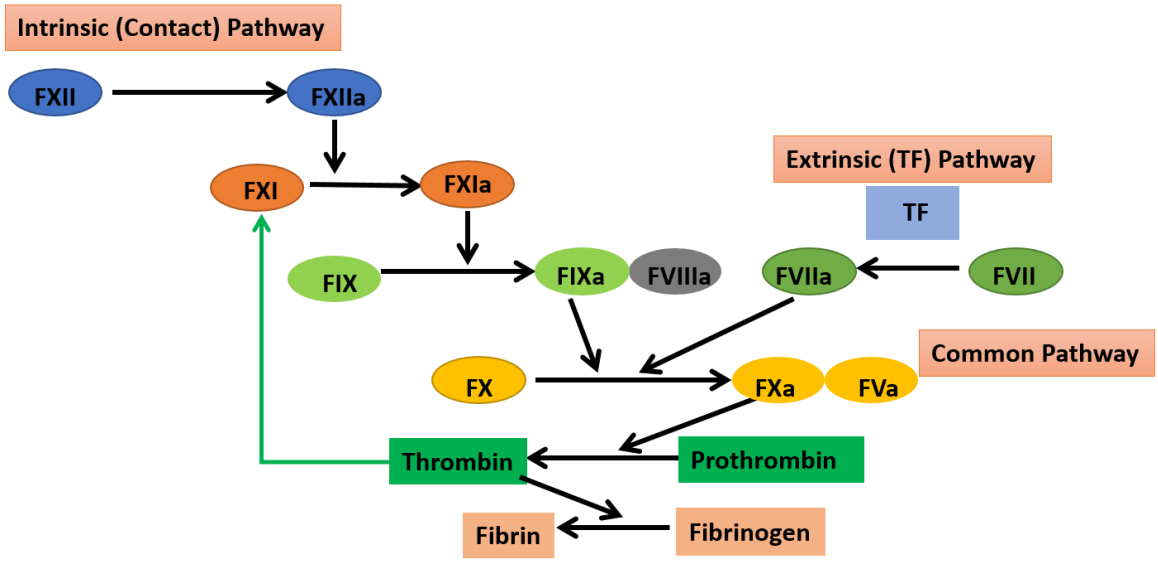


Figure 1: Classical model of coagulation cascade.

The classical model of coagulation depicts coagulation initiated by two pathways: intrinsic and extrinsic pathway of coagulation. The pathways represent a series of inactive zymogens that are activated leading to the generation of thrombin. Coagulation is initiated by the extrinsic and intrinsic pathway. The common represents the convergence of the intrinsic and extrinsic pathways, which both generate FXa. FXa converts prothrombin into thombin. Thrombin activates platelets and converts fibrinogen into insoluble fibrin monomers that self-associate to form a fibrin network.

or contact pathways, respectively. Both initiating steps trigger a series of enzymatic reactions that culminate in FXa generation at the common pathway (Mackman, 2004). TF binds FVIIa to form extrinsic tenase, which activates FIX and FX. Initially, FXa only produces small quantities of thrombin. However, once generated, thrombin feeds back and activates FVIII to FVIIIa and FV to FVa, which are important cofactors in the intrinsic tenase and prothrombinase complexes, respectively. Binding of FIXa and FVIIIa forms intrinsic tenase, which also activates FX (Mann, Butenas, and Brummel, 2003). The propagation phase of hemostasis is defined by the formation of intrinsic tenase through the intrinsic pathway, which is required for large scale thrombin generation on the surface of activated platelets (Davie and Ratnoff, 1964; Gailani and Renné, 2007). However, this process cannot be sustained indefinitely, as depletion of prothrombin effectively slows the amplification steps. The coagulation cascade is regulated by natural anticoagulants and thrombin generation is effectively terminated when the rate of inhibition exceeds the rate of thrombin formation (Wolberg and Campbell, 2008).

Extrinsic Pathway:

Initiation of coagulation by the extrinsic pathway is carried out by tissue factor (TF). TF is a transmembrane glycoprotein protein that is normally located in the subendothelial layers of vessels or on cells (Mackman, 2004). The vast

majority of FVII in blood is in the form of inactivate zymogen, with trace amounts in the activated form. Therefore, the activation of FVII is largely TF-dependent and occurs at the site of vascular injury after formation of a TF-FVIIa complex. Binding of FVIIa to TF induces a conformational change in the enzyme that augments its proteolytic activity by 1000-fold (Kirchhofer *et al.*, 1995). The TF-FVIIa complex then activates additional FVII to FVIIa by cleaving the peptide bond between Arg152 and Ile153 (Mackman, 2009). The catalytic function of the TF-FVIIa (extrinsic tenase complex) is responsible for FX activation in the presence of Ca^{2+} and phospholipid membranes. The TF-FVIIa complex cleaves the Arg-Ile bond in FX to produce FXa (Owens and Mackman, 2010).

Intrinsic Pathway:

The intrinsic pathway of coagulation is triggered by the exposure of FXII in blood to polyanionic activators that provide a platform or surface for assembly of the intrinsic pathway proteins (Renné and Gailani, 2007). FXII binds to polyanions and undergoes a conformational change, which renders the zymogen more susceptible to proteolysis. FXII undergoes autoactivation by cleavage of the Arg353-Val354 bond, which is promoted by the polyanion and Zn^{2+} to amplify its activation (Gailani and Renne, 2007). FXIIa then activates prekallikrein (PK) into kallikrein in the presence of high molecular weight kininogen (HK). Kallikrein further amplifies the activation of FXII in the presence of HK (Schmaier, 2016). FXIIa then activates FXI, which circulates in complex with HK. The homodimer

FXI is activated through the cleavage of the Arg369-Ile370 bond in each of its monomers, thus the “catalytic domain”, which comprises the light chain of factor XIa, is cleaved from the heavy chain region. The heavy and light chains of factor XIa remain associated through a disulfide bond (Yipeng Geng *et al.*, 2013). FXIa then proceeds to activate factor IX (FIX) in the presence Ca^{2+} (Gailani and Renne, 2007). FIXa combined with FVIIIa binds to the surface of activated platelets to form the intrinsic tenase complex (Mann, Butenas and Brummel, 2003). The intrinsic tenase complex is responsible for activating factor X (FX).

The term “contact” system/pathway generally refers to FXII, HK and PK, and this designation came about because these zymogens required “contact” with polyanions for activation. The contact system is also known as the plasma kallikrein-kinin system. In contrast, the term intrinsic pathway is more encompassing and in addition to the contact system, includes FXI, FIX and FVIII (Colman and Schmaier, 1997). This distinction is useful for understanding the relative contributions of the different components of the intrinsic pathway to pathophysiology.

Common Pathway:

After the intrinsic and extrinsic pathways generate FXa, the subsequent enzymatic complex is part of the common pathway (Monroe, Hoffman and Roberts, 2002). FXa in the prothrombinase complex activates the substrate, prothrombin to thrombin. Factor V is activated by thrombin and becomes a

cofactor for FXa. In the presence of Ca^{2+}, the factors assemble on the negatively charged plasma membrane of monocytes, activated platelets, or endothelial cells to form the prothrombinase complex (Krishnaswamys, 1990). The Gla-domains of FXa and prothrombin mediate membrane binding, whereas FVa binds via its lectin-like C domains (Gilbert *et al.*, 2012). When associated with the prothrombinase complex, the catalytic efficiency of FXa is increased by over 10^5-fold. Thus, under these conditions, FXa efficiently cleaves prothrombin to generate thrombin (Smiles *et al.*, 1998). Thrombin will directly catalyze fibrinogen cleavage, releasing fibrinopeptides to allow for fibrin clot formation by association then cross-linking by activating FXIII (Bereczky, Katona and Muszbek, 2003).

Activators of the Intrinsic Pathway:

In the intrinsic pathway, specifically the activation of FXI and FXII autoactivation, slow rates of activation by FXIIa or thrombin in purified systems indicate that activators are necessary (Zhong *et al.*, 2017). For decades, it was known that synthetic substances such as kaolin, silica, ellagic acid and dextran sulfate activate FXII and FXI in vitro. While these agents form the basis for the activated partial thromboplastin time (aPTT) assay, which is a global coagulation test used to assess the integrity of the intrinsic and common pathways of coagulation, the identity of potent physiologic activators of FXII remained elusive (Gailani & Renne, 2007). Dextran sulfate is an anionic polymer of sulfated glucose residues containing approximately 17% sulfur which is equivalent to

approximately 2.3 sulfate groups per glucosyl residue. The straight chains consist of α-1,6 glycosidic linkages between glucose molecules, while branches begin from α-1,3 linkages (Zeerleder *et al.*, 2002). Dextran sulfate demonstrates a sequestering interaction with proteins through hydrogen-bonding between the sulfate groups and amine groups of the protein that generates an insoluble complex. Dextran sulfate has been used for anticoagulant applications due to the ability of the polymer to precipitate fibrinogen and low-density lipoproteins from plasma solutions.

Recently, polyphosphate containing molecules such as inorganic polyphosphates (polyP), DNA and RNA have been shown to activate FXII. Whereas polyP is produced and stored in platelets and is released upon platelet activation, DNA and RNA are released from activated, damaged or dying cells (Smith *et al.*, 2006; Kannemeier *et al.*, 2007; Müller, Gailani and Renné, 2011). In addition, the observation that the intrinsic pathway also initiates coagulation induced by interaction of the blood with heart valves and catheters suggests that this pathway also contributes to thrombosis on medical devices (Yau *et al.*, 2012; Jaffer *et al.*, 2015). The discovery of these physiological agents and the phenomenon that medical devices induce thrombosis via this pathway has led to a renewed interest in the intrinsic pathway.

PolyP is class of linear polymer of phosphate molecules found in prokaryotic and eukaryotic organisms. In platelets, polyP is stored in the dense granules and is released upon activation. Platelet derived polyP consist of 60-100

phosphate units while bacterial-derived polyP consists of >500 phosphate units (Smith *et al.*, 2006). Bacterial polyP has been reported to play diverse roles such as regulating growth, stress responses, and virulence (Smith et al., 2010). Comparatively, the physiological roles of platelet polyP are less known, but polyP has been reported to promote calcification in osteoblasts (Kawazoe *et al.*, 2004), block metastasis of melanoma cells in a mouse model (Han *et al.*, 2007), induce apoptosis in plasma cells (Han *et al.*, 2007), and possibly serve as a regulatory factor in proliferative signaling pathways (Wang *et al.*, 2003). PolyP can induce the autoactivation of FXII and facilitate the activation of FXI by thrombin (Smith *et al.*, 2010). Bacterial-derived polyP is a potent activator of the contact pathway initiation, while short platelet-derived polyP plays a greater role in accelerating FXI and FV activation (Smith *et al.*, 2010). The importance of polyP can be observed in humans with dense granule release defects or delta storage pool deficiency, where platelets dense granule deficiencies is associated with bleeding diathesis (Masliah-Planchon, Darnige and Bellucci, 2013).

DNA and RNA, which are nucleic acids that have also been identified as promoters of coagulation by augmenting (auto-) activation of contact proteases (van der Vaart and Pretorius, 2008). DNA and RNA are normally contained within cells, but are expelled from cells that are stimulated or undergo necrosis. In rodent and baboon models of DVT elevated levels of circulating DNA and RNA can be found. (Fuchs *et al.*, 2010; Brill *et al.*, 2012). When neutrophils are activated with interleukin-8, a signaling pathway is triggered that leads to the

release of web-like structures called neutrophil extracellular traps (NETs), composed of DNA, histones and various cytoplasmic proteins. NETs are prothrombotic because they contain DNA and histones. DNA triggers contact activation of coagulation and histones trigger platelet activation and thrombin generation in a manner that is dependent on toll-like receptors and polyP (Kannemeier *et al.*, 2007; Fuchs *et al.*, 2010; Brill *et al.*, 2012).

Inhibitors of the Intrinsic Pathway:

The physiological inhibitors of FXIa include antithrombin (AT) and C1-inhibitor (C1INH). AT and C1INH are both members of the serine protease inhibitor (serpin) superfamily (Kolyadko *et al.*, 2014). AT inhibits FXIa as well as thrombin, FXa, FIXa, FXIIa, and FVIIa. Heparin catalyzes the inhibition rate of AT by binding to a specific cationic site on AT and inducing a conformation change that enhances its activity for inhibition of coagulation factors (Richards *et al.*, 1997). C1INH inhibits FXIa irreversibly by binding to the active site of the protease and disrupting its catalytic activity (Wuillemin *et al.*, 1995). Although C1INH is considered to be the major inhibitor of FXIIa, however, it is a slow inhibitor of FXIIa and is unable to inhibit FXIIa when it is bound to anionic surfaces (MacQuarrie *et al.*, 2011).

Thrombin is central in the clotting cascade and its formation and activity can be suppressed by natural anticoagulants including tissue factor pathway inhibitor (TFPI), AT, Protein C (PC) and Protein S (PS). TFPI plays a crucial role

in the regulation of coagulation by controlling thrombin production through the extrinsic pathway. TFPI inhibits the TF-FVIIa complex in a FXa dependent fashion by first binding to and inactivating FXa in a 1:1 stoichiometric complex (Lwaleed and Bass, 2006). TFPI binds to FXa most effectively when FXa is in the prothrombinase complex. The TFPI-FXa complex binds and inhibits the TF-FVIIa complex, thus switching off the extrinsic pathway (Lwaleed and Bass, 2006).

PC serves as an anticoagulant by supressing thrombin generation through the inactivation of FVIIIa and FVa (Esmon, 2003). PC is activated through the protein C pathway which is initiated when thrombin binds to thrombomodulin (TM), a thrombin receptor on endothelial cells (Esmon, 2003). The thrombin-TM complex binds to the endothelial cell protein C receptor, which in turn converts PC into the enzyme activated protein C (APC). APC binds to its cofactor PS resulting in a complex that can inactivate FVIIIa and FVa (Esmon, 2003).

As stated previously, AT inhibits many coagulation factors, including thrombin, in the presence of heparin. Heparin is released from secretory granules of mast cells at the site of tissue injury. (Walker and Royston, 2002) Heparin is a physiological anionic polysaccharide known as a glycosaminoglycan, and is composed of alternating residues of D-glucosamine and L-iduronic acid (Walker and Royston, 2002). It contains a specific pentasaccharide sequence that binds to and activates AT by inducing a conformational change. Heparin also bridges thrombin together with AT by simultaneously binding to exosite 2 on thrombin and AT, thus forming a ternary complex.

The Role of FXI in Hemostasis and Thrombosis:

Thrombosis is the formation of a blood clot inside a blood vessel, which obstructs the flow of blood through the circulatory system (Wolberg and Campbell, 2008). Causes of thrombosis include excessive blood coagulation, which results from disrupted blood flow, hypercoagulation of the blood, hypofibrinolysis, or atherosclerotic plaque rupture (Warkentin, 1995). Thrombosis can lead to cardiovascular disease, stroke, and venous thromboembolism (VTE) (Warkentin, 1995). The general consensus was that the same coagulation pathways initiate thrombosis and hemostasis. However, studies of patients with congenital deficiency of FXII, PK or HK have shown that despite impaired thrombin generation and fibrin formation in vitro, the patients have no bleeding diathesis (Müller, Gailani and Renné, 2011). This suggests that FXII is dispensable for hemostasis. In contrast, patients with FXI deficiency have a mild bleeding diathesis. The difference in bleeding phenotypes between FXII and FXI deficiency supports the model that FXI can be activated by other means, such as the back activation by thrombin (Seligsohn, 2009). This other mechanism of FXI activation might contribute to FXI's minor role in hemostasis in a contact pathway-independent manner.

The role of FXI in thrombosis is supported by epidemiological evidence in patients with congenital FXI deficiency and those with elevated levels of FXI. Patients with deficiency appeared to be protected from VTE and ischemic stroke, while patients with higher levels of FXI are at greater risks of VTE and ischemic

stroke than those with normal levels (Salomon *et al.*, 2008, 2010). In FXI deficient mouse models, thrombosis is attenuated and the thrombi that are formed are prone to fragmentation (Renné *et al.*, 2009). Similar results were seen in non-human primates where FXI was knocked down with antisense oligonucleotides, resulting in a concentration dependent attenuation of thrombosis (Zhang *et al.*, 2010). These results suggest that FXI is a key contributor to thrombosis and is important for thrombus stabilization, and growth, with a small effect on hemostasis.

Thrombin Structure:

Thrombin is a serine protease, which cleaves fibrinogen to form fibrin, and is also responsible for activation of protein C, platelet activation and feedback activation of FV, FVIII and FXI (Davie and Kulman, 2006). Thrombin is a α/β heterodimer composed of a 36 amino acid NH2-terminal A chain, and a 259 amino acid COOH-terminal B chain. The chains are connected by a disulfide bridge between Cys1 and Cys122, in addition to 3 other intrachain disulfide bonds (Davie and Kulman, 2006). The active site is located on the B chain in between two interacting beta barrels. The active site contains the serine protease catalytic triad Ser195, His57, and Asp102 and an oxyanion hole. Two basic regions that flank the active site, termed exosites 1 and 2, provide an additional level of regulation. Exosites 1 and 2 are also located on the B chain of thrombin (Davie and Kulman, 2006). Both exosites are positively charged, although,

exosite 1 also has hydrophobic character. It is becoming increasingly evident that all thrombin substrates and cofactors interact with at least one exosite. Exosite 1, which is also known as the fibrinogen recognition site is important for thrombin's interactions with FV, FVIII and PARs on platelets (Davie and Kulman, 2006). Of the two exosites, exosite 2 is more basic and was originally identified as the heparin binding site. Exosite 2 binds to heparin through the sulfate groups on the glycosaminoglycan and GpIbα binding on the platelet surface. Furthermore, thrombin is inhibited and cleared from the circulation by the serpins AT and HCII in a manner that is dependent on glycosaminoglycans (GAGs) such as heparin (Huntington, 2005). Therefore, the capacity of the exosites to bind both pro- and anti-coagulant substrates provides a dynamic molecular switch that directs thrombin activity and regulates coagulation.

For further analysis on thrombin exosites, thrombin variants and DNA aptamers are used to examine the function and structures of the exosites. Thrombin variants γ-thrombin and RA-thrombin are exosite 1 and 2 variants, respectively (Rezaie, 1999). γ-thrombin is generated by thrombin- or trypsin-mediated cleavage of the β-thrombin B2-chain at the Lys190-Gly191 bond. This cleavage results in γ-thrombin lacking an exosite 1. Although its ability to clot fibrinogen, cleave thrombospondin or activate protein C have been markedly decreased, it retains its ability to cleave substrates that are not exosite 1 dependent such as the chromogenic enzyme Spectrozyme-TH (Witting, Miller and Fenton, 1987). RA-thrombin contains mutations Arg93 to Ala, Arg97 to Ala,

and Arg101 to Ala in exosite 2 preventing it from binding to heparin (Charles T Esmon and Lollar, 1996). RA-thrombin is able to cleave fibrinogen normally, confirming it to be an exosite 2 variant only. The DNA aptamers HD1 and HD22 are single stranded nucleic acids with high-affinity to thrombin exosites 1 and 2, respectively (Muller *et al.*, 2008). By binding directly to the exosites, the aptamers have an inhibitory effect on the various activities of thrombin. By binding to exosite 1, HD1 is available attenuate fibrinogen cleavage (Kretz *et al.*, 2015). Also, HD1 binds with similar affinity to prothrombin, resulting in the inhibition of prothrombin activation (Kretz *et al.*, 2015). HD22 binds to exosite 2, which results in the inhibition of thrombin-mediated activation of platelets and FV/VIII (Müller *et al.*, 2008).

FXI Activation by Thrombin:

FXI deviates from the conventional waterfall cascade model of coagulation, by not only being activated through FXIIa mediated proteolysis, but also by thrombin. This alternative activation by thrombin was shown to occur only in the presence of a negatively charged surface such as dextran sulfate and sulfatides (Naito and Fujikawa, 1991). Only non-physiological surfaces were known to promote the activation of FXI by thrombin, until the activation was performed in the presence of activated platelets (Yun *et al.*, 2003). The platelet glycoprotein Ibα, found on platelet surfaces, plays a role in the co-localization of thrombin and FXI for an efficient activation of FXI (Yun *et al.*, 2003).

Polyphosphates released from the dense granules of activated platelets also have procoagulant activity by accelerating FXI activation by thrombin (Choi, 2011). The capacity of polyp to promote FXI activation by thrombin is chain length dependent where by longer polymer chains are more potent activators than shorter ones (Choi, 2011). In terms of the mechanism of how FXI is activated by thrombin, it appears that the two coagulation factors are co-localized on polyP allowing thrombin to activate FXI. Exosite 2 of thrombin and the anion-binding site on the apple 3 domain of FXI are reported to mediate binding to polyp (Mutch *et al.*, 2010; Y. Geng *et al.*, 2013). However, the role of exosite 1 and the effects of other physiological surfaces on FXI activation is required.

Purpose of this study:

The overall objective of this study was to better understand the mechanism by which FXI is activated by thrombin to determine the role of polyanions and the thrombin exosites in the activation. In order to accomplish this, we used the well-defined thrombin exosite 1 variant γ-thrombin and the exosite 2 mutant RA-thrombin. These thrombin variants were used in FXI activation assays and polyanion binding assays to determine the role of the thrombin exosites in FXI activation and their binding capabilities. DNA aptamers were also used to complement the thrombin variant and mutant data. We hypothesized that polyanions are necessary cofactors to the activation of FXI by thrombin to form a ternary complex. We further hypothesized that exosite 2 has

the dominant role in activating FXI while exosite 1 will have only minor effects on the activation.

Materials and Methods:

Materials:

Chromogenic substrates S2366, S2302 were purchased from Hyphen BioMed (Zac Neuville Université, Neuville sur Oise, France). The technothrombin thrombin generation assay fluorogenic substrate Z-Gly-Gly-Arg-AMC was purchased from Diapharma (Franklin, Ohio, USA), Human FXIa, α-thrombin, γ-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN, USA). RA-thrombin was provided by C. Esmon (University of Oklahoma Health Sciences Center). RA-thrombin is an exosite 2 mutant generated by substituting the following residues of thrombin: Arg93 → Ala, Arg97 → Ala, and Arg101 → Ala. Human FXI, FXa, and FVa were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Hirudin was purchased from Dade-Behring (Marburg, Germany). Unfractionated heparin from porcine intestinal mucosa (grade II; 166.9 USP units/mg) was purchased from Sigma Chemical Co. Heparin fractions of defined molecular weight (3788 and 2835 Da) were isolated from unfractionated heparin by gel filtration on a TSK G2000 SWXL column (30 cm×7.8 mm, Supelco) as previously described (Cosmi *et al.*, 1997). Dextran sulfate with a molecular weight of 500 kDa was obtained from Sigma-Aldrich, Inc. (Saint Louis, Missouri, USA); 5 kDa dextran sulfate was from Fluka Biochemika - Sigma-Aldrich, Inc. (Saint Louis, Missouri, USA). Oligonucleotides HD1 and HD22 were synthesized by the Molecular Biology and Biotechnology Institute at McMaster University (Hamilton, Canada). Short-chain polyphosphate (polyP70)

was purchased from BK Giulani GmBH (Ladenburg, Germany). Long-chain polyphosphate (HMW-polyP >1000 monomers) was a gift from Dr. James Morrissey. Quick-1 thrombin was kindly provided by Ruth Ann Henriksen, University of North Carolina School of Medicine. Quick-1 thrombin is an exosite 1 specific mutant with a Cys to Arg mutation at position 67 in exosite 1. RMZ was generously provided by Dr. Paul Kim whose lab generated the recombinant prothrombin using the methods of Côté *et al*., 1997. rMZ is generated by substituting the following residues of thrombin: Arg158 → Ala, Arg271 → Ala, and Arg284 → Ala.

Methods:

A. FXI activation by thrombin (chromogenic assay):

FXI activation by thrombin in the presence or absence of dextran sulfate (500 kDa) were performed in wells of 96-well plate (NUNC, Rochester, NY) and with reagents diluted in Tris-Buffered Saline (TBS) consisting of 20 mM Tris, 150 mM NaCl, 0.01% Tween20 and pH of 7.4. The reaction was initiated by the addition of 40 nM FXI, and 10 nM α-thrombin, in the presence or absence of 1 μg/ml dextran sulfate (500 kDa). Two controls were used; 40 nM of FXI and 10 nM of α-thrombin alone in TBS. The solutions were incubated at 37°C for 30 minutes and then 100 nM of hirudin was added to each well to inhibit α-thrombin activity. After an additional 5-minute incubation at 37°C, 400 μM of S-2366 was added, and absorbance at 405 nm was monitored every 10 seconds for 15

minutes using SpectraMax 340PC384 microplate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were plotted against time. The slopes (mOD/min) of the linear portion were converted to the concentration of FXIa generation (nM), by dividing the slopes by the specific activity of FXIa with 400 μM of S-2366 obtained in a separate experiment. The FXIa generation was then converted to percent activation of FXI.

B. SDS-PAGE analysis of FXI activation by thrombin:

Samples of α-thrombin, FXI, FXIa, and a mixture of FXI, α-thrombin, and dextran sulfate (500 kDA) were incubated at 37°C for 30 minutes. The samples were then denatured by boiling for 3 minutes. After loading 2-3 μg of protein onto a 4-15% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel (BIO-RAD Laboratories, Inc.). Electrophoresis was performed under denaturing conditions in the presence of SDS at 50 volts for 10 minutes and then 180 volts for an additional 40 minutes. Gels were fixed by 10-minute incubation in 40% methanol-10% acetic acid solution, followed by a series of three 5-minute washes in filtered Milli-Q H2O. Gels were stained with Bio-Safe Sypro Ruby stain (BIO-RAD Laboratories, Inc.) overnight, followed by destaining in a 10% methanol and 7% acetic acid solution, and washing for 60 minutes. Gels were then imaged using Image Lab Software (version 5.2.1.) on the Gel/ChemiDoc MP Imager System (BIO-RAD Laboratories, Inc.).

C. FXI activation by thrombin variants stimulated by various heparin fractions:

Heparin molecules that vary in molecular weight stimulated the activation of FXI by α-thrombin. 40 nM FXI was added in TBS with 10 nM of α-thrombin and incubated with 0- 50 µg/mL of unfractionated heparin, enoxaparin, Heparin MW 3788, Heparin MW 2835, or fondaparinux at 37°C for 30 minutes.

D. Biotinylation of Dextran Sulfate:

For biotinylation of 500 kDa and 5 kDa dextran sulfate, 500 mg of each dextran sulfate was incubated overnight at 37°C with 0.5 amine-PEG_2-biotin, 100 mM EDAC (1-ethyl-3-[3-dimetyhlamino-propyl]carbodiimide), 100 mM 2-[morpholino]ethanesulfonic acid (MES), pH 6.5. The 500 kDa and 5 kDa biotin-dextran sulfates were purified by size-exclusion chromatography using PD-10 desalting columns containing Sephadex G-25 (GE Healthcare, Pittsburgh, PA).

E. Biotinylation of polyphosphate:

For biotinylation of short and long chain polyP, 500 mg of the polyPs were incubated with 0.5 amine-PEG_2-biotin, 100 mM EDAC, 100 mM MOPS, pH 6.5 overnight at 37°C. The samples were purified by size-exclusion chromatography using a PD-10 desalting column. The biotin-polyP preparations were resolved by polyacrylamide gel electrophoresis using 15% urea-polyacrylamide gels in TBE

(90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3) and detected by staining with toluidine blue.

F. Surface Plasmon Resonance:

Biomolecular interaction analysis was performed on the Biacore T200 biosensor system (GE Healthcare, Chicago, IL). The affinity of FXI and the thrombin variants α-thrombin, γ-thrombin, or RA-thrombin for 500 kDA dextran sulfate was determined by Surface Plasmon Resonance (SPR). Biotinylated dextran sulfate was bound to a CM5 flow cell in a streptavidin coated sensor chips until a response of 200 resonance units (RU) was achieved. The immobilized biotin-dextran sulfate was titrated with increasing concentrations (0 to 4 μM) of the thrombin variants in 20 mM Hepes, 150 mM NaCl (pH 7.4), 0.01% Tween20 buffer at 10 μL/min for 200 seconds, and then washed with the HBS buffer for 500 seconds to monitor dissociation. Flow cells were then regenerated with 1.2 M NaCl. The sensograms were analyzed using BIAcore T200 evaluation software. The maximal (steady-state) RU values were plotted against the thrombin concentrations and the dissociation constant (K_D) was determined using SigmaPlot software (Version 11, California, USA) using the single rectangular hyperbola equation, $f(x) = y_0+a*x/(b+x)$. The binding affinities of FXI and the thrombin variants to long chain and short chain polyP were also determined as described above.

G. Effect of HD1 and HD22 on the binding of thrombin variants to polyanions:

The effect of the DNA aptamers HD1 and HD22 on binding of thrombin variants to dextran sulfate and polyp was assessed using SPR. 250 nM of α-thrombin, 2000 nM of γ-thrombin and RA-thrombin along with various concentrations of the aptamers (0 – 10 μM) were passed over the immobilized 500 kDa biotin-dextran sulfate or biotin-polyP70. The sensograms generated were analyzed using BIAcore T200 evaluation software and the maximal (steady-state) RU values were plotted against the aptamer concentrations. These data were used to determine the aptamer IC50 values for displacement using SigmaPlot software (Version 11, California, USA).

H. FXI activation by thrombin variants stimulated by polyanions:

FXI activation by α-thrombin and its exosite mutants, γ-thrombin and RA-thrombin, was assessed in the absence and presence of high and low molecular weight dextran sulfate concentrations. 40 nM FXI was incubated with 10 nM of α-thrombin, RA-thrombin, or γ-thrombin. The mixtures were incubated with 0- 50 μg/mL of 500 kDA or 5 kDa dextran sulfate at 37°C for 30 minutes. FXIa generation was measured by FXIa hydrolysis of S-2366 as described above. The FXI activation percentages were calculated and the analysis of the FXI activation was done using TableCurve 2D (Version 4, Jandel Scientific Software, SPSS Inc., Chicago, IL) to determine the half maximal response (EC50) of the activation

to dextran sulfate. PolyP70 was also used to stimulate the activation of FXI mediated by α-thrombin, RA-thrombin, γ-thrombin or Quick-1 thrombin. 40 nM FXI was added in TBS with 10 nM of the thrombin variants. The mixtures were incubated with 0- 50 µg/mL of polyP70 at 37°C for 30 minutes. 100 nM of hirudin was added to each well to inhibit thrombin, and after an additional 5 minute incubation at 37°C, 400 µM of S-2366 was added. FXIa generation and the EC50 for the activation were determined as described previously.

I. Activation of human prothrombin and recombinant prothrombin by the prothrombinase complex:

The activation of recombinant prothrombin (rMZ) is conducted by prothrombinase complex to form a stable activated meizothrombin (rMZa) (Côté *et al.*, 1997). A reaction mixture containing 1.4 µM of either prothrombin or rMZ, 5 mM $CaCl_2$, and 10 µM PCPS was prepared in 20 mM Tris-HCl, 0.15 NaCl, 0.1% Prionex, pH 7.4 at 25°C. The reactions were initiated by the addition of 0.5 nM of FXa and 10 nM FVa for 15 minutes. The reactions at their endpoint were dissolved in sample buffer and 2-mercaptoethnaol was added to reduce the proteins. The solutions were subjected to electrophoresis as described previously and stained with Sypro Ruby. The gels were imaged and analyzed using densitometry on Image Lab (Bio-Rad, California, USA). The activated rMZa was stored in -20°C.

J. Activation of FXI by rMZa in the absence or presence of polyP70:

The rMZ activated by the prothrombinase complex (rMZa) was used to activate FXI. 40 nM FXI was incubated with 10 and 40 nM of rMZa and 5 µg/mL of polyP70 in 20 mM Tris-HCl, 0.15 NaCl, 0.1% Prionex, pH 7.4 at 37°C for 30 minutes. 100 nM of hirudin was added to each well to inhibit the rMZa activity, and after an additional 5 minute incubation at 37°C, 400 µM of S-2366 was added. The activation of FXI was determined as described in the chromogenic assays previously and compared to FXI's activation mediated by α-thrombin. The activation of FXI by rMZa was also seen using electrophoresis and analyzed using densitometry as described above.

K. FXI activation by thrombin variants stimulated by polyanions in the presence or absence of DNA aptamers:

To measure the effects of thrombin exosite specific aptamers on FXI activation, 40 nM FXI and 10 nM α-thrombin were added to TBS. 1 µg/mL of 500 kDa or 5 µg/mL of polyP70 was added to the mixtures. After diluting the DNA aptamers in aptamer buffer consisting of TBS containing 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 0.1% polyethylene glycol. The mixtures were incubated with 0-10 µM of HD1 and HD22 was added and incubated at 37°C for 30 minutes. 100 nM of hirudin was added to each well to inhibit the thrombin activities, and after an additional 5-minute incubation at 37°C, 400 µM of S-2366 was added. FXIa

generation was calculated and the IC50s of the aptamers were determined using the hyperbolic decay equation f(x) = y0+(a*b)/(b+x).

L. Thrombin generation assay:

Thrombin generation in human plasma was measured by monitoring the cleavage of Z-Gly-Gly-Arg-AMC at 37°C using a SpectraMax Pro 5e microplate reader (Molecular Devices, Sunnyvale, CA). The plasma was supplemented with 415 µM Z-Gly-Gly-Arg-AMC, 5 µM PC/PS vesicles and 4 µM CTI to inhibit FXIIa activity and limit the activation of FXI by thrombin only (Matafonov *et al.*, 2011). The supplemented plasma was mixed with 10 nM final concentration of α-thrombin, γ-thrombin, or RA-thrombin to initiate the FXI activation along with 100 mM $CaCl_2$ and 5 µg/mL of polyP70. The fluorescence of the thrombin substrate at excitation λ 390 nm, emission λ 460 nm was monitored. Each set of conditions was run 3 times in duplicates. Lag time for thrombin generation, peak thrombin generation and area under the thrombin generation curve was calculated using the TECHNOTHROMBIN TGA Evaluation Software (Technoclone, Vienna, Austria).

Results:

FXI activation by thrombin (chromogenic assay):

To determine whether a negatively charged surface has an effect on FXI activation by thrombin, FXI was incubated with thrombin in the presence or absence of dextran sulfate. 40 nM of FXI and 10 nM of thrombin were incubated with or without 1 µg/mL of dextran sulfate 500 kDa. The incubation of 40 nM FXI with and without dextran sulfate was used to ensure that there is very minimal auto-activation of FXI occurring and that FXI has no proteolytic activity. The 10 nM thrombin negative control was used to ensure that 100 nM of hirudin was sufficient to completely inhibit the thrombin, so that the chromogenic substrate S-2366 is not being cleaved by thrombin. The activation of FXI by thrombin alone is very minimal with 2.1 nM FXIa generated compared with 25 nM in the presence of 1 µg/mL of dextran sulfate (Figure 2). These results support the previous findings that a negatively charged surface is required to promote the activation of FXI by thrombin (Naito and Fujikawa, 1991).

SDS-PAGE protein analysis of FXI activation by thrombin:

SDS-PAGE was also used to monitor the FXI activation by thrombin to provide a visual representation of the activation. 100 nM of FXI was incubated with 40 nM of thrombin in the presence of 1 µg/ml of dextran sulfate 500 kDa. This FXI activation sample along with samples of 2 µg of thrombin, FXIa, and FXI were subjected to SDS-PAGE analysis under non-reducing and reducing

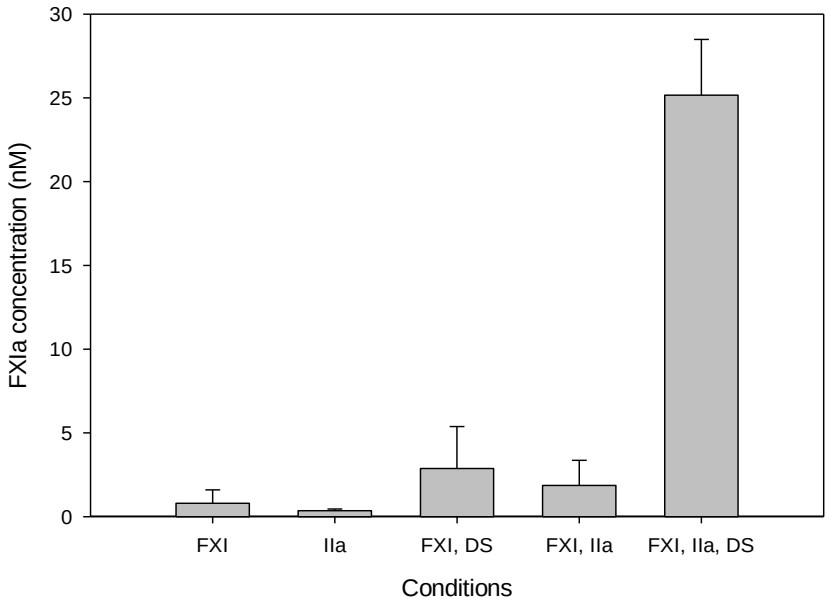


Figure 2: FXI activation by thrombin.

40 nM of FXI was incubated with 10 nM of thrombin (IIa) for 30 minutes at 37°C in the presence or absence of 1 µg/mL dextran sulfate (DS). FXIa activity was monitored by measuring hydrolysis of S-2366 at 405 nm and converted to concentration of FXIa generated. Controls used were 40 nM of FXI in the absence or presence of dextran sulfate and 10 nM of IIa. FXI activation by thrombin was 12 fold greater in the presence of dextran sulfate compared to the absence of it. Data are presented as mean ± standard deviation (n = 3 in duplicate).

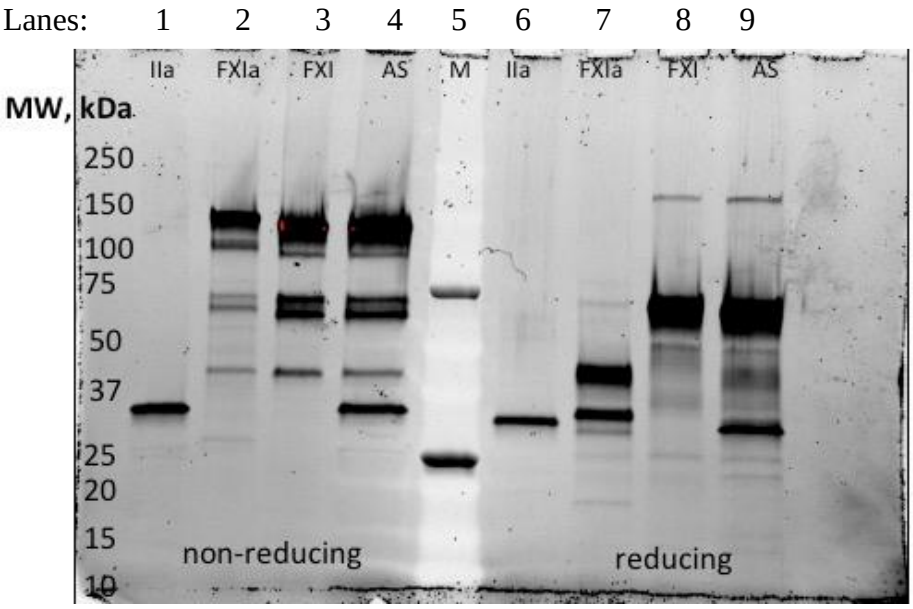


Figure 3: SDS-PAGE analysis of thrombin (IIa), FXIa, FXI, and the activation sample (AS)

Thrombin (lanes 1 and 6), FXIa (lanes 2 and 7), FXI (lanes 3 and 8) and activating sample (lanes 4 and 9) were subjected to SDS-PAGE analysis on a 4–15% polyacrylamide gradient gel under nonreducing and reducing conditions and stained using Sypro Ruby. The activating sample (AS) contains 100 nM FXI activated by 40 nM IIa with 1 μg/mL of dextran sulfate. The molecular weights of the mobility markers are shown on the left. FXI appears as a single band at ~160 kDa under non-reducing and ~80 kDa under reducing conditions. The heavy and light chains of FXIa are seen as single bands at ~50kDa and -30 kDa, respectively, under reducing conditions

conditions (Figure 3). Under non-reducing conditions, the bands of FXIa, FXI, and the activation sample had similar band patterns with each having distinct bands around 160 kDa and 75 kDa in size. This is due to the two monomers of FXI and FXIa, each 80 kDa in size, are held intact by disulfide bonds resulting in a band of 160 kDa. The smaller 75 kDa bands might be result of few homodimers splitting in the FXIa, FXI, and the FXI activation samples. Under reducing conditions, the disulfide bonds are broken and the bands of FXIa subunits can be compared with the FXI activation bands. The heavy chain (50 kDa) and light chain (30 kDa) bands of FXIa can be seen in the activation sample, but not in the FXI sample due to the heavy and light chains not being cleaved. Thus, the SDS-PAGE gel gives visual confirmation that FXI activation by thrombin has occurred in the presence of dextran sulfate.

FXI activation by thrombin variants stimulated by various heparin molecules:

In order to confirm that a template mechanism is necessary in the thrombin mediated FXI activation, various heparin molecules with well-defined sizes were used to stimulate the activation. Although heparin is physiologically an anticoagulant, it is a highly sulfated glycosaminoglycan and has the highest negative charge density of any known biological molecule (Muhl *et al.*, 2009). These negative charges allow both FXI and thrombin to bind to it, thereby facilitating the activation. Unfractionated heparin, enoxaparin, heparin MW 3788,

heparin MW 2835, and fondaparinux are heparin molecules that range in an average molecular weight from 1700 to 15000 Da. The titration of the heparin molecules in the FXI activation displayed peak activation of FXI at around 0.5 – 1 µg/mL of heparin. The maximum activation of FXI was plotted against the heparin molecular weight and a decrease in FXI activation was observed as the size of the heparin decreases (Figure 4). Peak activation of FXI by thrombin decreases by nearly 45% when the heparin molecules used to stimulate the activation are below a molecular weight of 4000. The correlation between FXI activation and size of heparin is not linear because the molecular weight of the heparins are only approximate averages. The unfractionated heparin and enoxaparin used contain heparin chains of varying molecular weights, which might result in the larger molecules contributing to greater activation. The heparin molecules differ in only in size; therefore, this suggests that a template mechanism is necessary for the activation of FXI by thrombin, and there is a minimum length of polyanion required for the activation to occur. The template mechanism was also noted by Choi, 2011, when they observed a bell shaped curve when activating FXI by thrombin in the presence of various concentrations of polyP.

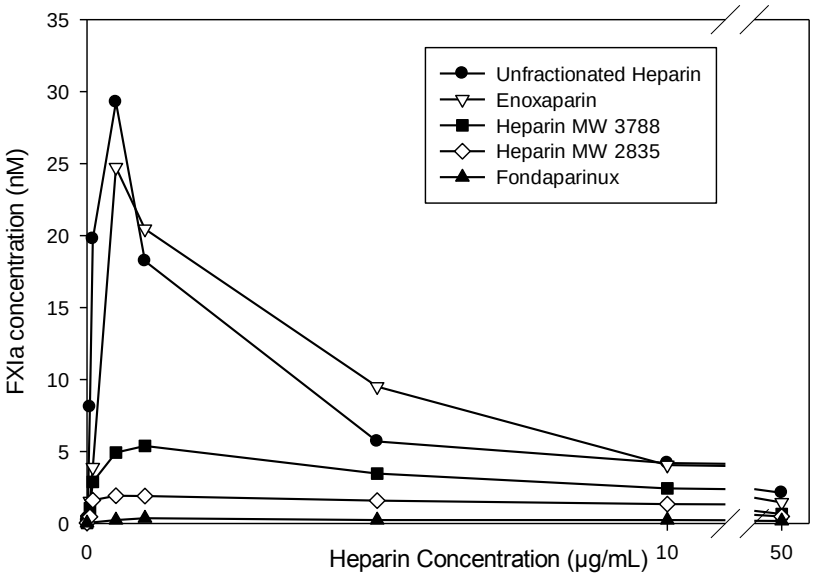


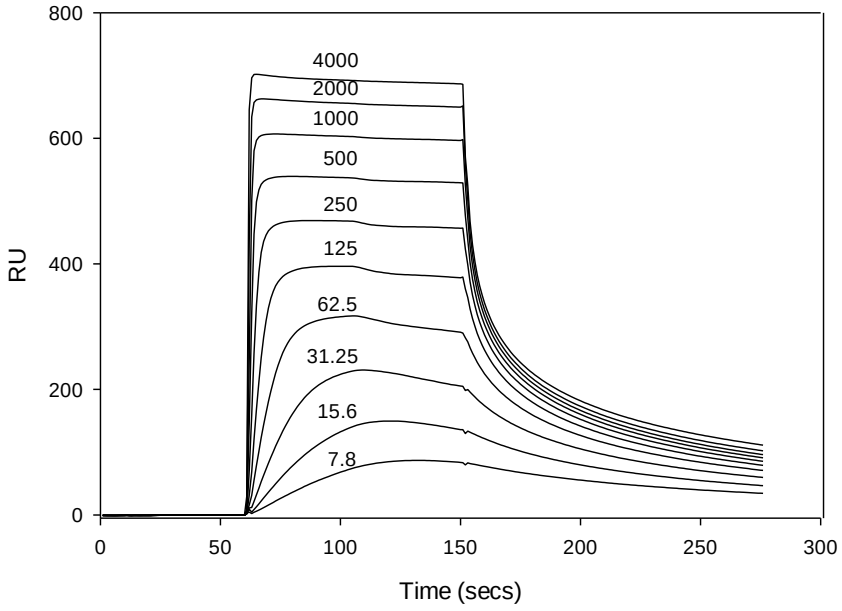
Figure 4: Effect of various heparin fractions on FXI activation by thrombin.

40 nM of FXI was incubated with 10 nM thrombin in the presence of 0- 50 µg/mL of various heparins. The amount of FXIa generated was quantified using a chromogenic assay and plotted versus the concentration of heparin.

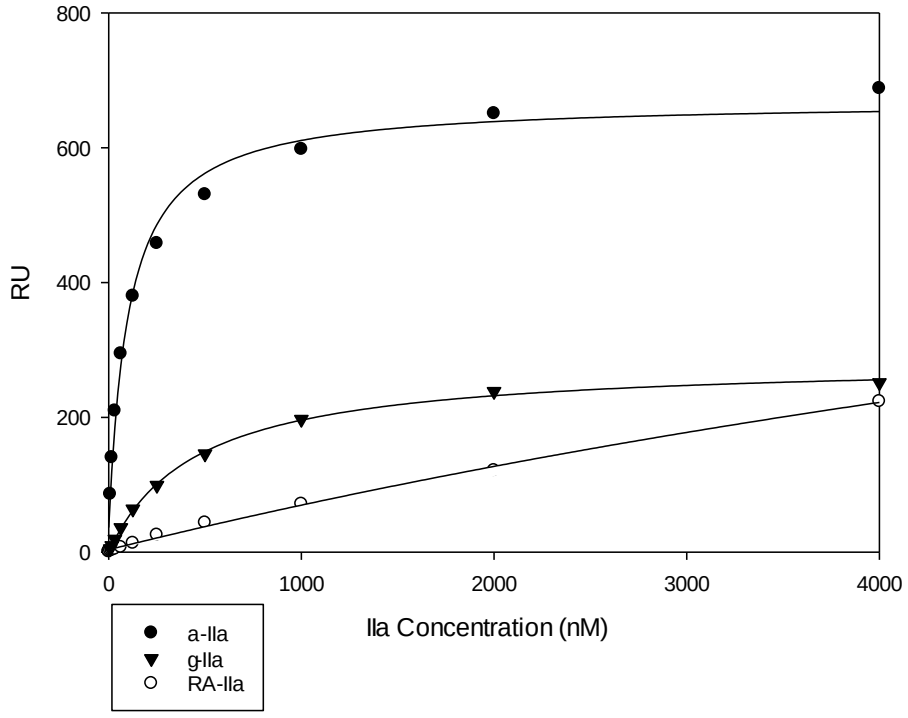
SPR analysis of FXI and thrombin variants binding to 500 kDa dextran sulfate:

To understand the interaction between thrombin and dextran sulfate, the affinity of the thrombin variants for dextran sulfate was determined using SPR. The thrombin variants were used to observe the effects of exosite 1 and 2 on the binding of thrombin to dextran sulfate. Aliquots of α-thrombin, γ-thrombin, and RA-thrombin (0 – 4000 nM) were injected at a flow rate of 30 µl/min for 200 s, followed by injections of HBS-0.05% Tw buffer for 200 s to monitor dissociation. The flow cells were then regenerated by an injection of a concentrated salt solution. An unmodified streptavadin-containing flow cell served as the reference control. The sensograms revealed fast association and slow dissociation phases for the thrombins binding to dextran sulfate (Figure 5A). Kd values were obtained by kinetic analysis of the on- and off-rates by globally fitting the binding data. Binding of thrombin and the thrombin variants were analyzed by steady-state analysis. Plotting the RU values at equilibrium from the sensograms against input concentrations shows α-thrombin and γ-thrombin bound to dextran sulfate with similar K_D values of 45 nM and 30 nM, respectively (Figure 5B). However, the total amount of γ-thrombin bound was significantly lower than that of α-thrombin. The small amount of γ-thrombin binding may be due to traces of α-thrombin present or an underestimation of the γ-thrombin concentration. This suggests that exosite 1 is necessary for the binding of thrombin to a polyanion due to the exosite 1 variant being unable to bind to

A


800
600
400
200
0
RU
0
50
100
150
200
250
300
Time (secs)
4000
2000
1000
500
250
125
62.5
31.25
15.6
7.8

B


800
600
400
200
0
RU
0
1000
2000
3000
4000
IIa Concentration (nM)
a-IIa
g-IIa
RA-IIa

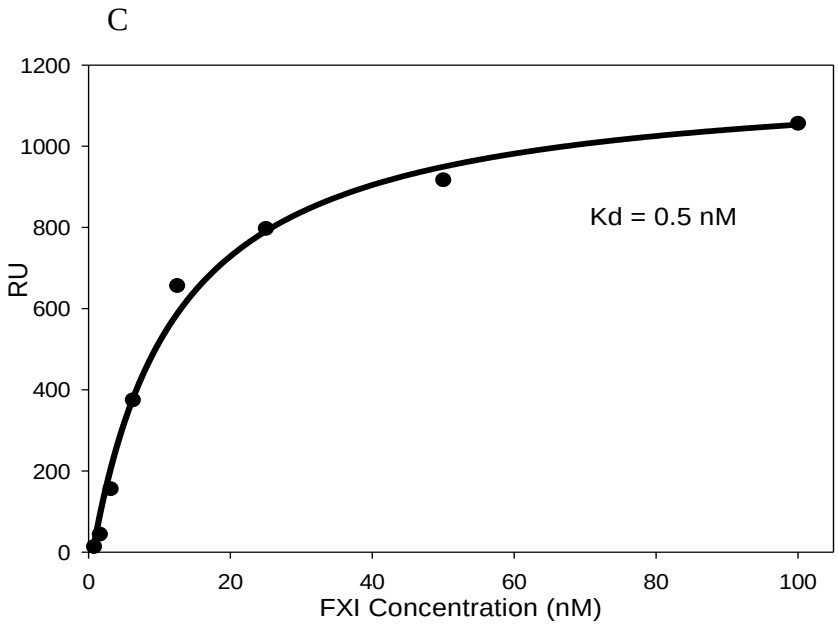


Figure 5: SPR analysis of thrombin variants and FXI binding to dextran sulfate.

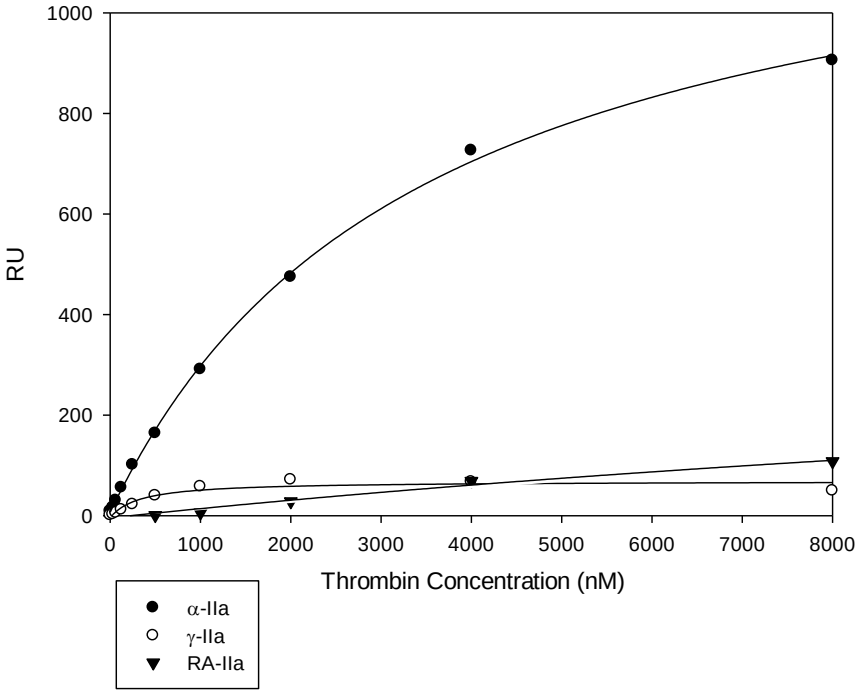
A) 500 kDa dextran sulfate was immobilized to 200 RU on separate flow cells of a CM4 BIAcore chip. α-thrombin (0 – 4 μM) was injected into flow cells for 200 s, and the cells were then washed with HBS buffer for 500 s to monitor dissociation. B) Increasing concentrations (0 – 4 μM) of α-thrombin, γ-thrombin, RA-thrombin and C) FXI (0 – 100 nM) were successively injected into flow cells. The RU at equilibrium was calculated from the generated sensograms and, after background correction, is plotted against the input protein concentrations. The lines represent nonlinear regression analyses of the data.

dextran sulfate. RA-thrombin had a much lower KD value of over 4000 nM compared with α-thrombin and γ-thrombin. These results are supportive of previous findings that exosite 2 plays the predominant role in the interaction of thrombin's with polyanions because the exosite 2 mutant RA-thrombin exhibited weak binding affinity to dextran sulfate. FXI also binds to dextran sulfate with a high affinity with a KD value of 0.5 nM (Figure 5C). The ability of both thrombin and FXI to tightly bind to dextran sulfate further suggests that polyanions stimulate the thrombin mediated FXI activation via a template mechanism.

SPR analysis of FXI and thrombin variants binding to polyP70 and HMW-polyP:

To further understand the interaction between the exosites of thrombin and polyanions, polyP70 and HMW-polyP were used in the binding assays. PolyP has shown to bind to exosite 2 on thrombin. The SPR analysis showed similar data between the thrombin variants' ability to bind to HMW polyP and polyP70 (Figure 6). The K_D values for α-thrombin to HMW polyP and polyP70 were 3.5 and 3.6 nM, respectively. The exosite 1 variant, γ-thrombin, exhibited sub-nanomolar binding affinity to the polyPs, while the exosite 2 mutant, RA-thrombin, had a much lower binding affinity. Although the dissociation constant of γ-thrombin suggested tight binding, the amount of γ-thrombin bound to the polyPs was less than 5% of that

A


1000
800
600
400
200
0
RU
0
1000
2000
3000
4000
5000
6000
7000
8000
Thrombin Concentration (nM)
α-IIa
γ-IIa
RA-IIa

B

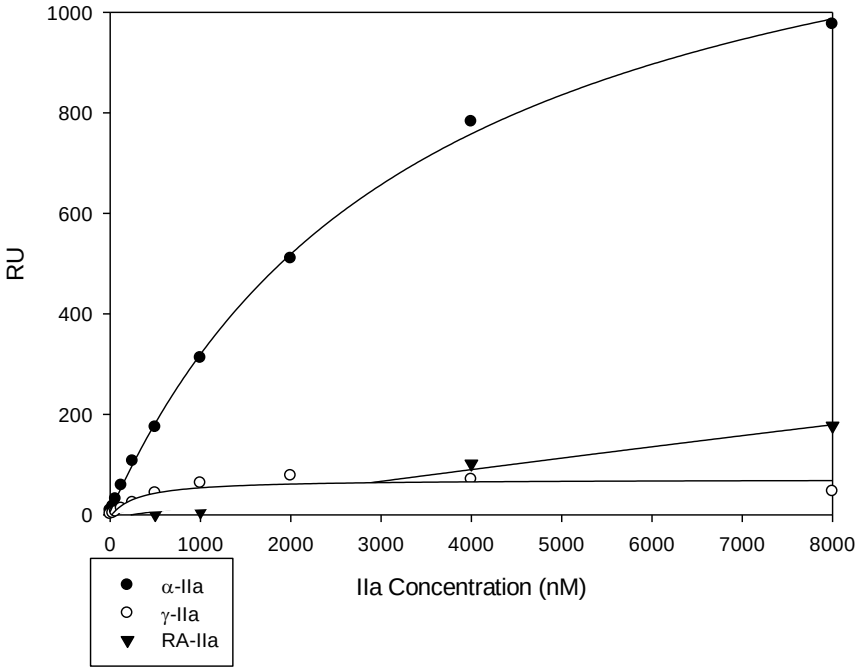

1000
800
600
400
200
0
RU
0
1000
2000
3000
4000
5000
6000
7000
8000
IIa Concentration (nM)
α-IIa
γ-IIa
RA-IIa

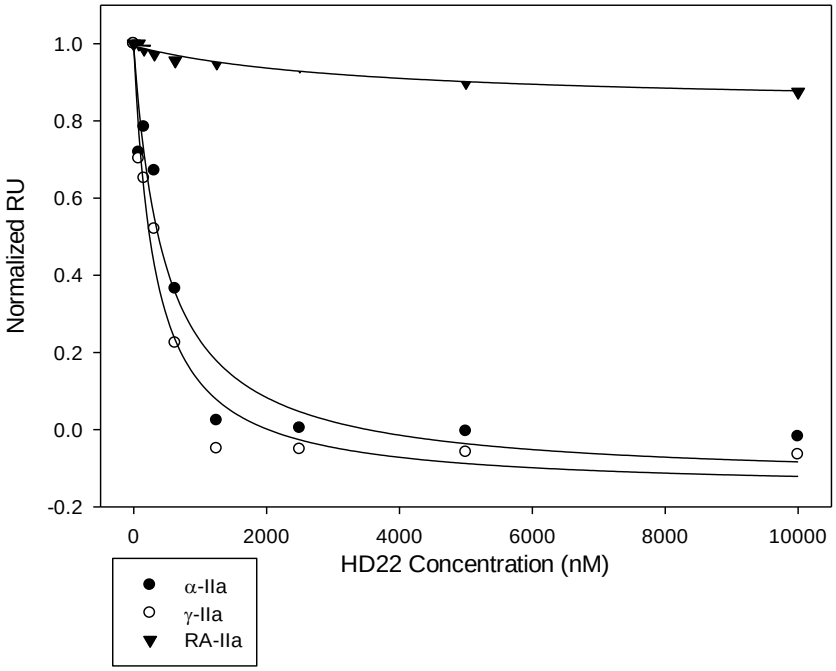
Figure 6: SPR analysis of thrombin variants binding to polyphosphates. Increasing concentrations (0 – 8 μM) of α-thrombin, γ-thrombin, RA-thrombin were successively injected into flow cells with immobilized biotin-polyP70 and -HMW-polyP for 200 seconds, followed by a wash with buffer to monitor dissociation. A) The amount of thrombin and its variants to A) polyP70 and B) HMW polyP at equilibrium (RU) after background correction is plotted against the input thrombin concentrations. The lines represent nonlinear regression analyses of the data.

of α-thrombin. These results were similar to the SPR analysis of thrombin and its variants binding to dextran sulfate. PolyP has been shown to preferentially bind to exosite 2, however, the inability of γ-thrombin to bind to the polyPs suggests that exosite 1 also has a significant role in thrombin's ability to bind to polyanions.

Competition of α, γ, and RA-thrombin binding to 500 kDA dextran sulfate by DNA aptamers HD1 and HD22:

The exosite 1 specific HD1 and exosite 2 specific HD22 DNA aptamers were used as competitors of exosite function. Binding assays were setup similarly to as described previously. However, along with thrombin variants, various concentrations of the DNA aptamers were also passed over the immobilized dextran sulfate. SPR was used to measure the binding of the thrombin to the dextran sulfate and the normalized binding was plotted as a function of aptamer concentration (Figure 7). The exosite 2 specific aptamer, HD22 greatly reduced α-thrombin and γ-thrombin's ability to bind to 500 kDa dextran sulfate (Figure 7A), due to competitively binding to exosite 2. HD22 had little effect on RA-thrombin's ability to bind to dextran sulfate due to its exosite 2 mutations already hindering its binding ability, therefore indicating the specificity of the aptamers to the exosites. Surprisingly, HD1 also competed with α-thrombin's ability to bind to dextran sulfate (Figure 7B). HD1 binds to exosite 1 on α-thrombin, however, with exosite 2 still available to bind to dextran sulfate, thrombin's ability to bind to the

A


Normalized RU
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
2000
4000
6000
8000
10000
HD22 Concentration (nM)
α-IIa
γ-IIa
RA-IIa

B

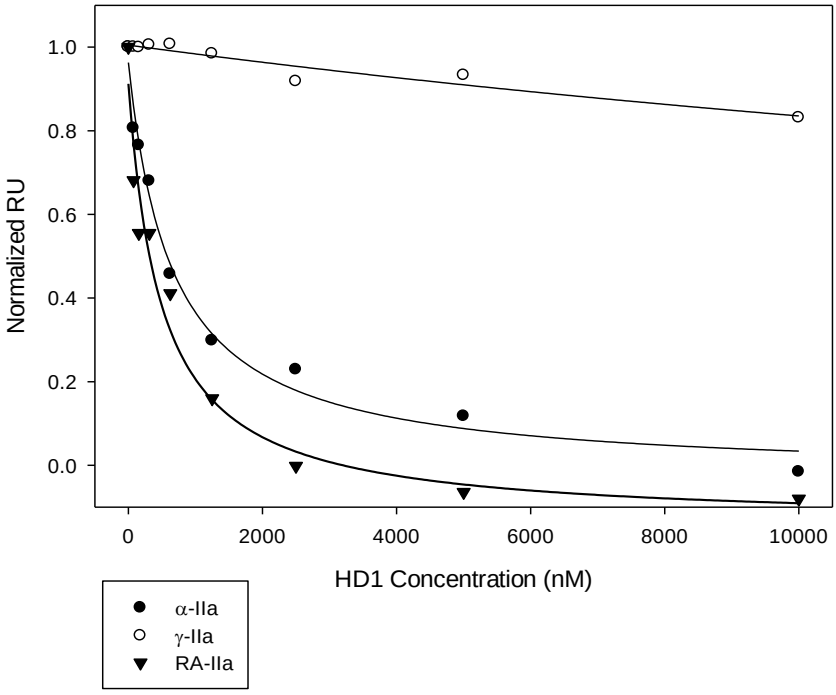

Normalized RU
1.0
0.8
0.6
0.4
0.2
0.0
0
2000
4000
6000
8000
10000
HD1 Concentration (nM)
α-IIa
γ-IIa
RA-IIa

Figure 7: Competition of α, γ, and RA-thrombin binding to 500 kDa dextran sulfate by exosite-specific aptamers.

The amount of α-thrombin (250 nM), γ-thombin (1000 nM), and RA-thombin (1000 nM) bound immobilized 500 kDa dextran sulfate in the presence of increasing concentrations (0 – 10 μM) of A) HD22 and B) HD1 was normalized to that determined in the absence of competitor. The normalized amount of thrombin and its variants bound at equilibrium (RU) after background correction is plotted against the input thrombin concentrations. The lines represent nonlinear regression analyses of the data.

polyanion should not be affected by HD1. Previous evidence suggests that exosite 2 plays the predominate role in the interaction between thrombin and polyanions, but the binding data displaying γ-thrombin's inability to bind to dextran sulfate and HD1 competing with dextran sulfate to bind to α-thrombin suggests exosite 1 also plays a significant role in polyanion binding.

Competition of α-thrombin binding to polyP70 by DNA aptamers HD1 and HD22:

Competitive binding assays were conducted similarly as before, this time substituting dextran sulfate with polyP70. SPR was used to measure the binding of α-thrombin to polyP70 in the presence of the DNA aptamers, and the binding was plotted as a function of aptamer concentration. HD1 and HD22 attenuated binding to α-thrombin adsorbed polyP70 in a concentration-dependent fashion with IC50s of 146.2 and 132.8 nM. Results were again similar to previous results with dextran sulfate, with HD22 displacing α-thrombin more than HD1, however, HD1 still had a profound effect on thrombin's ability to bind to polyP70 (Figure 8). PolyP has specificity to exosite 2 and competitive binding should only be seen in the presence of HD22. However, HD1's effect on thrombin's ability to bind to polyP70 provides further evidence that exosite 1 is involved in polyanion binding. HD1 may indirectly effect thrombin's ability to bind to polyP70 by having an allosteric effect on exosite 2 when bound to exosite 1.

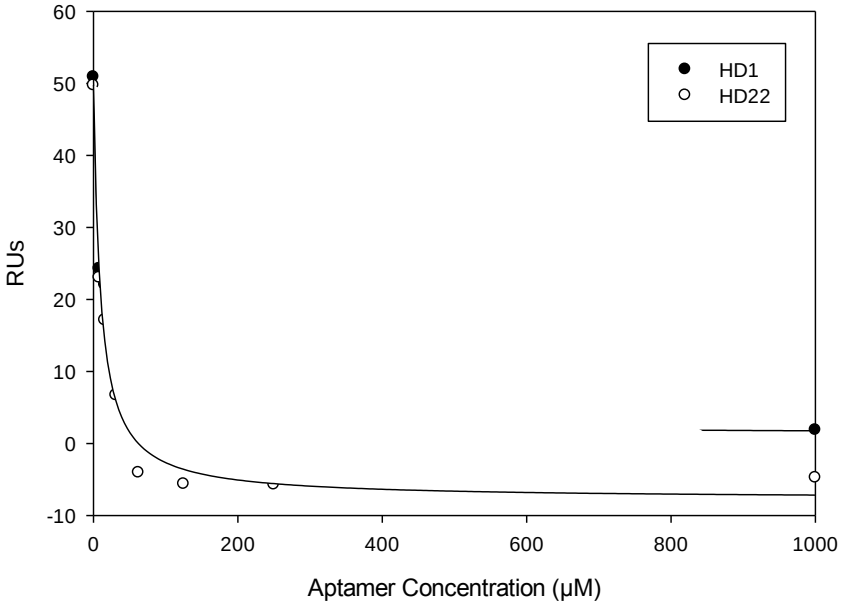


Figure 8: Competition of α -thrombin binding to polyP70 by exosite-specific aptamers.

The amount of α-thrombin (250 nM) bound immobilized polyP70 in the presence of increasing concentrations (0 – 10 μM) of HD1 and HD22 was normalized to that determined in the absence of competitor. The normalized amount of thrombin bound at equilibrium (RU) after background correction is plotted against the input thrombin concentrations. The lines represent nonlinear regression analyses of the data.

FXI activation by thrombin variants simulated by 500 kDa dextran sulfate:

FXI activation was conducted using α-thrombin, γ-thrombin, and RA-thrombin to determine the effects of exosite 1 and exosite 2 on the activation. 40 nM of FXI was incubated with 10 nM of α-thrombin, RA-thrombin, or γ-thrombin, in the presence of a range of 500 kDa dextran sulfate concentrations from 0-50 µg/mL. After measuring the FXIa mediated hydrolysis of chromogenic substrate S-2366, the amount of FXIa generated was calculated. The peak activation of FXI by α-thrombin in the presence of 500 kDa dextran sulfate, was 50% greater than activation by RA-thrombin, and 5% greater than that by γ-thrombin (Figure 9). The α-thrombin also exhibited a wider bell-shape profile than the thrombin variants. The bell-shaped curves again provide evidence that a ternary complex between FXI, thrombin, and a polyanion is required for the activation to occur. Using the peaks of the curves as maximum, the concentration at which half maximal stimulation (EC50) of FXI activation is achieved using the thrombin variants was calculated. The EC50 using α-thrombin in the presence of 500 kDa dextran sulfate was 0.01 µg/mL compared with 0.25 µg/mL and 0.34 µg/mL for γ-thrombin and RA-thrombin, respectively. These results show that exosite 2 is required for the activation of FXI by thrombin due to RA-thrombin having a decreased ability to activate FXI. α-thrombin and γ-thrombin have similar peak activations of FXI due to their exosite 2 still being intact. The bell-shaped curves again provide evidence that a ternary complex between FXI, thrombin, and a

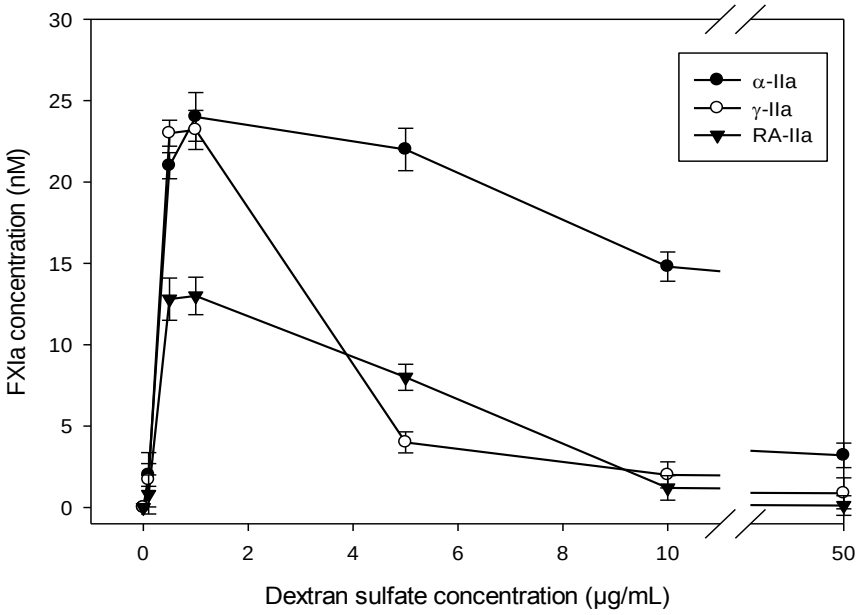


Figure 9: FXI activation by thrombin exosite variants stimulated by 500 kDa dextran sulfate.

40 nM FXI was activated by 10 nM α-thrombin, γ-thrombin, and RA-thrombin with increasing concentrations (0 – 50 μg/mL) of 500 kDa dextran sulfate. Thrombin activity was inhibited with hirudin before FXIa generation was measured by the hydrolysis of FXIa-directed S2366 chromogenic substrate and determined using the FXIa standard curve. Data are mean ± SD, of three determinations.

polyanion is required for the activation to occur.

FXI activation by thrombin variants simulated by polyP70:

FXI activation was once again conducted using α-thrombin, γ-thrombin, Q1-thrombin and RA-thrombin this time using polyP70 instead of dextran sulfate. In order to see if the proteolysis of exosite 1 in γ-thrombin does not have an effect on γ-thrombin's ability to activate thrombin substrates, the exosite 1 mutant, Q1-thrombin was included in this experiment. The single point mutation (Cys67 to Arg) in Q1-thrombin decreases its ability to bind to exosite 1 specific substrates, however, it does not alter the overall structure of thrombin (Henriksen and Mann, 1988). Although the peak activation of FXI by the various thrombins was slightly lower when stimulated by polyP70 (Figure 10) as opposed to the larger 500 kDa dextran sulfate (Figure 9), they followed a similar pattern. Peak activation of FXI by α-thrombin with polyP70 generated 22.8 nM of FXIa which is similar to the peak activation of FXI by γ-thrombin and Q1-thrombin (18.4 nM and 20.4 nM respectively). This suggests that thrombin and thrombin variants with

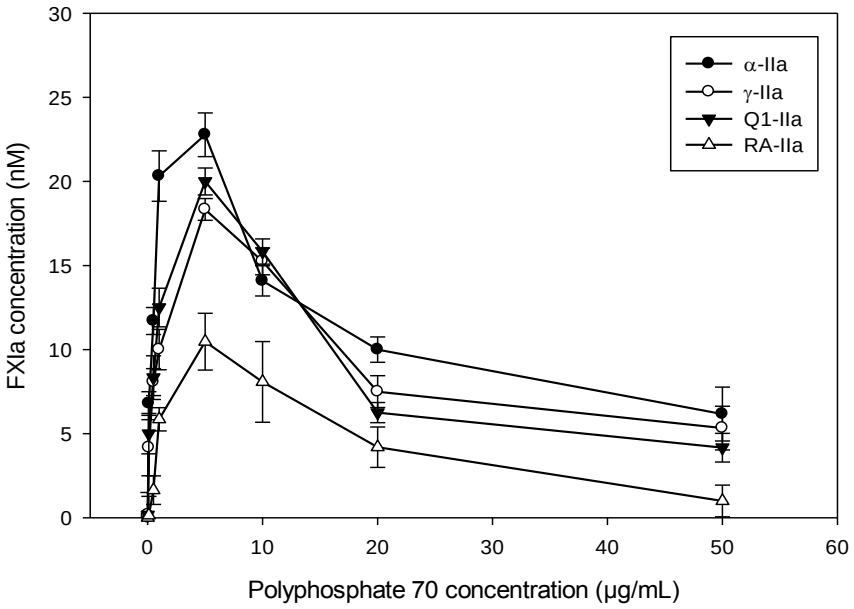


Figure 10: FXI activation by thrombin exosite variants stimulated by polyP70.

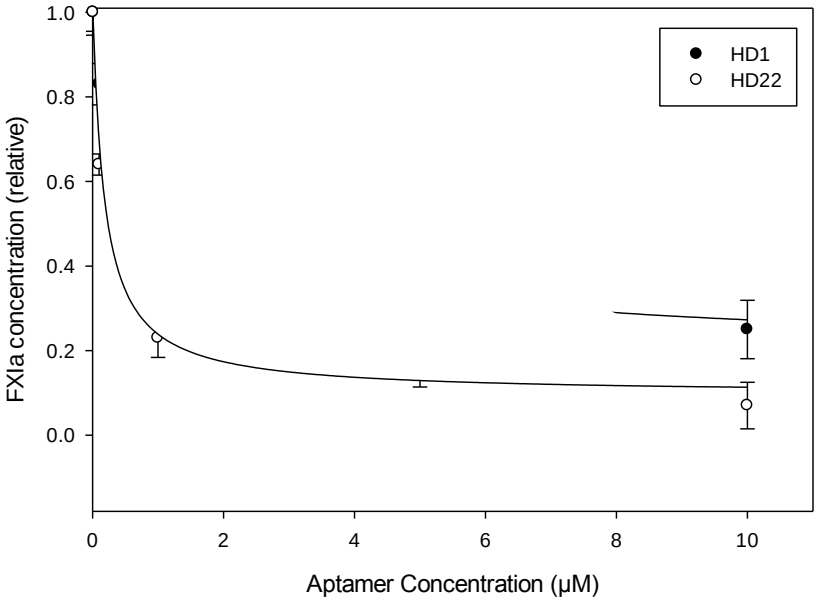
40 nM FXI was activated by 10 nM α-thrombin, γ-thrombin, Q1-thrombin and RA-thrombin with increasing concentrations (0 – 50 μg/mL) of 500 kDa dextran sulfate. Thrombin activity was inhibited with hirudin before FXIa generation was measured by the hydrolysis of FXIa-directed S2366 chromogenic substrate and determined using the FXIa standard curve. Data are mean ± SD, of three determinations.

an unaltered exosite 2 will have similar effectiveness to activate FXI. The activation of FXI mediated by RA-thrombin was more than 2-fold lower than with the other thrombin variants. These results further support the necessity of exosite 2 for the activation of FXI by thrombin due to RA-thrombin's limited ability to activate FXI. However, there is still FXI activation occurring by RA-thrombin, which means that residual binding to the polyP70 is occurring allowing the activation to occur, as seen previously in the binding assays (Figure 6A). The γ-gamma thrombin and Q1-thrombin had very similar results confirming that although γ-thrombin is proteolyzed, its active site is not altered.

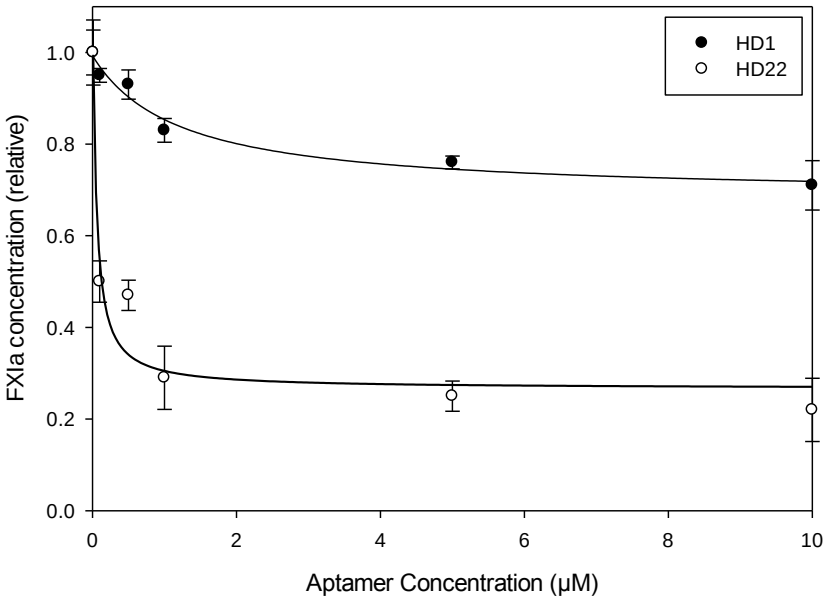
FXI activation by thrombin variants stimulated by 500 kDa dextran sulfate in the presence or absence of DNA aptamers HD1 and HD22:

DNA aptamers HD1 and HD22 were used to further analyze the roles of thrombin exosites 1 and 2 in FXI activation. 40 nM of FXI was added to 10 nM of α-thrombin along with 1 μg/ml of 500kDa dextran sulfate. The activation was done in the presence of increasing concentrations (0 – 10 μM) of each aptamer. The activation of FXI was analyzed using the chromogenic assay. In the presence of HD1 and HD22, a significant reduction in FXI activation by α-thrombin was observed (Figure 11A). Although both aptamers had an inhibitory effect, HD22, the aptamer that binds to exosite 2, had a significantly greater effect. With 500 kDa dextran sulfate stimulating the activation, HD22 inhibited FXI activation by more than two-fold compared with HD1 and was able to inhibit the

A


HD1
HD22
FXIa concentration (relative)
Aptamer Concentration (μM)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10

B


HD1
HD22
FXIa concentration (relative)
Aptamer Concentration (μM)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10

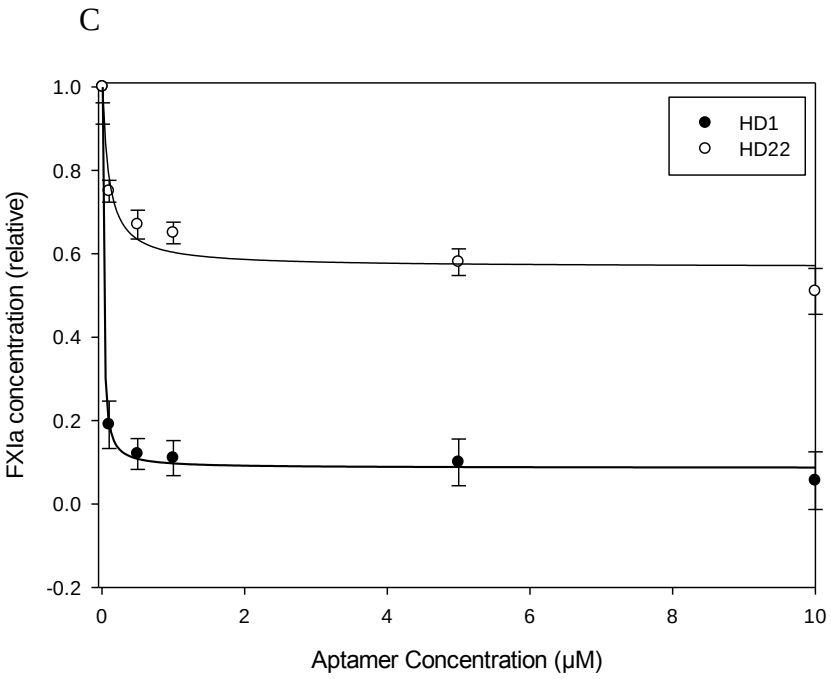


Figure 11: FXI activation by α-thrombin stimulated by 500-kDa dextran sulfate in the presence or absence of DNA aptamers HD1 and HD22.

40 nM of FXI was incubated with 10 nM of A) α-thrombin B) γ-thrombin C) RA-thrombin in presence of 1 μg/mL 500 kDa dextran sulfate and increasing concentrations (0 – 10 μM) of HD1 and HD22 for 30 minutes at 37ºC. Thrombin activity was inhibited with hirudin before FXIa generation was measured by the hydrolysis of FXIa-directed S2366 chromogenic substrate and determined using the FXIa standard curve. Data are mean ± SD, of three determinations.

activation by 50% at concentrations of 84 nM (IC50) in contrast to 424 nM for HD1 (IC50) (Figure 11A). As expected, HD22 had a profound inhibitory effect on FXI activation mediated by γ-thrombin due to HD22 competing with dextran sulfate from binding to its exosite 2 (Figure 11B). Thus, HD22 was able to prevent γ-thrombin from forming a ternary complex with FXI and dextran sulfate. Exosite 1 specific aptamer HD1 also inhibited α-thrombin's ability to activate FXI. These results along with the competitive binding of HD1 to α-thrombin confirms that HD1 prevents thrombin from binding to dextran sulfate; thus, inhibiting its ability to activate FXI. Although this suggests exosite 1 plays a role in FXI activation, the mechanism of exosite 1's role in FXI activation is unknown. The exosite 1 ligand may directly inhibit thrombin's ability to bind to polyanions, or it may have an allosteric effect on exosite 2. These results suggest that both exosites of thrombin play a role in FXI activation, however, exosite 2 has the more significant role due to the greater attenuation of FXI activation when exosite 2 is inhibited.

FXI activation by α-thrombin stimulated by polyP70 in the presence or absence of DNA aptamers HD1 and HD22:

Exosite-specific DNA aptamers HD1 and HD22 had similar inhibitory effects on the activation of FXI by α-thrombin stimulated by polyP70 as previous results with dextran sulfate. HD22 had a larger inhibitory effect on FXI activation mediated by α-thrombin than HD1 in the presence of polyP70 (Figure 12). This

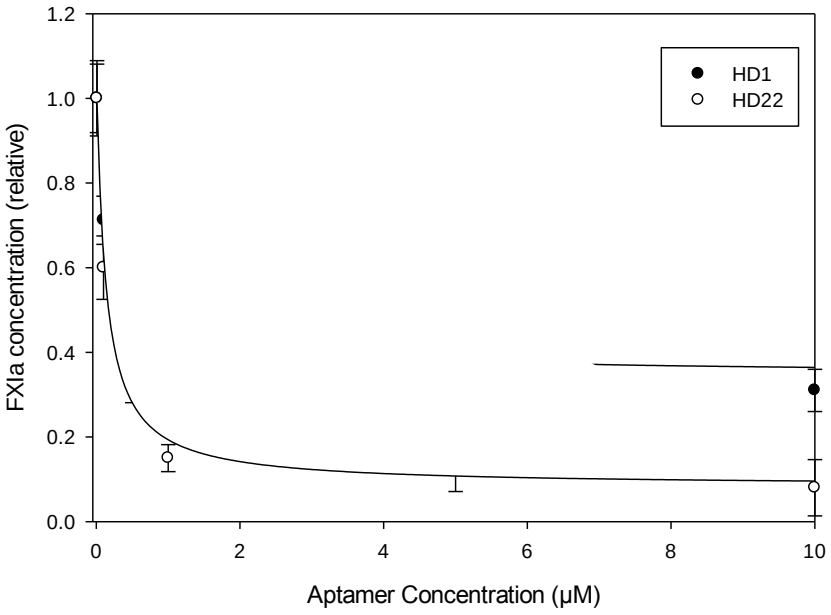


Figure 12: FXI activation by α-thrombin stimulated by polyP70 in the presence or absence of DNA aptamers HD1 and HD22.

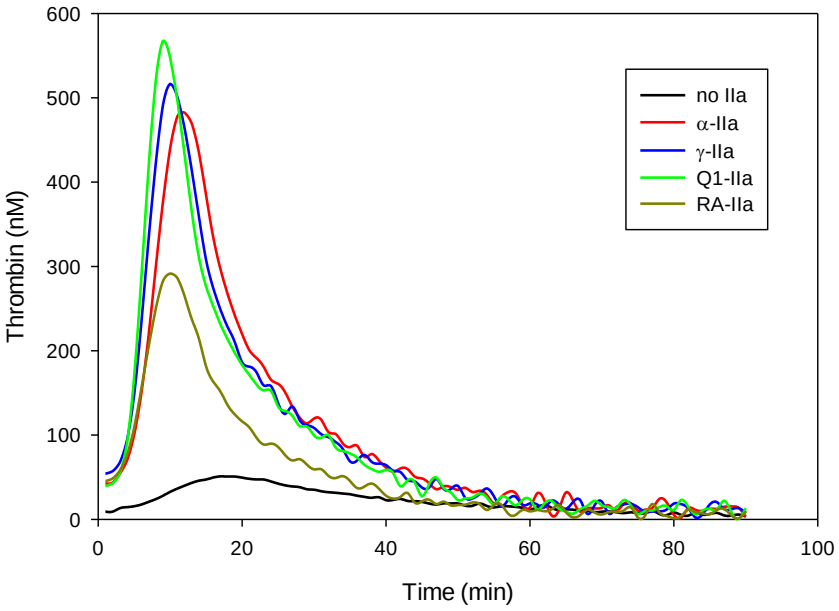
40 nM of FXI was incubated with 10 nM of α-thrombin in presence of 1 μg/mL 500 kDa dextran sulfate and increasing concentrations (0 – 10 μM) of HD1 and HD22 for 30 minutes at 37ºC. Thrombin activity was inhibited with hirudin before FXIa generation was measured by the hydrolysis of FXIa-directed S2366 chromogenic substrate and determined using the FXIa standard curve.. Data are mean ± SD, of three determinations.

provides further evidence that exosite 2 plays a greater role in the activation of FXI by thrombin. The inhibitory effects of HD1 on FXI activation by thrombin also suggests that exosite 1 has a role in FXI activation and can be targeted to inhibit the activation.

Thrombin Generation Assay:

The role of the thrombin exosites in FXI activation was tested in plasma by conducting a thrombin generation assay. Thrombin generation in human plasma was initiated via the intrinsic pathway by adding various thrombin variants and polyP70 to the plasma. The initiation of the intrinsic pathway was limited to FXI activation by thrombin, by adding 4 μM CTI to inhibit FXIIa activity. In order to minimize the cleavage of the fluorogenic substrate, only 10 nM α-thrombin, γ-thrombin, Q1-thrombin, or RA-thrombin was used to initiate the FXI activation. This concentration is very small compared with the 1.4 μM plasma concentration of prothrombin, and therefore insignificant in the detection of generated thrombin (Smiles *et al.*, 1998). As expected, the thrombin generation profile of normal plasma revealed enhanced thrombin generation upon addition of 10 nM thrombin and 5 μg/mL of polyP70. Very little thrombin generation occurred without the added thrombin (Figure 13A). The various parameters of the thrombin generation profile such as peak thrombin concentration and the endogenous thrombin potential were determined. The peak thrombin concentration generated by α-thrombin, γ-thrombin, and Q1-thrombin are almost 2-fold greater than the peak thrombin concentration generated by RA-thrombin (Figure 13B). The same

A


600
500
400
300
200
100
0
Thrombin (nM)
0
20
40
60
80
100
Time (min)
no IIa
α-IIa
γ-IIa
Q1-IIa
RA-IIa

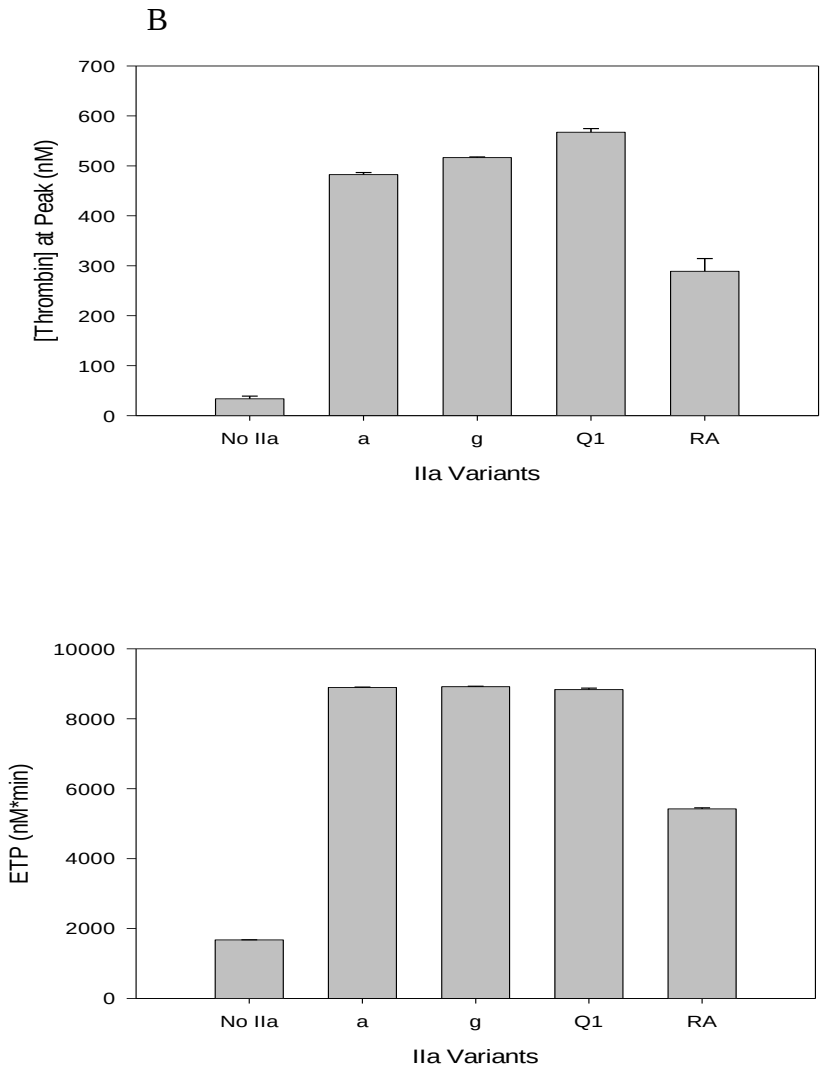


Figure 13: Thrombin generation assay.

Plasma was incubated with 415 µM Z-Gly-Gly-Arg-AMC, 5 µM PC/PS vesicles and 4 µM CTI before thrombin generation was initiated with 100 mM $CaCl_2$ and 5 µg/mL of polyP70, and 10 nM final concentration of α-thrombin, γ-thrombin, or RA-thrombin. A) Represents the data of the thrombin generation profile B) The peak thrombin concentration and the endogenous thrombin potential of thrombin generation assay.

results are seen for the endogenous thrombin potential, where α-thrombin, γ-thrombin, and Q1-thrombin produce significantly more thrombin than RA-thrombin. These results are similar to the results of functional assays of FXI activation by the thrombin variants in a purified system (Figure 10). Due to RA-thrombin's limited ability to activate FXI, less FXIa was produced compared with the thrombin variants, resulting in the decreased thrombin generation. Together these data suggest that a compromised exosite 2 attenuates the activation of FXI, leading to a decrease in thrombin generation in human plasma.

Activation of FXI by rMZa stimulated by polyP70:

Due to the residual binding and activation of FXI by the exosite 2 mutant (Figures 5, 6, 9 and 10), RA-thrombin, a rMZa was explored as an additional exosite 2 variant. This recombinant human meizothrombin is a stable analogue of meizothrombin, and in a conformation such that exosite 2 is masked. Therefore, there are no exposed positively charged residues in exosite 2 capable of binding to polyanions in rMZa (Côté *et al.*, 1997). Compared with rMZa, RA-thrombin has five of the total eight positively charged residues capable of binding to polyanions, making the rMZa the better exosite 2 variant. Prothrombinase complex consisting of 0.5 nM FXa, 10 nM FVa, 5 mM CaCl2, and 10 μM PCPS mediated the activation of prothrombin and rMZ. Over 90% of the prothrombin and rMZ was activated as detected by the densitometry (not shown). The 10 nM of the activated proteases were then incubated with 40 nM FXI and 5 μg/mL of

polyP70. The rMZa was inhibited by hirudin and the amount of FXIa generation was measured using the chromogenic substrate, S-2366. FXI was also incubated with 40 nM of rMZa due rMZa having approximately one-fourth of the enzymatic activity with thrombin substrates compared to α-thrombin. 26.4 nM of FXIa was generated by the thrombin was which was similar to previous results of FXI's activation by α-thrombin. Although the 10 nM of rMZa was not able significantly activate FXI, 40 nM of rMZa was able to activate 22.8 nM of FXI (Figure 14). These results show that with a lack of exosite 2 the activation of FXI can still take place. rMZa has an intact exosite 1, which suggests that at least one exosite is necessary for the thrombin to bind to a polyanion and activate FXI.

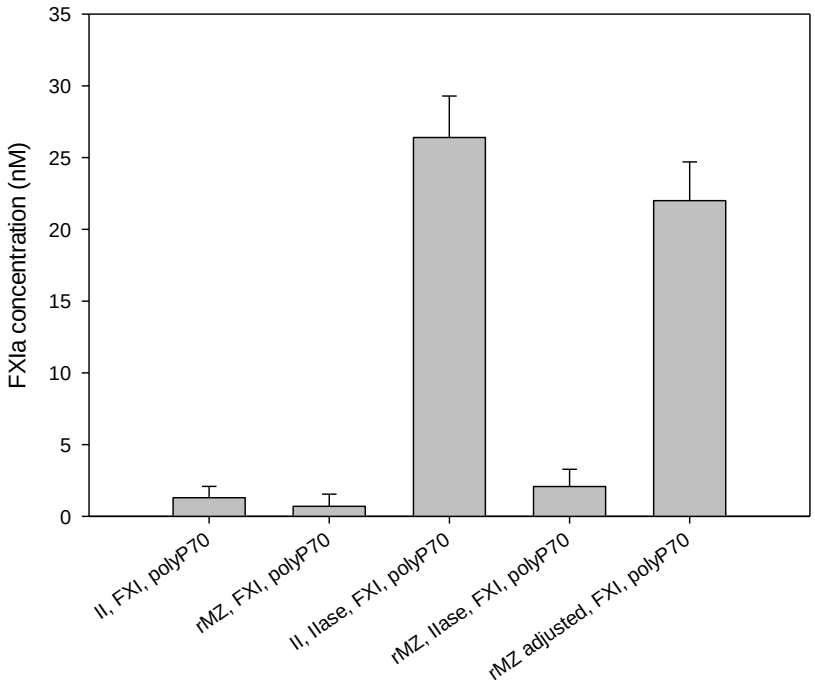


Figure 14: FXI activation by prothrombin and rMZ in the presence of prothrombinase.

10 nM of prothrombin (II) and rMZ activated by prothrombinase (IIase) and incubated with 40 nM FXI and 5 µg/mL polyP70. rMZa adjusted represents the 40 nM of rMZa activated by prothrombinase. The activation of FXI by rMZa and thrombin is detected using a chromogenic assay. Data are mean ± SD, of three determinations.

Discussion:

In the past, the intrinsic pathway (including the contact system) has received little interest because it was considered redundant for in hemostasis. However, over the recent years, it garnered renewed interest because of its role in thrombosis. FXI, a procoagulant factor in the intrinsic pathway, has been shown to have a minor role in hemostasis because patients with congenital FXI deficiency have only a little to mild bleeding diathesis (Seligsohn, 2009). However murine studies demonstrated that inhibition of FXI attenuated clot formation, suggesting it has a significant role in thrombosis (Van Montfoort and Meijers, 2014). These studies indicate that FXI is a potential target for antithrombotic agents with a low risk of inducing haemorrhage.

FXI is primarily activated by FXIIa, however it can also be activated by thrombin in the presence of polyanions. Many artificial surfaces have been identified as activators of FXII via the contact system including glass, silica, kaolin, ellagic acid, and dextran sulfate (Colman and Schmaier, 1997). Recently, physiological surfaces such as inorganic polyphosphate (polyP), DNA, and RNA have been identified as potential cofactors for FXI activation by thrombin and as activators of FXII. The mechanism of this alternative activation of FXI requires further research for the progression of developing antithrombotic agents that target FXI. In this study, we seek to identify the mechanism of how thrombin activates FXI by exploring the role of thrombin's exosites in binding to polyanions and activating FXI.

FXI activation by thrombin was known to occur only in the presence of non-physiological surfaces such as dextran sulfate. Recently polyP was identified as a potential physiological cofactor for this reaction by providing a surface onto which FXI and thrombin can bind (Choi, 2011). A characteristic feature of thrombin are two distinct electropositive surface regions, termed exosite 1 and exosite 2, that contribute to the specificity of thrombin by mediating the recognition of its substrates, inhibitors and receptors. Only exosite 2 has been implicated in the binding of thrombin to polyP (Mutch *et al.*, 2010). However, the role of exosite 1 in the activation requires further investigation for a complete understanding of the mechanism of FXI activation by thrombin.

Using a chromogenic assay and SDS-PAGE for visual support, the activation of FXI by thrombin was analyzed. While FXI incubated with thrombin alone exhibited little to no activation, the addition of dextran sulfate increased FXI activation by 12-fold. In the initial chromogenic assays, we tested the activation of FXI in various settings such in the presence and absence of thrombin, dextran sulfate, or both. We found that without the presence of a protease and polyanion, FXI exhibits little to no activation. This finding was contrary to previous findings (Ivanov *et al.*, 2017) that FXIIa or thrombin has similar effects on the rate of FXI activation relative to autoactivation of FXI in the presence of a polyanion. After repeating the experiment using the methods of Ivanov *et al.* no differences were observed from our previous results. However, our results are supported by Gajsiewicz et al. who showed the auto-activation of FXI in the presence of RNA

was much lower compared than the activation of FXI by thrombin in the presence of RNA (Gajsiewicz, Smith and Morrissey, 2017). These differences will need to be explored further to determine whether FXI is capable of undergoing auto-activation at a rate comparable with its activation by other proteases. Our finding that a polyanion is necessary for the efficient activation of FXI by thrombin suggests that a ternary complex consisting of FXI, thrombin, and the polyanion must form in order for the activation to occur.

To provide further support for a template mechanism in the heparin fractions of varying molecular weight were used to stimulate the activation. Heparin fractions of varying sizes were used in an attempt to identify the minimum length of polyanion required to stimulate the activation. The heparin titration indicates that the increase in activation correlates with a increase in cofactor concentration. But more importantly, there is a decrease in activity with the higher concentrations of the cofactor, reflecting the formation of non productive binding complexes, suggesting a ternary complex mechanism. The decrease in the size of heparin used lead to the decrease in FXI activation provided further evidence that the activation occurs via a template mechanism and suggests that there is a minimum length of polyanion required to stimulate the activation.

For the template mechanism to occur, both FXI and thrombin must be able to bind to the polyanions. To confirm the binding and to observe which exosite is

contributes to the binding, we used SPR to quantify the binding affinities of FXI, α-thrombin and its variants, γ-thrombin (exosite 1 variant), and RA-thrombin (exosite 2 mutant) for 500 kDa dextran sulfate. FXI, α-thrombin, and γ-thrombin displayed nanomolar binding affinities, however, RA-thrombin had a K_D value of over 4000 nM and did not achieve saturation. This confirmed that exosite 2 of thrombin plays a predominant role in binding to polyanions. However, although γ-thrombin had a tight binding affinity, the total amount of binding to the immobilized dextran sulfate was extremely low with less than 200 RU bound. Similar results were observed with the binding of thrombin and its variants to long-chain and short-chain polyP. Both polyPs were able to bind α-thrombin and γ-thrombin with high affinity, while RA-thrombin did not achieve saturation. These results are similar to previous findings by Mutch *et al*., which suggested indicated that thrombin binds to polyp via exosite 2. The reduced amount of γ-thrombin bound to the polyanions suggests that the exosite 1 variant is unable to bind to the polyanions. Our findings contrast with other results (Mutch *et al.*, 2010), which reported comparable binding of both α-thrombin and γ-thrombin to immobilized polyP. Furthermore, their exosite 1 mutant (Tyr71Ala, His66Ala) also displayed essentially the same binding affinity to polyP as wildtype thrombin, while their exosite 2 mutants displayed weak binding affinities. Our data suggest that both exosites are necessary for thrombin to bind to polyanions, however, the discrepancy between our findings and others will have to be explored further. The

exosite 1 variant Quick 1 thrombin can be used in the binding assays to confirm whether results of γ-thrombin in the binding assays were accurate.

The chromogenic FXI activation assays showed that α-thrombin and γ-thrombin had similar activation of FXI, while activation by RA-thrombin was almost two-fold lower. This provides further evidence that disrupting exosite 2 on thrombin, attenuate its capacity to activateFXI. However, there is still a considerable amount of activation occurring, which we suspect is due to the residual binding of RA-thrombin to the dextran sulfate. This could be responsible for the near 40% activation of FXI by RA-thrombin. The activation of FXI by the thrombin variants in the presence of polyP70 showed similar results to the activation in the presence of 500 kDa dextran sulfate. Q1-thrombin was also included to demonstrate that the proteolysis of exosite 1 in γ-thrombin did not have any influence on its catalytic activity. Q1-thrombin and γ-thrombin displayed similar activation results. Our data showing decreased FXI activation by RA-thrombin compared with the exosite 1 variants supports the hypothesis that exosite 2 plays the predominant role in FXI activation. However, these findings suggest that either exosite can support the activation of FXI, while our binding experiments showed that both exosites are necessary for binding to occur. In order to further investigate the role of the exosites in binding to polyanions and FXI activation, we explored the effects of exosite 1 and 2 specific aptamers on the various assays. These aptamers were used to provide insight on how exosite specific ligands can affect the binding and activity of thrombin.

Both the binding and activation of FXI was significantly reduced by HD1 and HD22. The exosite 1 and 2 specific aptamers attenuated α-thrombin's ability to bind to dextran sulfate leading to the inhibition FXI activation by thrombin. HD22 affected γ-thrombin's ability to bind to dextran and activate FXI, while HD1 only affected RA-thrombin. This confirmed that the DNA aptamers were specific for their corresponding exosites. With IC50 values of 0.42 μM for HD1 and 0.08 μM for HD22, the exosite 2 specific aptamer had a greater inhibitory effect on FXI activation with an over 4-fold decrease in activation of FXI compared with HD1. The effects of the DNA aptamers on thrombin's ability to bind to polyP and activate FXI in the presence of polyP were similar to those with dextran sulfate. Once again, our findings differ from those of Mutch *et al*., (2010) who showed that the high affinity exosite 1 ligand, the C-terminal dodecapeptide of hirudin, had no discernible effect on thrombin binding to immobilized polyP. Their data exclude a role for exosite 1 in polyP binding, while ours demonstrate that both exosites are capable of binding to polyP and are involved in the activation of FXI.

Our finding that the exosite 1 specific aptamer, HD1, attenuates α-thrombin's ability to bind to polyanions differs from our FXI activation by γ-thrombin. In both experiments, exosite 1 is compromised, but the binding of exosite 1 ligand attenuates FXI activation, while the proteolysis of exosite 1 does not. This could be a result of ligands binding to specific exosites inducing an allosteric effect on the other exosite. Our lab has previously shown that the two exosites have mutual influence over the other's ability to bind to ligand through

reciprocal, allosteric modulation of ligand affinity between the two exosites (Fredenburgh, Stafford and Weitz, 1997; Petrera *et al.*, 2009). The inhibition of HD1 on thrombin's ability to bind to polyanions and activate FXI suggests that the exosite 1 ligand may have allosteric influence on exosite 2, thereby preventing it from binding to a surface, or HD1 may be sterically hindering thrombin from binding to dextran sulfate.

To demonstrate that exosite 2 has influence on the activation of FXI in the presence of polyP in human plasma, thrombin generation assays were conducted. The thrombin generation was initiated by the various thrombins in the presence of polyP70. CTI was added to inhibit FXIIa. This activated the intrinsic pathway by activating FXI by thrombin and bypassing FXII. The addition of RA-thrombin to the plasma resulted in significantly less thrombin generation than the addition of the other variants, once again confirming the predominant role exosite 2 has in FXI activation. Q1-thrombin and γ-thrombin showed similar thrombin generation as α-thrombin suggesting that exosite 1 is expendable for the activation of FXI by thrombin.

To address the residual activation of FXI by RA-thrombin rMZa was used. rMZa is a stable form of meizothrombin and contains a masked exosite 2. RA-thrombin has three substitutions: Arg^{93} to Ala, Arg^{97} to Ala, and Arg^{101} to Ala, however, it still contains important residues capable of binding to polyanions such as Arg^{98}, Lys^{248}, Lys^{252}, Asp^{255} and Glu^{256} (Esmon and Lollar, 1996). Despite having a masked exosite 2, the adjusted concentration (40 nM) of rMZa was able

to achieve 57% activation of the FXI in the presence of polyP which is similar to the amount of activation of FXI by α-thrombin. This provided further evidence that exosite 2 may not be essential for FXI activation if exosite 1 is present. The shortcoming of this experiment is that the decreased catalytic activity of rMZa renders it difficult to determine the appropriate concentration of rMZa to use in the FXI activation assay to produce activation similar to that achieved with to α-thrombin. Increasing the concentration of rMZa from 10 nM to 40 nM in the activation may have overinflated rMZa's ability to activate FXI.

The discovery of the role of FXI in thrombosis has led to development of anticoagulants that target FXI. Our experiments have shown potential ways to attenuate thrombin-mediated FXI activation. We found that a polyanion is required as a cofactor in the activation to serve as a template for both thrombin and FXI to co-localize on. Enzymes that catalyze the hydrolytic cleavage of phosphodiester linkages in polyp and DNA/RNA backbone could be utilized to inhibit the activation. Recombinant Escherichia coli exopolyphosphatase (PPX) specifically degrades polyphosphate. PPX has been shown to abolish procoagulant platelet activity in a factor XII-dependent manner, thereby reducing fibrin accumulation and impeding thrombus formation in blood under flow (Labberton *et al.*, 2016). By degrading polyp, PPX could effectively disrupt the formation of ternary complexes between FXI, thrombin, and polyp, therefore, inhibiting the activation. Our aptamer data have shown that either exosite can be targeted to inhibit thrombin's ability to activate FXI. Exosite 1 may be the better

target to inhibit because of its procoagulant role. Exosite 1 is known to interact with fibrinogen, FV, FVIII, and PARs on platelets for their activation by thrombin (Huntington, 2005). By specifically inhibiting exosite 1 using an aptamer, thrombin's ability to undergo these procoagulant activations, in addition to FXI activation, will be inhibited. Furthermore, by leaving exosite 2 uninhibited, it can still allow thrombin to be regulated by antithrombin in a manner that is dependent on heparin.

We have successfully shown that thrombin-mediated FXI activation stimulated by polyanionic surfaces requires the formation of a ternary complex. We also showed that although both exosites are necessary to bind to polyanions, but only one exosite is necessary for the activation of FXI by thrombin. Of the two exosites, exosite 2 has the predominant role on FXI activation. This was hypothesized to be due to the role of exosite 2 in binding to other negatively charged polymers such as glycoaminoglycans. Although exosite 1 is important in thrombin's interaction with other factors such fibrinogen, FV, and FVIII, we found that it did not interact with FXI. The exosite 1 variants used established similar activation of FXI as α-thrombin. However, exosite 1 ligands did display the ability to inhibit FXI activation suggesting a possible steric or allosteric mechanism. Therefore, either exosite 1 or 2 may be targeted to attenuate FXI activation by thrombin.

Future Directions:

Having explored the role of thrombin exosites in the activation of FXI, the role of the apple domains of FXI have yet to be determined in this activation. This will provide insight on the involvement of FXI in the mechanism of the activation. Using FXI mutants that have mutations in the various domains of FXI, the chromogenic and binding assays conducted previously can be utilized to determine how FXI binds to surfaces and which domains are necessary for the activation.

The differences between our findings and Mutch et al., (2010) must be explored further. They found that mutations that targeted positively charged residues in exosite 2, attenuated thrombins ability to bind to polyP. However, all of the exosite 1 mutants that they used bound to polyP with affinities similar to that of α-thrombin. When conducting the binding assay with γ-thrombin, we found that the exosite 1 variant had minimal binding to the polyanions, suggesting that exosite 1 does have a role in interacting with the polyanions. In order to confirm our findings, we could repeat our experiments using other exosite 1 variants such as Q1-thrombin. The proteolysis of exosite 1 in γ-thrombin may have significantly decreased its molecular weight, thus, skewing the concentration we calculated to inject into the flow cells. SDS-PAGE can also be used to confirm the molecular weight of γ-thrombin. If the size of the protein has been significantly reduced, we could repeat the binding assays using a higher range of concentrations of γ-thrombin, to compensate for the decreased molecular weight.

In addition, the allosteric effects of the exosite 1 ligand on thrombin's ability to activate FXI and bind to surfaces will be explored using similar experiments conducted previously by Fredenburg *et al*., (1997). Performing titrations with hirudin-(54–65) and sF2 (ligands for exosites 1 and 2 of thrombin, respectively), we will explore whether exosite 1 ligands inhibit FXI sterically or through an allosteric mechanism.

The main objective of anticoagulant therapy is to attenuate thrombosis without affecting hemostasis. That is why FXI is a potential target for the development of anticoagulants. In order to completely inhibit FXI, all activation of FXI must be attenuated. Our studies provide further insight on the mechanism of how thrombin activates FXI and how to attenuate the activation by targeting the thrombin exosites.

References:

Bereczky, Zsuzsanna, Eva Katona, and László Muszbek. n.d. “Fibrin Stabilization (Factor XIII), Fibrin Structure and Thrombosis.” *Pathophysiology of Haemostasis and Thrombosis* 33 (5–6): 430–37.

Boon, G. Daniel. 1993. “An Overview of Hemostasis.” *Toxicologic Pathology* 21 (2): 170–79. https://doi.org/10.1177/019262339302100209.

Brill, A., T. A. Fuchs, A. S. Savchenko, G. M. Thomas, K. Martinod, S. F. De Meyer, A. A. Bhandari, And D. D. Wagner. 2012. “Neutrophil Extracellular Traps Promote Deep Vein Thrombosis in Mice.” *Journal of Thrombosis and Haemostasis* 10 (1): 136–44.

Cesarman-Maus, Gabriela, and Katherine A. Hajjar. 2005. “Molecular Mechanisms of Fibrinolysis.” *British Journal of Haematology* 129 (3): 307–21.

Choi, S. H., S. A. Smith, and J. H. Morrissey. 2011. “Polyphosphate Is a Cofactor for the Activation of Factor XI by Thrombin.” *Blood* 118 (26): 6963–70.

Choi, Sharon H., Stephanie A. Smith, and James H. Morrissey. 2011. “Polyphosphate Is a Cofactor for the Activation of Factor XI by Thrombin.” *Blood* 118 (26).

Chu, Arthur J, and Arthur J. 2011. “Tissue Factor, Blood Coagulation, and beyond: An Overview.” *International Journal of Inflammation* 2011: 367284.

Colman, R W, and A H Schmaier. 1997. “Contact System: A Vascular Biology Modulator with Anticoagulant, Profibrinolytic, Antiadhesive, and Proinflammatory Attributes.” *Blood* 90 (10): 3819–43.

Cosmi, B, J C Fredenburgh, J Rischke, J Hirsh, E Young, and J I Weitz. 1997. “Effect of Nonspecific Binding to Plasma Proteins on the Antithrombin Activities of Unfractionated Heparin, Low-Molecular-Weight Heparin, and Dermatan Sulfate.” *Circulation* 95 (1): 118–24.

Côté, H C, L Bajzar, W K Stevens, J A Samis, J Morser, R T MacGillivray, and M E Nesheim. 1997. “Functional Characterization of Recombinant Human Meizothrombin and Meizothrombin(DesF1). Thrombomodulin-Dependent Activation of Protein C and Thrombin-Activatable Fibrinolysis Inhibitor (TAFI), Platelet Aggregation, Antithrombin-III Inhibition.” *The Journal of Biological Chemistry* 272 (10): 6194–6200.

Davie, Earl, and John Kulman. 2006. “An Overview of the Structure and Function

of Thrombin." *Seminars in Thrombosis and Hemostasis* 32 (S 1): 003-015.

Davie, Earl W., and Oscar D. Ratnoff. 1964. "Waterfall Sequence for Intrinsic Blood Clotting." *Science* 145 (3638).

Esmon, Charles T. 2003. "The Protein C Pathway." *Chest* 124 (3 Suppl): 26S–32S.

Esmon, Charles T, and Pete Lollar. n.d. "Involvement of Thrombin Anion-Binding Exosites 1 and 2 in the Activation of Factor V and Factor VIII*."

Fredenburgh, J C, A R Stafford, and J I Weitz. 1997. "Evidence for Allosteric Linkage between Exosites 1 and 2 of Thrombin." *The Journal of Biological Chemistry* 272 (41): 25493–99.

Fuchs, TA, A Brill, … D Duerschmied - Proceedings of the, and undefined 2010. n.d. "Extracellular DNA Traps Promote Thrombosis." *National Acad Sciences*. Accessed December 4, 2018.

Furie, Bruce, and Barbara C Furie. 2005. "Thrombus Formation in Vivo." *The Journal of Clinical Investigation* 115 (12): 3355–62.

Gailani, D., and T. Renne. 2007. "Intrinsic Pathway of Coagulation and Arterial Thrombosis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 27 (12): 2507–13.

Gailani, David, and Thomas Renné. 2007. "Intrinsic Pathway of Coagulation and Arterial Thrombosis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 27 (12): 2507–13.

Gajsiewicz, Joshua M, Stephanie A Smith, and James H Morrissey. 2017. "Polyphosphate and RNA Differentially Modulate the Contact Pathway of Blood Clotting." *The Journal of Biological Chemistry* 292 (5): 1808–14.

Geng, Y., I. M. Verhamme, S. A. Smith, Q. Cheng, M. Sun, J. P. Sheehan, J. H. Morrissey, and D. Gailani. 2013. "Factor XI Anion-Binding Sites Are Required for Productive Interactions with Polyphosphate." *Journal of Thrombosis and Haemostasis* 11 (11): 2020–28.

Geng, Yipeng, Ingrid M Verhamme, Stephen B Smith, Mao-Fu Sun, Anton Matafonov, Qiufang Cheng, Stephanie A Smith, James H Morrissey, and David Gailani. 2013. "The Dimeric Structure of Factor XI and Zymogen Activation." *Blood* 121 (19): 3962–69.

Gilbert, Gary E, Valerie A Novakovic, Randal J Kaufman, Hongzhi Miao, and Steven W Pipe. 2012. “Conservative Mutations in the C2 Domains of Factor VIII and Factor V Alter Phospholipid Binding and Cofactor Activity.” *Blood* 120 (9): 1923–32.

Han, Kyu Yeon, Bok Sil Hong, Yae Jin Yoon, Chang Min Yoon, Yoon-Keun Kim, Young-Guen Kwon, and Yong Song Gho. 2007. “Polyphosphate Blocks Tumour Metastasis via Anti-Angiogenic Activity.” *The Biochemical Journal* 406 (1): 49–55.

Henriksen, Ruth Ann, and Kenneth G. Mann. 1988. “Identification of the Primary Structural Defect in the Dysthrombin Thrombin Quick I: Substitution of Cysteine for Arginine-382.” *Biochemistry* 27 (26): 9160–65.

Huntington, J. A. 2005. “Molecular Recognition Mechanisms of Thrombin.” *Journal of Thrombosis and Haemostasis* 3 (8): 1861–72.

Ivanov, Ivan, Ruhama Shakhawat, Mao-fu Sun, S. Kent Dickeson, Cristina Puy, Owen McCarty, Andras Gruber, Anton Matafonov, and David Gailani. 2017. “Nucleic Acids as Cofactors for Factor XI and Prekallikrein Activation: Different Roles for High-Molecular-Weight Kininogen.” *Thrombosis and Haemostasis* 117 (04): 671–81.

Jaffer, I. H., J. C. Fredenburgh, J. Hirsh, and J. I. Weitz. 2015. “Medical Device-Induced Thrombosis: What Causes It and How Can We Prevent It?” *Journal of Thrombosis and Haemostasis* 13 (June): S72–81.

Kannemeier, Christian, Aya Shibamiya, Fumie Nakazawa, Heidi Trusheim, Clemens Ruppert, Philipp Markart, Yutong Song, et al. 2007. “Extracellular RNA Constitutes a Natural Procoagulant Cofactor in Blood Coagulation.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (15): 6388–93.

Kawazoe, Y., T. Shiba, R. Nakamura, A. Mizuno, K. Tsutsumi, T. Uematsu, M. Yamaoka, M. Shindoh, and T. Kohgo. 2004. “Induction of Calcification in MC3T3-E1 Cells by Inorganic Polyphosphate.” *Journal of Dental Research* 83 (8): 613–18.

Kirchhofer, Daniel, Arabinda Guha, Yale Nemerson, William H. Konigsberg, Francis Vilbois, Christiane Chène, David W. Banner, and Allan D’Arcy. 1995. “Activation of Blood Coagulation Factor VIIa with Cleaved Tissue Factor Extracellular Domain and Crystallization of the Active Complex.” *Proteins: Structure, Function, and Genetics* 22 (4): 419–25.

Kolyadko, V. N., V. A. Korneeva, F. I. Ataullakhanov, and M. A. Panteleev. 2014. "Molecular Mechanisms of Thrombosis. Fundamental and Applied Aspects of the Contact Activation." *Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology* 8 (4): 279–89.

Kretz, Colin A, Alan R Stafford, James C Fredenburgh, and Jeffrey I Weitz. 2015. "HD1, a Thrombin-Directed Aptamer, Binds Exosite 1 on Prothrombin with High Affinity and Inhibits Its Activation by Prothrombinase." *The Journal of Biological Chemistry* 290 (8): 4813.

Krishnaswamys, Sriram. 1990. "Prothrombinase Complex Assembly CONTRIBUTIONS OF PROTEIN-PROTEIN AND PROTEIN-MEMBRANE INTERACTIONS TOWARD COMPLEX FORMATION*" 265 (7): 3708–18.

Labberton, Linda, Ellinor Kenne, Andy T. Long, Katrin F. Nickel, Antonio Di Gennaro, Rachel A. Rigg, James S. Hernandez, et al. 2016. "Neutralizing Blood-Borne Polyphosphate in Vivo Provides Safe Thromboprotection." *Nature Communications* 7 (1): 12616.

Lwaleed, Bashir A, and Paul S Bass. 2006. "Tissue Factor Pathway Inhibitor: Structure, Biology and Involvement in Disease." *The Journal of Pathology* 208 (3): 327–39. https://doi.org/10.1002/path.1871.

Mackman, N. 2004. "Role of Tissue Factor in Hemostasis, Thrombosis, and Vascular Development." *Arteriosclerosis, Thrombosis, and Vascular Biology* 24 (6): 1015–22.

Mackman, Nigel. 2009. "The Role of Tissue Factor and Factor VIIa in Hemostasis." *Anesthesia and Analgesia* 108 (5): 1447–52.

MacQuarrie, J. L., A. R. Stafford, J. W. Yau, B. A. Leslie, T. T. Vu, J. C. Fredenburgh, and J. I. Weitz. 2011. "Histidine-Rich Glycoprotein Binds Factor XIIa with High Affinity and Inhibits Contact-Initiated Coagulation." *Blood* 117 (15): 4134–41.

Mann, Kenneth G., Saulius Butenas, and Kathleen Brummel. 2003. "The Dynamics of Thrombin Formation." *Arteriosclerosis, Thrombosis, and Vascular Biology* 23 (1).

Masliah-Planchon, Julien, Luc Darnige, and Sylvia Bellucci. 2013. "Molecular Determinants of Platelet Delta Storage Pool Deficiencies: An Update." *British Journal of Haematology* 160 (1): 5–11.

Matafonov, Anton, Suryakala Sarilla, Mao-fu Sun, John P Sheehan, Vladimir

Serebrov, Ingrid M Verhamme, and David Gailani. 2011. “Activation of Factor XI by Products of Prothrombin Activation.” *Blood* 118 (2): 437–45.

Monroe, Dougald M., Maureane Hoffman, and Harold R. Roberts. 2002. “Platelets and Thrombin Generation.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 22 (9).

Montfoort, Maurits L van, and Joost C M Meijers. 2014. “Recent Insights into the Role of the Contact Pathway in Thrombo-Inflammatory Disorders.” *Hematology. American Society of Hematology. Education Program* 2014 (1): 60–65.

Muhl, Lars, Sebastian P. Galuska, Katariina Öörni, Laura Hernández-Ruiz, Luminita-Cornelia Andrei-Selmer, Rudolf Geyer, Klaus T. Preissner, Felix A. Ruiz, Petri T. Kovanen, and Sandip M. Kanse. 2009. “High Negative Charge-to-Size Ratio in Polyphosphates and Heparin Regulates Factor VII-Activating Protease.” *FEBS Journal* 276 (17): 4828–39.

Müller, Felicitas, David Gailani, and Thomas Renné. 2011. “Factor XI and XII as Antithrombotic Targets.” *Current Opinion in Hematology* 18 (5): 349–55.

Müller, J., D. Freitag, G. Mayer, And B. Pötzsch. 2008. “Anticoagulant Characteristics of HD1-22, a Bivalent Aptamer That Specifically Inhibits Thrombin and Prothrombinase.” *Journal of Thrombosis and Haemostasis* 6 (12): 2105–12.

Mutch, N. J., T. Myles, L. L. K. Leung, And J. H. Morrissey. 2010. “Polyphosphate Binds with High Affinity to Exosite II of Thrombin.” *Journal of Thrombosis and Haemostasis* 8 (3): 548–55.

Naito, K, and K Fujikawa. 1991. “Activation of Human Blood Coagulation Factor XI Independent of Factor XII. Factor XI Is Activated by Thrombin and Factor XIa in the Presence of Negatively Charged Surfaces.” *The Journal of Biological Chemistry* 266 (12): 7353–58.

Owens, A. P., and N. Mackman. 2010. “Tissue Factor and Thrombosis: The Clot Starts Here.” *Thrombosis and Haemostasis* 104 (3): 432–39.

Petrera, Nicolas S, Alan R Stafford, Beverly A Leslie, Colin A Kretz, James C Fredenburgh, and Jeffrey I Weitz. 2009. “Long Range Communication between Exosites 1 and 2 Modulates Thrombin Function.” *The Journal of Biological Chemistry* 284 (38): 25620–29.

Renné, T., C. Oschatz, S. Seifert, F. Müller, J. Antovic, M. Karlman, And P. M.

Benz. 2009. “Factor XI Deficiency in Animal Models.” *Journal of Thrombosis and Haemostasis* 7 (July): 79–83.

Renné, Thomas, and David Gailani. 2007. “Role of Factor XII in Hemostasis and Thrombosis: Clinical Implications.” *Expert Review of Cardiovascular Therapy* 5 (4): 733–41.

Rezaie, Alireza R. 1999. “Role of Exosites 1 and 2 in Thrombin Reaction with Plasminogen Activator Inhibitor-1 in the Absence and Presence of Cofactors.”

Richards, E. M., M. M. Makris, P. Cooper, And F. E. Preston. 1997. “In Vivo Coagulation Activation Following Infusion of Highly Purified Factor XI Concentrate.” *British Journal of Haematology* 96 (2): 298–297.

Salomon, O., D. M. Steinberg, N. Koren-Morag, D. Tanne, and U. Seligsohn. 2008. “Reduced Incidence of Ischemic Stroke in Patients with Severe Factor XI Deficiency.” *Blood* 111 (8): 4113–17.

Salomon, O., D. M. Steinberg, M. Zucker, D. Varon, A. Zivelin, and U. Seligsohn. 2010. “Patients with Severe Factor XI Deficiency Have a Reduced Incidence of Deep-Vein Thrombosis.” *Thrombosis and Haemostasis* 105 (2): 269–73.

Schmaier, A. H. 2016. “The Contact Activation and Kallikrein/Kinin Systems: Pathophysiologic and Physiologic Activities.” *Journal of Thrombosis and Haemostasis* 14 (1): 28–39.

Seligsohn, U. 2009. “Factor XI Deficiency in Humans.” *Journal of Thrombosis and Haemostasis* 7 (July): 84–87.

Smiles, A M, E M Macy, P A Sakkinen, E G Bovill, K G Mann, and R P Tracy. 1998. “Variability in Plasma Prothrombin Concentration: Implications for Use in Epidemiology.” *Blood Coagulation & Fibrinolysis : An International Journal in Haemostasis and Thrombosis* 9 (6): 525–31.

Smith, S. A., S. H. Choi, R. Davis-Harrison, J. Huyck, J. Boettcher, C. M. Rienstra, J. H. Morrissey, and James H Morrissey. 2010. “Polyphosphate Exerts Differential Effects on Blood Clotting, Depending on Polymer Size.” *Blood* 116 (20): 4353–59.

Smith, S. A., N. J. Mutch, D. Baskar, P. Rohloff, R. Docampo, and J. H. Morrissey. 2006. “Polyphosphate Modulates Blood Coagulation and Fibrinolysis.” *Proceedings of the National Academy of Sciences* 103 (4): 903–8.

Smith, Stephanie A, Sharon H Choi, Rebecca Davis-Harrison, Jillian Huyck, John Boettcher, Chad M Rienstra, Chad M Reinstra, and James H Morrissey. 2010. “Polyphosphate Exerts Differential Effects on Blood Clotting, Depending on Polymer Size.” *Blood* 116 (20): 4353–59.

Vaart, Maniesh van der, and Piet J. Pretorius. 2008. “Circulating DNA.” *Annals of the New York Academy of Sciences* 1137 (1): 18–26.

Walker, C P R, and D Royston. 2002. “Thrombin Generation and Its Inhibition: A Review of the Scientific Basis and Mechanism of Action of Anticoagulant Therapies.” *British Journal of Anaesthesia* 88 (6): 848–63.

Wang, Lihong, Cresson D Fraley, Jesika Faridi, Arthur Kornberg, and Richard A Roth. 2003. “Inorganic Polyphosphate Stimulates Mammalian TOR, a Kinase Involved in the Proliferation of Mammary Cancer Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (20): 11249–54.

Warkentin, T E. 1995. “Hemostasis and Atherosclerosis.” *The Canadian Journal of Cardiology* 11 Suppl C (May): 29C–34C.

Witting, J I, T M Miller, and J W Fenton. 1987. “Human Alpha- and Gamma-Thrombin Specificity with Tripeptide p-Nitroanalide Substrates under Physiologically Relevant Conditions.” *Thrombosis Research* 46 (4): 567–74.

Wolberg, Alisa S., and Robert A. Campbell. 2008. “Thrombin Generation, Fibrin Clot Formation and Hemostasis.” *Transfusion and Apheresis Science* 38 (1): 15–23.

Wuillemin, WA, M Minnema, JC Meijers, D Roem, AJ Eerenberg, JH Nuijens, H ten Cate, and CE Hack. 1995. “Inactivation of Factor XIa in Human Plasma Assessed by Measuring Factor XIa-Protease Inhibitor Complexes: Major Role for C1-Inhibitor.” *Blood* 85 (6).

Yau, Jonathan W., Alan R. Stafford, Peng Liao, James C. Fredenburgh, Robin Roberts, John L. Brash, and Jeffrey I. Weitz. 2012. “Corn Trypsin Inhibitor Coating Attenuates the Prothrombotic Properties of Catheters in Vitro and in Vivo.” *Acta Biomaterialia* 8 (11): 4092–4100.

Yun, Thomas H, Frank A Baglia, Timothy Myles, Duraiswamy Navaneetham, José A López, Peter N Walsh, and Lawrence L K Leung. 2003. “Thrombin Activation of Factor XI on Activated Platelets Requires the Interaction of Factor XI and Platelet Glycoprotein Ib Alpha with Thrombin Anion-Binding

Exosites I and II, Respectively." *The Journal of Biological Chemistry* 278 (48): 48112–19.

Zeerleder, Sacha, Thomas Mauron, Bernhard Lämmle, and Walter A Wuillemin. 2002. "Effect of Low-Molecular Weight Dextran Sulfate on Coagulation and Platelet Function Tests." *Thrombosis Research* 105 (5): 441–46.

Zhang, Hong, Ester C. Löwenberg, Jeffrey R. Crosby, A. Robert MacLeod, Chenguang Zhao, Dacao Gao, Chris Black, et al. 2010. "Inhibition of the Intrinsic Coagulation Pathway Factor XI by Antisense Oligonucleotides: A Novel Antithrombotic Strategy with Lowered Bleeding Risk." *Blood* 116 (22).

Zhong, C., L. Zhang, L. Chen, L. Deng, and R. Li. 2017. "Coagulation Factor XI Vaccination: An Alternative Strategy to Prevent Thrombosis." *Journal of Thrombosis and Haemostasis* 15 (1): 122–30.