IDENTIFICATION OF A FVA BINDING SITE ON PROTHROMBIN

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Abstract

Thrombin is generated by the prothrombinase complex consisting of the enzyme factor (F) Xa, cofactor FVa, a negatively charged lipid surface and calcium ions. While FXa alone generates thrombin, the reaction rate increases by 5 orders of magnitude upon formation of prothrombinase. Previous work indicated that the kringle 2 domain of prothrombin is involved in binding FVa. Based on unpublished data that identified seven potential residues of the kringle 2 domain as the binding site for FVa (S160, Q177, Q179, R181, L182, V184 and T185), a variant with the latter six residues simultaneously mutated was generated (Q177A, Q179A, R181A, L182T, V184T, and T185A; PT6). Activation kinetics and thrombin generation of PT6 were compared with wild-type prothrombin (WTPT). Prothrombin activation along with DAPA showed a significant decrease in catalytic efficiency both in the presence and absence of FVa, but not without the membrane surface. Thrombin-activated platelets were found to have a similar effect to synthetic membrane for overall catalytic efficiency. SDS-PAGE analyses revealed a 40% decrease in meizothrombin potential compared with the wild-type, suggesting a decrease in a FVa-specific prothrombin activation by cleavage at Arg320. In the absence of FVa, initial rates of WTPT and PT6 consumption were similar while the overall thrombin generation was 48% lower with PT6, suggesting that the initial cleavage at Arg271 was unaltered while the secondary cleavage at Arg320 was reduced. Total thrombin generation was significantly decreased in PT6 compared with WTPT in a FVa-dependent manner, which is in contrast with the activation kinetics data. This suggests that simplified initial rate analyses may be insufficient in investigating a complicated multi-

component enzyme such as prothrombinase. Taken together, the six residues of kringle 2 domain are important for efficient cleavage at Arg320 both in a FVa-dependent and FVa-independent manner.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACD – acid-citrate-dextrose buffer

BHK – baby hamster kidney

$CaCl_2$ – calcium chloride

Da - Daltons

DAPA – dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide

DMEM – Dulbecco's modified eagle's medium

DOAC – direct oral anticoagulant

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

F – factor

Fgn – fibrinogen

Fn – fibrin

GLA – γ-carboxyglutamic acid

HEPES – N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid

HBS – HEPES buffered saline

HBST – HEPES buffered saline + 0.01% Tween 80

K_{cat} – turnover number of an enzymatic reaction

K_d – dissociation constant

K_i – inhibitory constant

K_m – Michaelis-Menten constant

Lnk – linker region

mIIa – meizothrombin

MTH – modified Tyode's buffer

F1.2 – fragment 1.2

F1.2-A – fragment 1.2:A-chain

PCPS – small unilamellar phospholipid vesicles composed of 75% phosphatidylcholine and 25% phosphatidylserine

Pre1 – prethrombin-1

Pre2 – prethrombin-2

PT6 – prothrombin mutant with residues 177, 179, 181 and 185 substituted with alanine and residues 182 and 184 substituted with threonine

RFU – relative fluorescence units

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS – tris buffered saline

TF – tissue factor

Tris – Tris[hydroxymethyl]aminomethane

NET – neutrophil extracellular trap

VTE – venous thromboembolism

WTPT – wild-type prothrombin

Declaration of Academic Achievement

Alexander Friedmann contributed to conception and design of studies, performed all experiments, analyzed and interpreted the data, and performed statistical analyses.

Dr. Paul Y. Kim contributed to conception and design of studies, obtained funding to support the studies, and critically reviewed and obtained results.

I. Introduction

Hemostasis

Upon vascular damage, bleeding is stopped by the formation of a stable blood clot, a process referred to as hemostasis. This is a tightly regulated process as too much clotting leads to pathological thrombosis and insufficient clotting leads to bleeding diatheses. Heart disease and stroke are caused by arterial thrombosis. In 2010, 7 million deaths occurred from ischemic heart disease and 5.9 million deaths occurred from stroke, accounting for 1 in 4 deaths worldwide. Furthermore, ischemic heart disease was the leading cause of disability-adjusted life years, a measure of mortality and morbidity (Raskob et al. 2014). Venous thromboembolism (VTE) is a serious and potentially life-threatening condition where thrombosis occurs in the veins. When this occurs in the deep veins of the legs, it is called deep vein thrombosis. These thrombi may fragment and mobilize in the circulation and can become lodged in the smaller veins of the lungs, causing pulmonary embolism. Accurate measurement of total deaths caused by VTE is more difficult, but it is estimated that 300,000 deaths annually can be attributed to VTE in the United States alone (Raskob et al. 2014).

Traditional therapies such as warfarin and heparin have been used for many years to treat these conditions. These drugs, however have many food/medication interactions and require regular physician follow-up leading to increased healthcare costs. Direct oral anticoagulants (DOACs) are a newer class of drugs which tend to have less frequent major bleeding and better prognosis for stroke prevention in atrial fibrillation and treatment of VTE, while having less food interaction and requiring less frequent

therapeutic level monitoring than earlier drugs (Steiner, Weitz, and Veltkamp 2017; Gunasekaran and Parashara 2015). Unfortunately, kidney disease is a common comorbidity for patients requiring DOACs and some of these drugs rely heavily on renal clearance. Bleeding is also still a serious consideration for prescribing physicians (Xiong et al. 2015). Additionally, there is a need for rapid and reliable tests to measure DOAC levels in clinical settings and current methods are unreliable or not universally available (Steiner, Weitz, and Veltkamp 2017). Therefore, further understanding of the mechanisms of thrombosis is key to opening doors to further improvements in its treatment and prevention.

Coagulation Cascade

The coagulation cascade is the main mechanism driving hemostasis. Hemostasis has two general phases: primary hemostasis and secondary hemostasis. Primary hemostasis is the initial response to vascular injury. Exposure of subendothelial cells and extracellular matrix (ECM) components to circulating blood stimulates platelet adhesion, activation and aggregation, inducing the formation of a loose platelet plug (Hugenholtz, Porte, and Lisman 2009). Secondary hemostasis occurs when a series of proteolytic reactions, often referred to as the coagulation cascade, leads to fibrin (Fn) generation (Y. Wang et al. 2014). Thrombin is a key enzyme in this process as it catalyzes the cleavage of soluble fibrinogen (Fgn) to an insoluble Fn mesh. Thrombin generation also leads to cross-linking of Fn polymers, causing increased clot stability (Versteeg et al. 2013). The coagulation cascade can be divided into two general pathways: the intrinsic pathway and the extrinsic pathway (Figure 1).

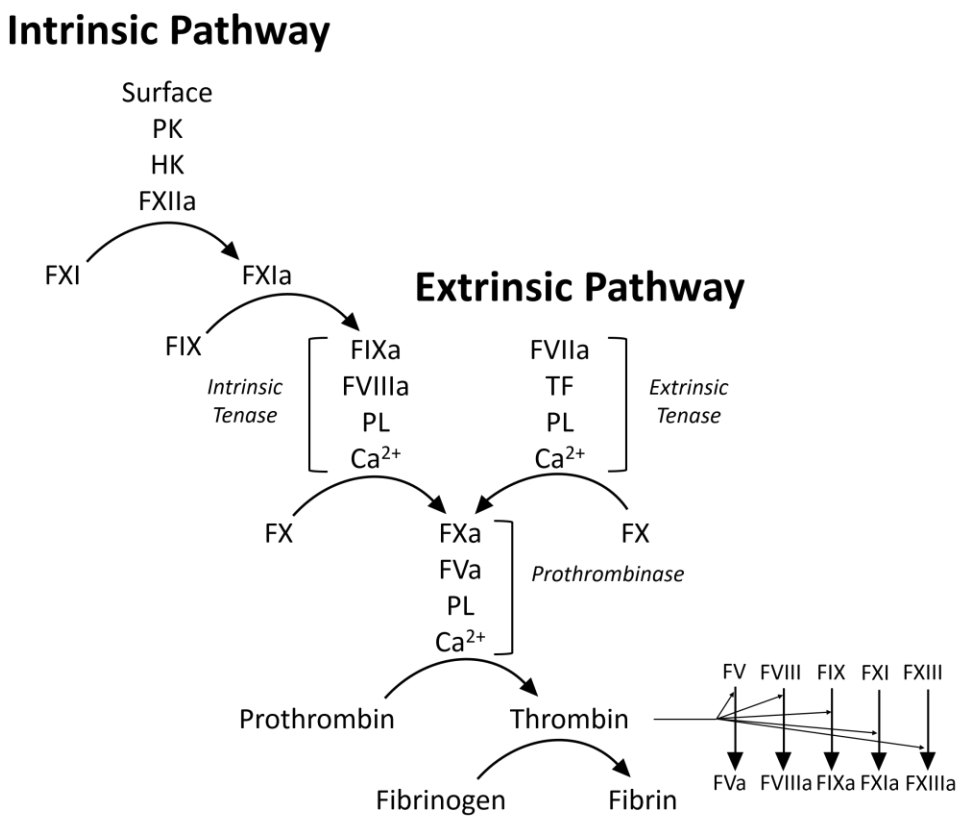

Figure 1 - The coagulation cascade.

The coagulation cascade includes both the extrinsic and intrinsic pathways. In the extrinsic pathway, exposed TF combines with FVIIa on a membrane surface in the presence of calcium ions to form the extrinsic tenase complex. The intrinsic pathway is initiated by the presence of negatively charged/foreign surfaces or thrombin-mediated activation of FXI, resulting in formation of intrinsic tenase. Extrinsic and intrinsic tenase complexes activate prothrombin to thrombin, which then cleaves soluble Fgn to Fn. Thrombin feeds back to up-regulate its own production through cleavage of FV, FVIII, FIX, FXI and FXIII, as shown. Additional cross-talk between pathways has been omitted for the sake of simplicity.

Coagulation in secondary hemostasis begins in the extrinsic pathway. The extrinsic pathway of blood coagulation is initiated when tissue factor (TF) is exposed to circulating blood. TF is a transmembrane glycoprotein expressed on subendothelial cells such as fibroblasts and smooth muscle cells. Normally these cells are not exposed to circulating blood, but are exposed upon damage to the vascular endothelium. TF expression is upregulated upon vascular damage, exposure to clotting factors and inflammatory cytokines (Butenas and Mann 2002; Peña, Arderiu, and Badimon 2012). TF acts as a cofactor for circulating factor VIIa (FVIIa), which both triggers coagulation and causes autoactivation of factor VII (FVII) (Neuenschwander, Fiore, and Morrissey 1993). FVIIa has very little catalytic activity in the absence of TF, so these processes are TF-dependent (Petrovan and Ruf 2001). TF then combines with FVIIa to form the TF-FVIIa complex on the membrane surface of endothelial cells, platelets or microparticles to form the extrinsic tenase complex. This cleaves factor X (FX) to active factor X (FXa), leading to subsequent thrombin and Fn generation (Butenas and Mann 2002). This is referred to as the initiation phase of coagulation (Hoffman and Monroe 2001).

The intrinsic pathway, also referred to as the contact pathway, can be initiated by collagen, polyphosphates, neutrophil extracellular traps (NETs) and other negatively charged surfaces (Versteeg et al. 2013; Renné and Gailani 2007). These molecules activate the factor XII (FXII) zymogen to active FXII (FXIIa) in small quantities. FXIIa cleaves prekallikrein to active kallikrein which can further activate anion-bound FXII. FXIIa converts FXI to active FXIa, which then cleaves FIX to FIXa. The protease FIXa complexes with its thrombin-cleaved cofactor FVIIIa and the FX substrate (Versteeg et

al. 2013) forming a complex referred to as intrinsic factor tenase, cleaving FX to FXa (Butenas and Mann 2002). At this step of FX activation, the intrinsic pathway integrates with the extrinsic pathway and enters the common pathway.

In the common pathway FX becomes activated to FXa by the intrinsic or extrinsic tenase complexes. FXa can then associate with prothrombin, active factor V (FVa), Ca^{2+} and a negatively charged phospholipid membrane surface, to form the prothrombinase complex. This is the enzymatic complex responsible for activating the majority of prothrombin to thrombin (Versteeg et al. 2013). Thrombin can then cleave soluble Fgn to insoluble Fn, forming a mesh which acts to stabilize the platelet plug (Hugenholtz, Porte, and Lisman 2009; Butenas and Mann 2002). Thrombin also cleaves FXIII to FXIIIa which cross-links fibrin polymers, further stabilizing the blood clot (Versteeg et al. 2013).

During the initiation phase the TF-FVIIa complex generates picomolar quantities of FXa and FIXa which generates a very small amount of thrombin. This initial amount of thrombin generation begins the amplification phase, where thrombin upregulates its own production by activating FXI, FIX and helping activate FV and FVIII (Butenas and Mann 2002). This causes a 10^5-10^6-fold enhancement in thrombin generation (Butenas et al. 2007) by increasing the proteolytic activity in both the intrinsic and common pathways. Thrombin also activates platelets, causing the release of procoagulant molecules, and allowing platelet membranes to serve as binding sites for procoagulant proteins (Butenas et al. 2007).

Thrombin is clearly a central enzyme in the coagulation cascade. It is involved in the initiation, amplification and stabilization of blood clots. Traditional anticoagulant drugs such as warfarin and heparins, and NOACs work to ultimately prevent the production and activity of thrombin. Aside from its major role in coagulation, thrombin is a key effector in many physiological pathways including embryonic development, acute and chronic inflammation, atherosclerosis, neuropathology and tumour biology (Danckwardt, Hentze, and Kulozik 2013). Therefore, understanding the mechanisms behind thrombin generation is crucial for advancing medicine in numerous fields.

Prothrombinase Complex

FXa is the catalytic component of the prothrombinase complex. While FXa alone can activate prothrombin to thrombin, formation of the prothrombinase complex with its cofactor FVa and calcium ion on a negatively charged membrane surface enhances the catalytic efficiency of thrombin generation by ~5 orders of magnitude (Qureshi et al. 2009). The phospholipid membrane decreases the apparent K_m of FXa for prothrombin by approximately 100-fold by binding all components of the complex, bringing the enzyme, cofactor and substrate closer in proximity. FVa serves as a cofactor for FXa, increasing the k_{cat} for prothrombin activation by approximately 3000-fold. FVa has been shown to bind to the phospholipid membrane with a 10-fold higher affinity than FXa and prothrombin (Nesheim, Kettner, et al. 1981) and binds the negatively charged phospholipid membrane in a calcium-independent fashion (Koppaka et al. 1997). Within the prothrombinase complex, FXa and membrane-bound FVa are found in a 1:1

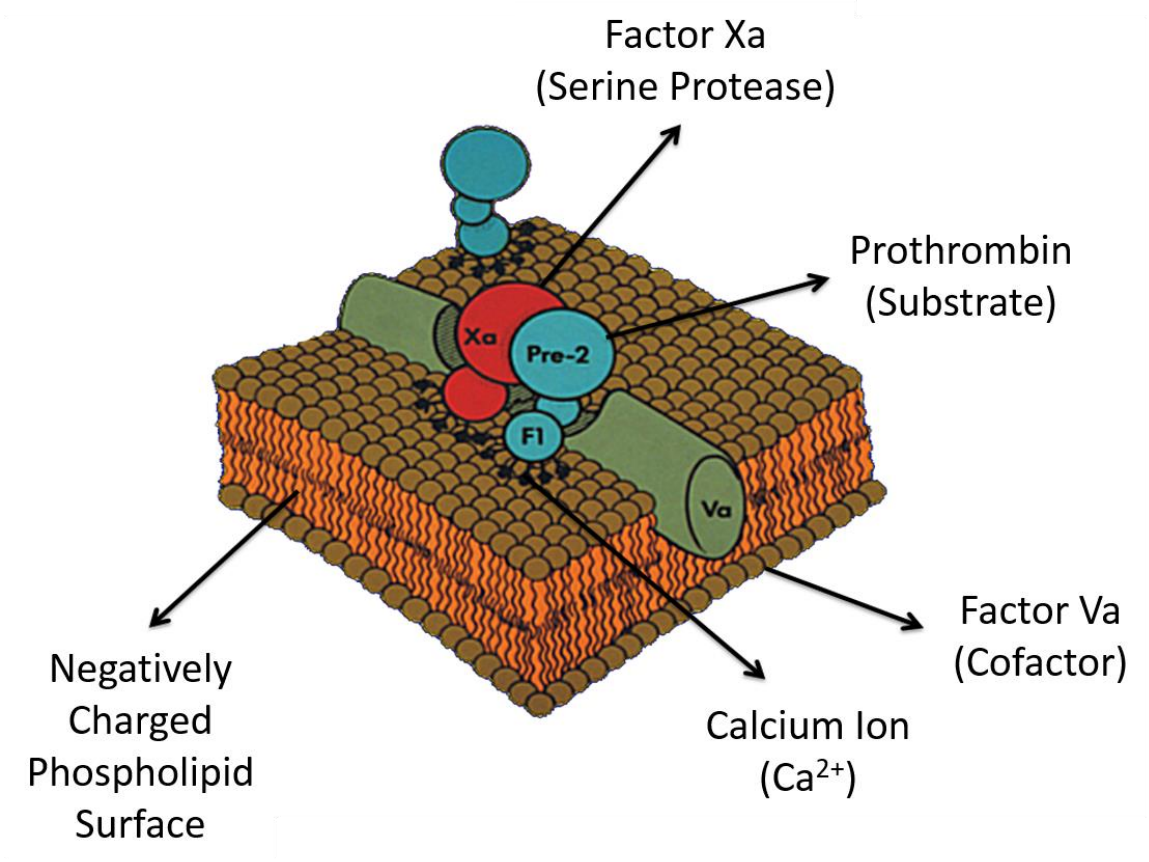

Figure 2 - Prothrombinase complex.

Prothrombinase consists of the prothrombin substrate, the serine protease FXa and the cofactor FVa assembling on a negatively charged membrane surface in the presence of calcium ions. Phospholipids bind prothrombin, FXa and FVa, bringing them closer in proximity to each other. FVa induces a conformational change in prothrombin, making it a better substrate for catalysis by FXa. Figure is adapted from Nesheim et al, 1980 (Nesheim et al. 1980).

stoichiometric ratio and have a high binding affinity, with a K_d value of 7.0×10^{-10} M. These binding affinities were obtained from experiments using synthetic phospholipid vesicles, meant to be representative of the activated cell surface.

This enzymatic complex has been shown to assemble in the absence of prothrombin as the substrate (Nesheim, Kettner, et al. 1981; Brufatto and Nesheim 2003). Due to these relative rates of prothrombin cleavage, the fully formed prothrombinase complex, as opposed to FXa alone, is understood to be the physiologically relevant activator of prothrombin (Mann, Jenny, and Krishnaswamy 1988; Krishnaswamy 2013) and therefore developing an in-depth understanding of this enzymatic complex is of utmost importance.

Prothrombin

Prothrombin is a single-chain glycoprotein which is synthesized in the liver before being released into circulation. Its gene is located on chromosome 11 (Lancellotti, Basso, and De Cristofaro 2013). It is activated by FXa to form the central clotting enzyme, thrombin. Prothrombin has a molecular mass of 72,000 Daltons (Da) and consists of 4 major domains: γ-carboxyglutamic acid (GLA) domain, kringle 1, kringle 2 and the catalytic domain (Degen and Davie 1987). These 4 domains can be organized into 3 structural regions. Fragment 1 contains the GLA domain (spanning residues 1-40), and kringle 1 domain (spanning residues 41-155) while Fragment 2 encompasses residues 156 through 271, which includes the kringle 2 domain. The catalytic domain spans residues 272-579, which contains the thrombin A-chain and the B-chain

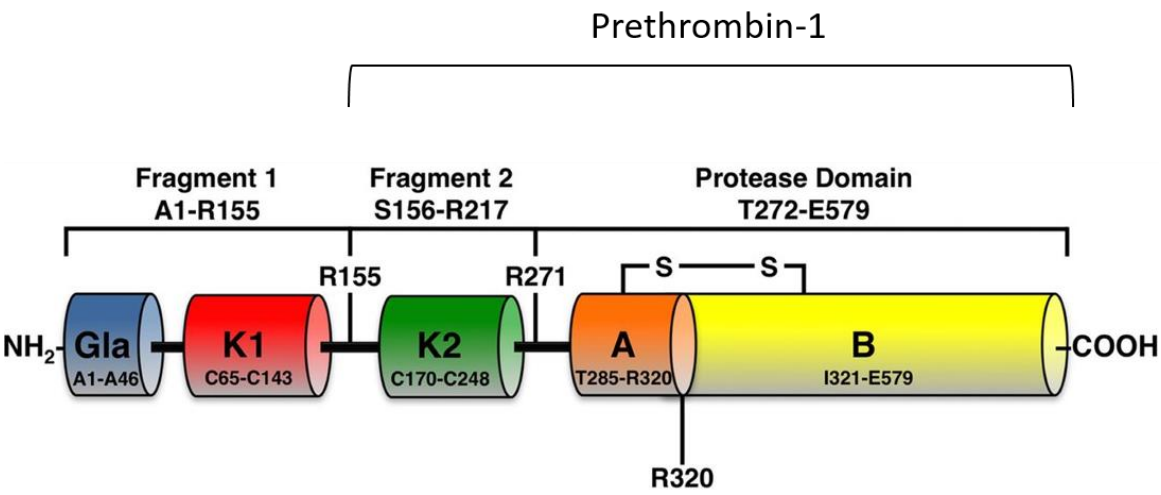

Figure 3 - Domain organization of prothrombin.

Prothrombin consists of four domains: γ-carboxyglutamic acid (GLA) domain, kringle 1, kringle 2 (labelled as K1 and K2 respectively) and the protease domain, moving from N-terminus to C terminus. Fragment 1 consists of GLA and kringle 1 and fragment 2 consists of kringle 2. Prothrombin is activated by cleavage at residues Arg^{271} and Arg^{320}, releasing fragment 1.2 and thrombin. Cleavage at Arg^{155} releases fragment 1 and generates prethrombin-1. Linker regions Lnk1, Lnk2 and Lnk3 are represented as black lines. Figure adapted from Pozzi et al, 2013 (Pozzi et al. 2013).

(Deguchi et al. 1997; Haynes et al. 2012; Pozzi et al. 2014). Three linker regions have been characterized as well, denoted Lnk1, Lnk2 and Lnk3. Lnk1 (residues 47-64) connects the GLA domain to kringle 1, Lnk2 (residues 144-169) connects kringle 1 to kringle 2 and Lnk3 (residues 249-284) connects kringle 2 to the A chain (Pozzi et al. 2014). This domain organization can be seen in Figure 3.

The GLA domain of prothrombin plays a critical role in its Ca^{2+}-dependent membrane binding. Prothrombin has 10 glutamic acid residues, which are modified to γ-carboxyglutamic acid upon vitamin K-dependent post-translational modification in the liver. These residues tightly bind calcium, altering the conformation of the GLA domain from a disordered structure, to a more ordered structure (Soriano-Garcia et al. 1989). This calcium-induced structural change allows prothrombin to bind phosphatidylserine-containing membranes, co-localizing the protein with FXa and FVa (Dombrose et al. 1979). The extent of γ-carboxylation of these residues has been shown to be crucial for overall prothrombin function. There appears to be a threshold-like effect of GLA-deficiency on the kinetics of prothrombin activation, where insufficient γ-carboxylation (7 or less GLA residues) results in a substantial increase in K_m values (Malhotra, Nesheim, and Mann 1985).

The catalytic domain of prothrombin contains proexosites-1 and 2 (termed exosites-1 and 2 on thrombin), which are highly rich in basic residues. These regions have shown to be important in determining substrate specificity for thrombin activity. For example, Fgn and protease-activated receptor-1 must interact with exosite-1 on thrombin to become functional (Wu et al. 1991). Cofactors FV and FVIII must also interact with

exosite-1 on thrombin in order to become activated (Myles et al. 2001; Myles, Yun, and Leung 2002; Lollar 1996), showing that this site is important for amplification of coagulation. It has also been reported that proexosite-1 may be important for the interaction of prothrombin with FXa and FVa in the prothrombinase complex (Chen, Yang, and Rezaie 2003).

Factor Xa

FX is the zymogen for FXa. It is a vitamin-K dependent glycoprotein synthesized in the liver and has a molecular mass of approximately 58,900 Da, containing 15% carbohydrate (Di Scipio et al. 1977). Its gene is located on chromosome 13 (Scambler and Williamson 1985). FX has a plasma concentration of 170 nM and a plasma half-life of 40 h (Walsh and Ahmad 2002). The zymogen takes the form of a two-chain protein, having both a light chain (17,000 Da) and a heavy chain (49,000 Da) linked by a single disulfide bond. The light chain of FXa, located on its N-terminus, contains a GLA domain and two epidermal growth factor (EGF)-like domains. The GLA domain contains 11 GLA residues, important for Ca^{2+}-dependent binding which causes a conformational change allowing for membrane binding (Miao et al. 1992). The heavy chain contains the activation peptide and the C-terminal serine protease domain, responsible for catalytic activity (Miao et al. 1992; Chevreux et al. 2015; Steen et al. 2008). This structure bears resemblance to that of prothrombin, having a high degree of identity in its DNA coding sequence, however with EGF domains in place of kringle domains (Leytus et al. 1984).

FX is cleaved by either the extrinsic or intrinsic tenase complex to form FXa. A tripeptide containing residues 140-142 is removed upon cleavage, separating the heavy

and light chain. Full activation of FX occurs upon cleavage at Arg^{52}, liberating the 52 amino acid activation peptide from the heavy chain (Hertzberg 1994). The GLA domain of FX has shown to be important during its activation by the extrinsic tenase complex (Pinotti et al. 2002; Rudolph et al. 1996) and Arg^{386} during activation by the intrinsic tenase complex (Baroni et al. 2015; Vanden Hoek et al. 2012). FXa is prone to inactivation by circulating antithrombin (AT) and tissue factor pathway inhibitor (TFPI). Inactivation by AT is rapid, but requires heparin as a cofactor. Inactivation by TFPI does not require any cofactor (Bianchini et al. 2002).

Factor Va

FV is the zymogen for FVa and has a molecular mass of 330,000 Da. Its gene is located on chromosome 1 (H. Wang et al. 1988). FV has a plasma concentration of 20-30 nM (Wiencek et al. 2013; Walsh and Ahmad 2002) and a plasma half-life of 36 h (Walsh and Ahmad 2002). FVa is a key element in the prothrombinase complex, serving as a cofactor for FXa. FV must be cleaved to FVa in order to exhibit its pro-coagulant cofactor activity, as FV does not bind FXa (Wiencek et al. 2013). FV consists of domains in the structural order: A1, A2, B, A3, C1, C2. Cleavage by thrombin and FXa releases the heavily glycosylated B domain as two activation fragments, producing FVa. Cleavage occurs sequentially at Arg^{709}, Arg^{1018} and Arg^{1545} to generate the heavy chain (domains A1-A2) and light chain (domains A3-C1-C2) of FVa (Wiencek et al. 2013; Elisabeth Thorelli, Kaufman, and Dahlback 1997; Dharmawardana, Olson, and Bock 1999). Cleavage at Arg^{709} and Arg^{1545} separates the B region from the heavy and light chains respectively, and cleavage at Arg^{1018} separates the B region into 2 fragments (E Thorelli,

Kaufman, and Dahlbäck 1998). Sulfation of tyrosine residues within the A2 domain of FV has shown to be important for efficient cleavage of FV by thrombin, but not by FXa (Pittman et al. 1994). It has been traditionally believed that very small amounts of thrombin generated during the initiation phase of coagulation were responsible for initial cleavage of FV, however it has recently been suggested that FXa may be responsible for this initial cleavage (Schuijt et al. 2013).

Upon cleavage, the heavy and light chain then associate with each other, allowing FVa to gain pro-coagulant activity, and are held together by non-covalent Ca^{2+}-dependent interaction (Steen et al. 2008). Release of the B domain exposes FXa binding sites (Steen 2002). FVa binds to the membrane surface with a very high affinity through Ca^{2+}-independent interaction between the FVa light chain and anionic phospholipids (Koppaka et al. 1997). FVa binds membranes through its C1 and C2 domains. C2 is responsible for initial binding of FVa to the phospholipid membrane. Hydrophobic residues on protruding loops of the C2 domain embed into the phospholipid membrane, while phosphatidyl serine groups on the membrane can embed within these loops. Basic residues on C2 also interact with the negatively charged phosphate groups on the membrane (Macedo-Ribeiro et al. 1999). C1, with similar homology to C2, binds the phospholipid membrane and appears to be key to the assembly of a fully functional prothrombinase complex (Majumder et al. 2008). The binding of FVa to both FXa and the membrane forms a more stable complex, allowing for optimal interaction with prothrombin.

Membrane Surface

Formation of the prothrombinase complex occurs on negatively charged membrane surfaces. Physiologically, phosphatidylserine (PS) is the major negatively charged phospholipid present on procoagulant membranes and accounts for 8-15% of the total phospholipid within cell membranes (Zwaal, Comfurius, and Bevers 2005; Das and Plow 2011). PS provides a surface for vitamin K-dependent clotting factors to assemble on along with their cofactors (Halliez et al. 2015). Membrane binding of FVa and FXa reduces the dissociation constant for this interaction from micromolar to nanomolar ranges. Binding of FVa to the membrane surface has shown to greatly enhance the affinity of FXa for the membrane surface (Krishnaswamy 1990). Binding of FXa to the membrane surface substantially reduces the inhibition capabilities of antithrombin III (ATIII) towards FXa (Teitel and Rosenberg 1983). The membrane surface therefore leads to a significant increase in the rate of thrombin generation and clot formation. Unilamellar vesicles, composed of 75% PC and 25% PS (referred to as PCPS) are used extensively in research to mimic the surface of activated cells.

Activated platelets are thought to be the main surface for assembly of prothrombinase, although other blood cell populations, such as monocytes, neutrophils, lymphocytes, microparticles, red blood cells and the activated endothelium have been shown to support prothrombinase binding and activity (Mann, Krishnaswamy, and Lawson 1992; Rosing et al. 1985; Tracy, Eide, and Mann 1985; Rodgers and Shuman 1983; Ivanciu, Krishnaswamy, and Camire 2014). In quiescent cells, PS is solely located on the inner (cytoplasmic) leaflet of the cell membrane, whereas the outer leaflet of the

membrane is composed mostly of phosphaditylcholine (PC) and sphingomyelin (Sph) (Zwaal, Comfurius, and Bevers 2005). This distribution is maintained by membrane lipid transporters flippase, floppase and scramblase. Flippase shuttles PS from the outer membrane leaflet to the inner leaflet in an adenosine triphosphate (ATP)-dependent manner. Floppase transports non-PS phospholipids, such as PC from the inner leaflet to the outlet leaflet in an ATP-dependent manner. Scramblase is a nonselective lipid transporter which rapidly moves phospholipids back and forth between the outer and inner leaflets of the lipid bilayer (Hankins et al. 2015).

Platelet activation by thrombin or collagen causes PS to be shuttled to the surface of the membrane, allowing it to interact with circulating clotting factors. The mechanism for this involves activation of phospholipid scramblase and inhibition of flippase. This results in PS being randomly shuttled to the outer membrane leaflet by scramblase, while transport to the inner leaflet by flippase is inhibited, thereby increasing its concentration on the outer membrane leaflet (Zwaal, Comfurius, and Bevers 2005). Impairment of PS expression after platelet activation can lead to bleeding disorders. For example, Scott syndrome is an inherited bleeding disorder caused by a defect in the gene coding for scramblase (Halliez et al. 2015).

Prothrombin Activation by Prothrombinase

Assembly of the full prothrombinase complex is required for efficient prothrombin cleavage. Kinetic studies have demonstrated that formation of prothrombinase begins with FXa and FVa forming separate binary complexes (FXa-PL and FVa-PL) with the membrane surface. This is the rate-limiting step in prothrombinase

assembly (Krishnaswamy, Jones, and Mann 1988). Formation of the full prothrombinase complex occurs rapidly from the high-affinity interaction with FXa-PL and FVa-PL, shown to have a K_d of approximately 10^{-9} M (Krishnaswamy 1990). The formation of initial binary complexes is essential as the K_d values of FXa and FVa in solution are estimated to be in the 10^{-5}-10^{-6} M range (Krishnaswamy 1990; Skogen, Esmon, and Cox 1984). Stopped-flow kinetic studies indicate that FXa-PL and FVa-PL interaction appears to occur under two-dimensional conditions demonstrating the ability of the membrane to reduce degrees of freedom in protein orientation (Krishnaswamy, Jones, and Mann 1988). This increases the frequency of productive collisions between FXa and FVa. Both the heavy and light chain of FVa have been shown to interact with FXa (Steen et al. 2002), leading to a conformational change in FXa to better align its active site with the prothrombin substrate (Bianchini et al. 2005).

Activation of prothrombin to thrombin by FXa requires 2 separate cleavages at residues Arg^{271} and Arg^{320} (Figure 4). As such, there are two possible pathways of generating thrombin (Bradford, Orcutt, and Krishnaswamy 2013). In the presence of the fully-formed prothrombinase complex, cleavage at Arg^{320} is kinetically favoured and is therefore cleaved first to generate the intermediate meizothrombin (mIIa) (Adams and Huntington 2015). mIIa then undergoes a conformational change (Krishnaswamy et al. 1987a) from a zymogen to a protease-like structure prior to its subsequent cleavage at Arg^{271} to liberate thrombin (Haynes et al. 2012; Adams and Huntington 2015). In the absence of FVa, cleavage at Arg^{271} is kinetically favoured resulting in formation of prethrombin-2 (Pre2) as the intermediate (Adams and Huntington 2015). Subsequent

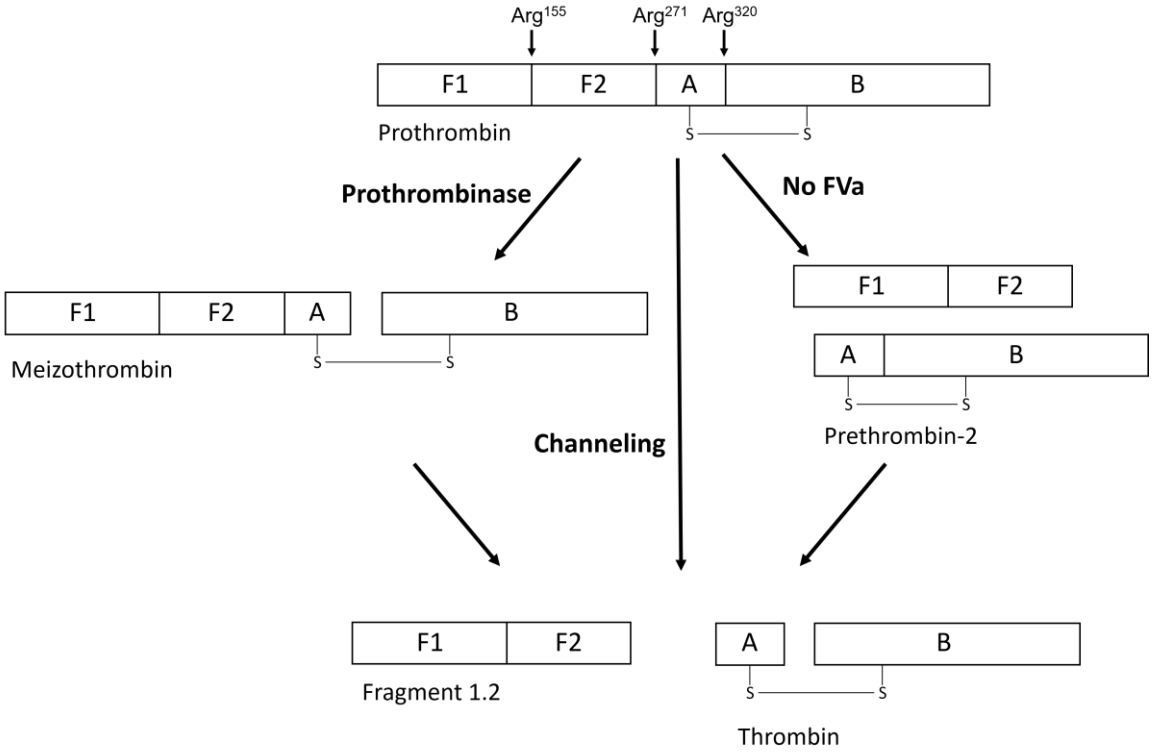

Figure 4 - Pathways for prothrombin cleavage.

This diagram displays the two pathways in which prothrombin can be cleaved by prothrombinase. In the presence of FVa prothrombin is activated via the mIIa intermediate, with initial cleavage at Arg^{320}. In the absence of FVa prothrombin is activated via a Pre2 intermediate, with initial cleavage at Arg^{271}. Channeling can also occur, where prothrombin is processed directly to thrombin without the release of intermediates. All cleavage pathways result in the generation of α-thrombin and F1.2. Figure adapted from Haynes et al., 2012 (Haynes et al. 2012).

cleavage at Arg^{320} then activates Pre2 to thrombin. Furthermore, channeling has also been observed during prothrombin activation, whereby thrombin is generated directly without the release of the intermediates mIIa or Pre2 (Boskovic, Bajzar, and Nesheim 2001).

Cleavage at Arg^{155} releases fragment 1 from both prothrombin and meizothrombin. This is thought to be a down-regulation step and has a number of effects on the rate of thrombin formation. Cleavage at Arg^{155} on prothrombin generates prethrombin-1 (Pre1), a less effective substrate for FXa. This cleavage also results in membrane-bound fragment 1 which may act to competitively reduce the amount of anionic phospholipid available for further prothrombin cleavage. Cleavage of meizothrombin at Arg^{155} removes the fragment 1 domain, generating a species known as meizothrombin des-F1. This removes meizothrombin from the phospholipid surface and substantially reduces its cleavage to thrombin (Nesheim et al. 1988).

Prothrombinase activity is specific to prothrombin, as the activity of FXa on peptidyl substrates does not appear to be affected by the presence of phosphatidylserine-containing membrane or formation of the full prothrombinase complex (Buddai et al. 2002; Lottenberg et al. 1986; Walker and Krishnaswamy 1993). This indicates that interactions of the prothrombinase constituents with prothrombin away from the scissile bond play a major role in prothrombin cleavage and therefore understanding these interactions are key to understanding how thrombin is generated.

Prothrombin-Factor Va Interaction

FVa has been shown to bind prothrombin through its heavy chain (Guinto and Esmon 1984). This interaction causes a conformational change in prothrombin resulting

in a better alignment of its scissile bond with the catalytic region of FXa (Bianchini et al. 2002), making prothrombin more suitable as a substrate for proteolysis by FXa (Deguchi et al. 1997; Walker and Krishnaswamy 1993; Yegneswaran et al. 2002). FVa compresses the flexible Lnk2 region (the region connecting the 2 kringle domains) of prothrombin to optimally align the scissile bonds of prothrombin with FXa (Pozzi et al. 2014). Part of the FVa interaction with prothrombin is believed to occur near the C-terminal of the FVa heavy chain. This region, which has a high homology with the protein hirudin, contains several sulfated tyrosine residues and has shown to be responsible for thrombin-mediated activation of FV (Hortin 1990; Pittman et al. 1994). A number of regions on prothrombin have been implicated in FVa interaction. Bolstein *et al*. demonstrated that the GLA domain is likely involved in this interaction. They showed that a GLA domain-like prothrombin fragment spanning residues 1-46, binds FVa using kinetic and fluorescence-quenching studies (Blostein et al. 2000). Basic residues within proexosite-1 appear to interact with FVa during Pre1 activation. Chen *et al*. demonstrated this by showing that mutation of these residues impaired FXa catalytic efficiency towards Pre1 in the presence, but not absence of FVa. This work also demonstrated that hirugen, a hirudin variant which specifically interacts with proexosite-1, inhibits wild-type Pre1 activation but had no inhibitory effect on their Pre1 mutant lacking these basic residues (Chen, Yang, and Rezaie 2003). Prothrombin residues 473-487, immediately adjacent to proexosite-1, have recently been implicated in FVa interaction using peptide inhibition and spectroscopic analyses (Yegneswaran et al. 2004).

Both kringle domains of prothrombin have been shown to interact with FVa (Deguchi et al. 1997; Kotkow et al. 1995), despite conflicting results regarding kringle 1 (Kotkow et al. 1995). The kringle 2 domain of prothrombin has been shown interact with FVa in the prothrombinase complex. Church *et al.* developed a monoclonal antibody specific to the fragment 2 region of prothrombin, which showed a significant FVa-dependent inhibition of prothrombin activation and prevented cleavage of prothrombin to mIIa (Church, Ouellette, and Messier 1991). Church *et al.* were not able to definitively conclude that their antibody directly blocked the FVa binding site, as the observed inhibition may have been due to steric hindrance or altered peptide conformation. Regardless of this fact, their data gave early insight to the specific binding site of FVa on prothrombin.

Kotkow *et al.* generated prothrombin mutants lacking either kringle 1 or kringle 2, which retained 50% and 10% of the procoagulant activity of wild-type prothrombin, respectively (Kotkow, Furie, and Furie 1993), suggesting that prothrombin likely utilizes both kringle domains to properly interact with the prothrombinase complex for efficient thrombin generation. Kotkow *et al.* later showed that this inhibition was FVa-dependent and was caused by the kringle 2-lacking prothrombin having a 7-fold lower k_{cat} value and 4 to 5-fold higher apparent K_m value compared to plasma-derived prothrombin. 90º light scattering experiments showed that the prothrombin mutant lacking kringle 2 showed no significant binding to FVa (Kotkow et al. 1995). The latter finding is in contrast to the more recent data previously discussed, suggesting additional sites outside of kringle 2 to

play a role in FVa binding. Nonetheless, this data strengthens the argument that kringle 2 is involved in this interaction.

Unpublished data from Nicole Brufatto and Michael Nesheim (Queen's University, Kingston, Ontario, Canada) demonstrated that the area spanning prothrombin residues 171-190 is a probable site of interaction with FVa. Peptidyl mimicry experiments were carried out using small synthesized pieces of prothrombin fragment 2. Two fragments containing residues 171-181 and 179-190 decreased initial rates of prothrombin activation in a concentration- and FVa-dependent manner. Inhibition was incomplete and non-competitive. 90º light scattering experiments measuring equilibrium binding of prothrombin and FVa showed that increasing concentrations of each of these fragments diminished the prothrombin-FVa interaction. A single peptide fragment spanning residues 171-190 showed complete inhibition of thrombin generation. Heteronuclear NMR was used in collaboration with Dr. Feng Ni (National Research Council, QC) to study the effect of the FVa heavy chain on prothrombin fragment 2. The majority of fragment 2 was unperturbed, but 7 residues showed high perturbance in the presence of FVa. These residues include Ser^{160}, Gln^{177}, Gln^{179}, Arg^{181}, Leu^{182}, Val^{184}, and Thr^{185}, whereby 6 of the 7 residues are within a nine-residue span in the loop of kringle 2 domain. Therefore, this study proposes to investigate the role of these residues in expressing FVa cofactor activity during prothrombin activation by FXa.

II. Hypothesis

The 7 residues of prothrombin fragment 2 (S^{160}, Gln^{177}, Gln^{179}, Arg^{181}, Leu^{182}, Val^{184}, Thr^{185}) are important in expressing FVa cofactor activity during prothrombin activation by FXa.

III. Specific Aims

1) Generate a prothrombin mutant with 6 simultaneous mutations at Gln^{177}, Gln^{179}, Arg^{181}, Leu^{182}, Val^{184} and Thr^{185}. Residues 177, 179, 181 and 185 are substituted with alanine and residues 182 and 184 are substituted with threonine (PT6).
2) Quantify activation kinetics of wild-type prothrombin (WTPT) and PT6 by FXa in the prothrombinase complex assembled either on PCPS vesicles or thrombin-activated platelets, as well as without FVa, without the membrane surface, and by FXa alone.
3) Quantify the formation of intermediates and products of these reactions via time-course SDS-PAGE analysis.

IV. Experimental Procedures

Materials

QuikChange Lightening Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA, USA). DNA primers were synthesized by Integrated DNA Technologies® (Coralville, IA, USA). QIAprep® Spin Miniprep Kit and Plasmid Maxi Kit were purchased from Qiagen (Hilden, Germany). Lipofectamine® 3000

Transfection Kit was purchased from Life Technologies-Invitrogen (Carlsbad, CA, USA). Baby hamster kidney cells and the pNUT vector, used for mammalian expression, were provided by Dr. Ross MacGillivray (University of British Columbia). Methotrexate (Mayne Pharma Inc., Montreal, Quebec Canada) and Vitamin K1 were purchased from Hamilton General Hospital. Q-Sepharose Fast Flow anion-exchange resin and Mono-Q HR 5/5 column were obtained from GE Healthcare (Burlington, Ontario Canada). Gibco® D-MEM/F-12 media, newborn calf serum and Opti-MEM I media, 0.5% Trypsin-EDTA and Antibiotic-Antimycotic solution were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Phosphatidyl-L-serine, phosphatidyl-L-choline, and XAD-2 resin were obtained from Sigma. Matched-Pair Antibody Set for ELISA of Human Prothrombin Antigen, was purchased from Affinity Biologicals™ Inc (Ancaster, ON, Canada). The fluorescent α-thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as previously described (Nesheim, Prendergast, and Mann 1979). Phospholipid vesicles composed of 75% PC and 25% PS (PCPS) were prepared as previously described (Bloom, Nesheim, and Mann 1979). FXa and FVa were purchased from Haematologic Technologies, Inc (Essex Junction, VT, USA). FVa was also isolated from human plasma as described previously (Kalafatis and Beck 2002; Nesheim, Katzmann, et al. 1981) and was activated by incubating with 2nM thrombin for 10 min at 37ºC. Activation was stopped with 1 μM DAPA. Human plasma-derived wild-type prothrombin was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Integrilin was purchased from Kingston General Hospital (Kingston, ON). Mini-PROTEAN® TGX™ and Criterion™ TGX™ precast gels, Coomassie Brilliant Blue G-

250 stain and Bio-Rad Protein Assay reagent were purchased from Bio-Rad (Mississauga, ON, Canada).

Site-Directed Mutagenesis

For our PT6 mutant, site-directed mutagenesis was carried out on WT prothrombin in the pNUT expression vector using the QuikChange Lightening Site-Directed Mutagenesis kit. DNA primers were constructed to mutate the selected residues to alanine/threonine. After site-directed mutagenesis, DNA was isolated using the QIAprep® Spin Miniprep Kit and was sequenced. Upon confirmation of mutations, a larger quantity of DNA was isolated using Plasmid Maxi Kit. All DNA was sequenced at Robarts Research Institute (London, ON, Canada).

Protein Expression and Purification

Upon confirmation of successful mutations by DNA sequencing, DNA was transfected into baby hamster kidney (BHK) cells using the Lipofectamine® 3000 Transfection kit. Transfected cells were grown in DMEM/F-12 media, supplemented with 5% newborn calf serum. Media contained 400 μM methotrexate to select against cells lacking pNUT vectors. Multiple plates of cells were grown and plates with the highest level of prothrombin expression were isolated. Prothrombin expression level was measured using the Matched-Pair Antibody Set for ELISA of Human Prothrombin Antigen (FII). Cells were either frozen in liquid nitrogen for further usage or grown in high volume using DMEM/F-12 in triple-layered tissue culture flaks. Upon reaching

confluency cells were grown in Opti-MEM I media, containing 50 μM $ZnCl_2$, 10 μg/mL Vitamin K and 1X Antibiotic-Antimycotic solution. Media was collected in 48 h intervals. Prothrombin variants were purified as described by Kim and Nesheim (Kim and Nesheim 2007).

Briefly, collected media was loaded onto XAD_2 and Q-Sepharose columns in tandem at room temperature. The columns were then disconnected and Q-sepharose was washed with 400-500 mL of 0.02 M Tris, 0.15 M NaCl, pH 7.4 (TBS) and prothrombin was eluted in 2 mL fractions using 0.02 M Tris, 0.5 M NaCl, pH 7.4. Bradford assay was used to identify the fractions containing the highest protein content, which were pooled and subjected to barium-citrate precipitation. After centrifugation and removal of supernatant, the barium-citrate pellet was dissolved in a minimal volume of 0.5 M EDTA, pH 8.0 and dialyzed four times against 500 mL TBS. The dissolved protein was subjected to fast-protein liquid chromatography using a Mono-Q column and functionally γ-carboxylated prothrombin was eluted using a 0-30 mM $CaCl_2$ gradient. Protein from the first peak was pooled, subjected to precipitation by 80% ammonium sulfate and stored at -20ºC in 50% glycerol.

Thrombin Generation Measured by DAPA Fluorescence

Prothrombin activation was quantified using DAPA, a fluorescent molecule which shows a great increase in both intensity and lifetime of fluorescence when bound to thrombin, allowing for continuous monitoring of thrombin generation (Nesheim, Prendergast, and Mann 1979). DAPA is also a potent inhibitor of thrombin with a K_i of approximately 10^{-7} M (Nesheim, Prendergast, and Mann 1979). Because the thrombin

that is generated catalyzes the cleavages at Arg^{155}-Ser^{156} and Arg^{284}-Thr^{285}, inclusion of DAPA limits autodegradation at these locations and thus simplifies analyzing the kinetics of prothrombin activation by prothrombinase (Krishnaswamy et al. 1987b; Nesheim, Prendergast, and Mann 1979). Fluorescence was measured by a SpectraMax M2 fluorescent plate reader, with an excitation wavelength of 280 nm, emission wavelength of 545 nm and an emission cutoff at 530 nm. Opaque 96-well plates were pre-treated with 0.02 M HEPES, 0.15 M NaCl, pH 7.4 (HBS) with 1% Tween 80 (HBST) to prevent non-specific protein binding for at least 1 h and were then washed thoroughly with distilled deionized water prior to use.

Prothrombin at varying concentrations (0, 0.25, 0.5, 0.75, 1.0 and 1.4 μM) was diluted into HBST with 0.1% prionex and was then mixed with $CaCl_2$ (5 mM), FVa (20 nM), DAPA (10 μM) and either PCPS (50 μM), thrombin-activated platelets (5×10^7 mL^{-1}) or no membrane surface. When activated platelets were used as the membrane surface, reaction mixtures included integrilin (9 μM) to prevent platelet aggregation. This mixture was then added to empty wells and left in the plate reader for 15 min to equilibrate the initial signal. The mixture was then transferred to FXa-containing wells to initiate the reaction, and the fluorescence was monitored every 20 s. FXa concentrations were as follows: 20 pM for activation by FXa/FVa/PCPS, 20 nM for activation by FXa/FVa, FXa/PCPS, and FXa, and 100 pM for activation by FXa/FVa/activated platelets. Additionally, experiments were carried out with FVa as the limiting factor. Under these conditions, FXa was included at 1 nM and FVa was included at 20 nM, with $CaCl_2$ and PCPS remaining at the same concentrations used previously.

Initial rates, measured as the first 10-20% change in fluorescence, were measured along with total change in fluorescence signal. Total fluorescence change was then plotted against initial prothrombin concentration to generate a standard curve to convert relative fluorescence units (RFUs) to absolute thrombin concentration, and the slope of this line (RFU slope) was determined using the slope function in Microsoft® Excel. Thrombin generation rates were calculated using (Equation 1):

$$Thrombin\ generation rate = \frac{Initial\ rate}{RFU\ \ slope \times [FXa]}$$

(Equation 1)

Thrombin generation rates were then plotted against prothrombin concentrations. Using non-linear regression analysis in Sigma-Plot® 11.0, the K_m and k_{cat} values for prothrombin activation were estimated. Three trials were conducted (n=3) in duplicate, and values were averaged between all 6 reactions. Equal variance t-tests were conduced to determine statistically significant differences between WTPT and PT6.

An additional set of experiments was carried out in activating prothrombin with FXa/FVa, FXa/PCPS and FXa alone using decreased FXa concentrations in order to minimize the production and accumulation of Pre1 by FXa. Under these conditions, experiments were initiated with 5 nM FXa in the presence of $CaCl_2$ (5 mM), DAPA (10 μM), with or without FVa (20 nM) and/or PCPS (50 μM). Initial rates of these experiments were measured with 1 μM prothrombin substrate. The raw data from these prothrombin activation experiments was smoothed using TableCurve® 2D v5.01. DAPA

fluorescence values were converted to thrombin concentration by dividing DAPA fluorescence by the RFU slope calculated previously. The point-to-point slope of thrombin concentration against time was plotted to give thrombin generation plots. The area under the curve of this plot was calculated by splitting each curve into 0.5 min intervals and calculating the area under each interval, yielding total thrombin produced.

SDS-PAGE Time-Course Analysis

Prothrombin (1.4 μM) in a mixture of PCPS (50 μM), $CaCl_2$ (5 mM), DAPA (10 μM), buffered with HBST + 0.1% prionex, was activated by 20 pM FXa in the presence of FVa (20 nM) and by 20 nM FXa in the absence of FVa. FXa (100 pM) was used to activate reactions with thrombin-activated platelets (5×10^7 mL^{-1}) in place of PCPS. Samples were removed at respective time points, and reactions were stopped by adding samples into acetic acid at a final concentration of 0.134 N. Reactions with platelets were centrifuged at 10,000 RPM for 10 min to pellet platelets, and reaction supernatants were transferred to new tubes. Samples were dried down using SpeedVac, reconstituted in sample buffer and loaded onto Bio-Rad Criterion™ TGX™ 8-16% gels. These gels were resolved, stained for at least 1 h with Bio-Rad Coomassie Brilliant Blue G-250 and destained for at least 30 min with deionized water prior to imaging.

Quantitative densitometry was carried out using Bio-Rad Image Lab™ Version 5.2.1. Band intensities were summed for each well and normalized to the prothrombin band of lane 1 (1.4μM). These values were then converted into concentration by using (Equation **2**, where x is the concentration of the fragment, I_b is band intensity of the fragment band, I_a is intensity of the 1.4 μM prothrombin band and C is the correction

coefficient for the respective fragment when using Coomassie Blue. Correction coefficients were 0.849, 0.4468, 0.3695, 0.6265 and 0.5492 for Pre1, F1.2-A chain (F1.2A), F1.2, Pre2 and B-chain, respectively, as determined by Kim & Nesheim (Kim and Nesheim 2007). Calculated values were then plotted against reaction time to investigate the appearance of intermediates and products.

$$x = \left(\frac{I_b}{I_a} \right) 1.4C$$

(Equation 2)

Platelet Isolation and Activation

Modified Tyrode's Buffer (134mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES and 1 mM $MgCl_2$, pH 7.3; MTH) and Acid-Citrate-Dextrose (28.48 g/L sodium citrate, 20 g/L glucose and 15 g/L citric acid; ACD) buffer were made, filtered and stored in the fridge. Prior to drawing blood, glucose was added to MTH buffer to a final concentration of 5 mM and was preheated along with ACD buffer to 37ºC. 45 mL of blood was drawn from consenting human donors via venipuncture into 5 mL sodium citrate solution (4% w/v). 5 mL of ACD buffer was added to the blood and inverted to mix. Blood was centrifuged at 200 × g for 20 min, platelet rich plasma was carefully removed and mixed with 10 µL of 1 mg/mL prostacyclin (PGI_2). Platelet rich plasma was centrifuged at 1100 × g for 11 min and the platelet poor plasma supernatant

was discarded. The platelet pellet was re-suspended in 15 mL MTH with 2 mL ACD and 10 μL of 1 mg/mL PGI_2 and centrifuged again at 1100 × g for 11 min. The supernatant was discarded and the final washed platelet pellet was resuspended in 3.5 mL of MTH buffer. The final platelet concentration was measured by counting a 1:100 dilution of the platelet solution on a hemocytometer. Isolated platelets were diluted to a final concentration of $1x10^8$ mL^{-1} and allowed to rest at room temperature for 30 min prior to use. Integrilin was added to generate a final concentration of 9 μM in reactions, equal to approximately 18 μM in isolated platelets. Platelets were then activated by incubation with 1 unit/mL thrombin for 5 min at room temperature. Activation was stopped by addition of DAPA to a final concentration of 10 μM.

V. Results

Prothrombin Isolation

Prothrombin derivative PT6 was isolated from the expression media by first subjecting to XAD_2 and Q-Sepharose columns in tandem. PT6 was eluted from Q-Sepharose in 2 mL fractions and the protein contents were quantified using the Bradford assay (Figure 5). To select for prothrombin with functional γ-carboxylation, the samples were subjected to the Mono Q column and eluted using a 0 to 30 mM $CaCl_2$ gradient (Figure 6), and the first peak was pooled for ammonium sulphate precipitation. PT6 was dissolved in 50% glycerol and stored at -20ºC. Concentration was determined by absorbance and a single band was verified via SDS-PAGE.

Comparing Activation of WTPT and PT6

Because DAPA only fluoresces upon forming the thrombin-DAPA complex or the mIIa-DAPA complex, DAPA is a great tool for accurately quantifying thrombin generation in real-time (Figure 7). When prothrombinase was the enzyme under limiting FXa conditions (Figure 7A), WTPT generated roughly twice as much thrombin compared with the PT6, with the initial rates of PT6 being ~25% slower than WTPT. In the absence of FVa (Figure 7B), the relative differences between WTPT and PT6 appeared to be similar as when prothrombinase was the activator. The total levels of thrombin generated, however, were ~33% of prothrombinase for WTPT and PT6, despite 1000-fold higher level of FXa present. The initial rates were taken from these profiles to calculate thrombin generation rates using Equation 1, and these rates were subsequently plotted with respect to the initial concentration of prothrombin (Figure 8). Non-linear regression was

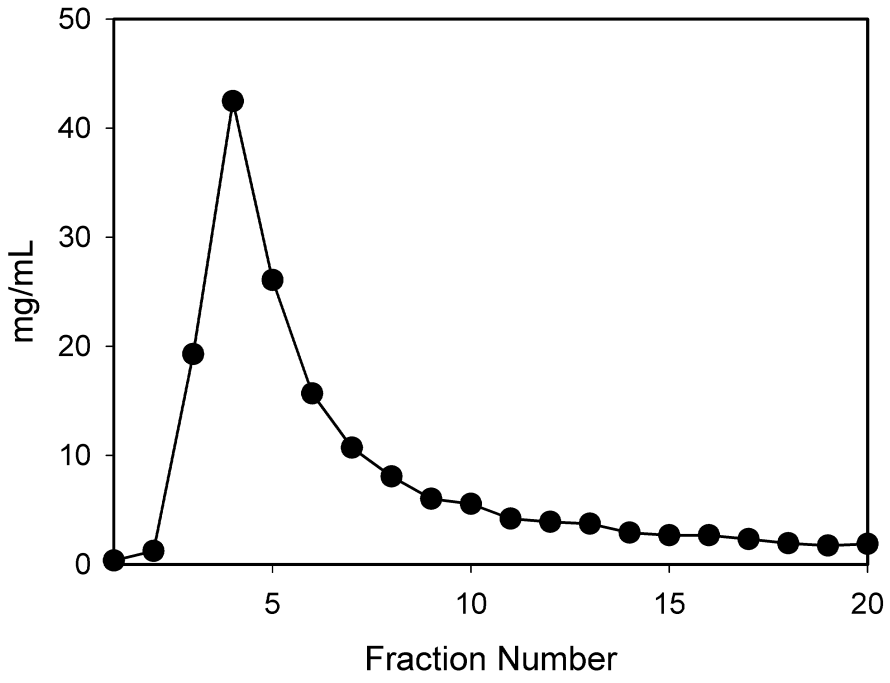

Figure 5 - Q-Sepharose elution profile for prothrombin.

Media was loaded onto XAD_2 and Q-Sepharose columns in tandem, washed with TBS and eluted into 2 mL fractions using 0.02 M Tris, 0.5 M NaCl, pH 7.4. Bradford assay was used to quantify protein content.

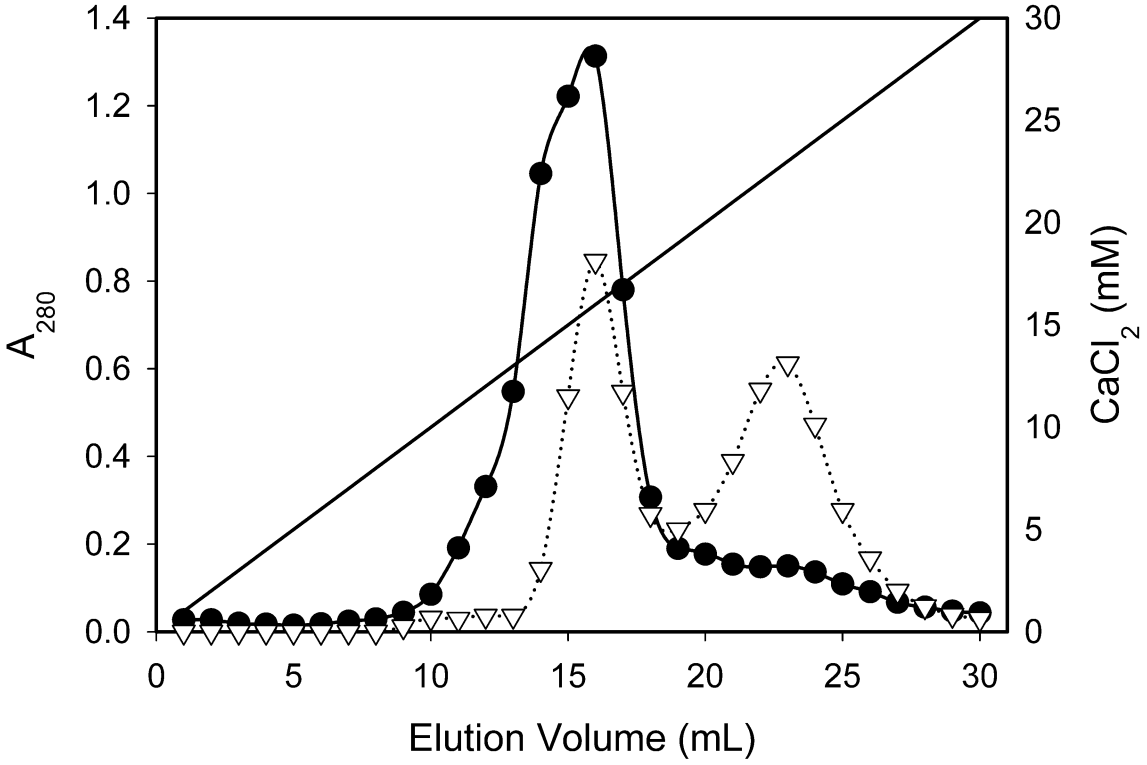

Figure 6 - Mono-Q elution for functionally γ-carboxylated prothrombin.

Dialyzed protein sample was loaded onto Mono-Q column, and eluted with a 0 to 30 mM $CaCl_2$ gradient in TBS, shown by the solid line. Absorbance of fractions were determined and the first peak (10-19 mL of elution volume) was pooled. Absorbance readings for PT6 eluent from current work (●) and prothrombin eluent from previous work in our lab (▽) are superimposed to demonstrate the two peaks separated due to γ-carboxylation (Kim and Nesheim 2007).

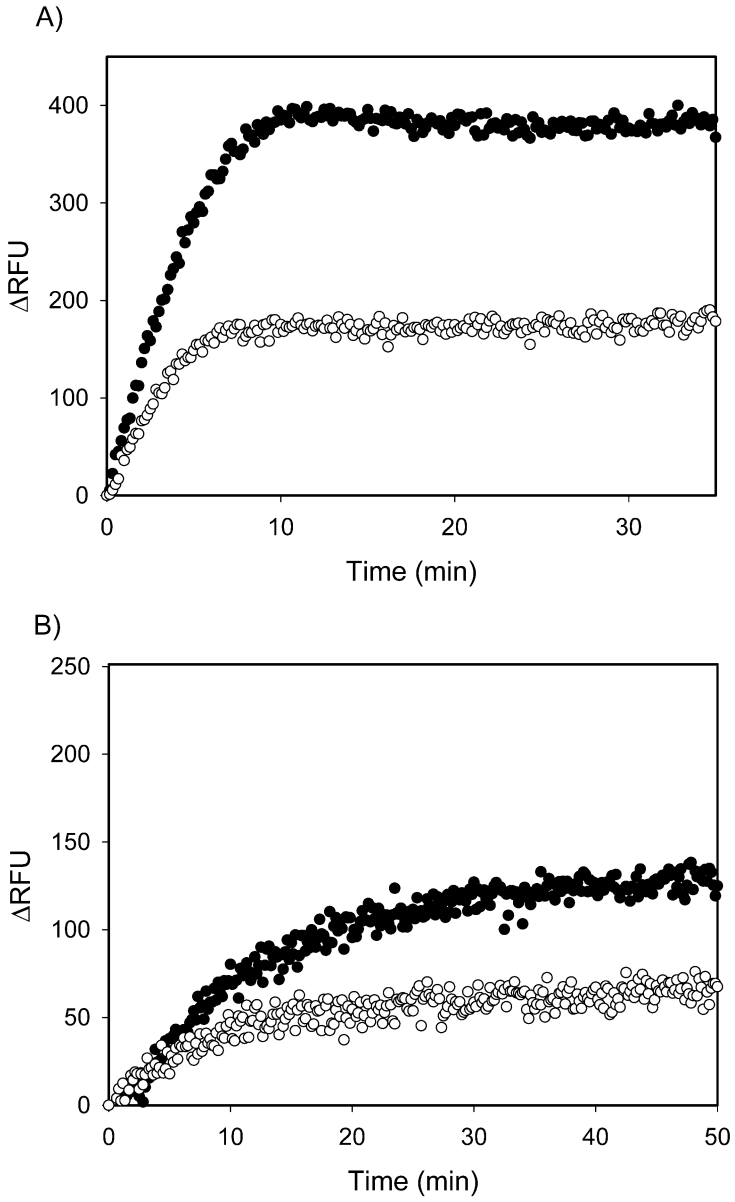

Figure 7 - Raw profiles of thrombin generation measured by thrombin-DAPA or mIIa-DAPA complex formation.

WTPT (●) and PT6 (○) activation by *(A)* prothrombinase or *(B)* FXa and PCPS without FVa were measured over time through fluorescence resonance energy transfer (FRET) upon formation of the thrombin-DAPA or mIIa-DAPA complex.

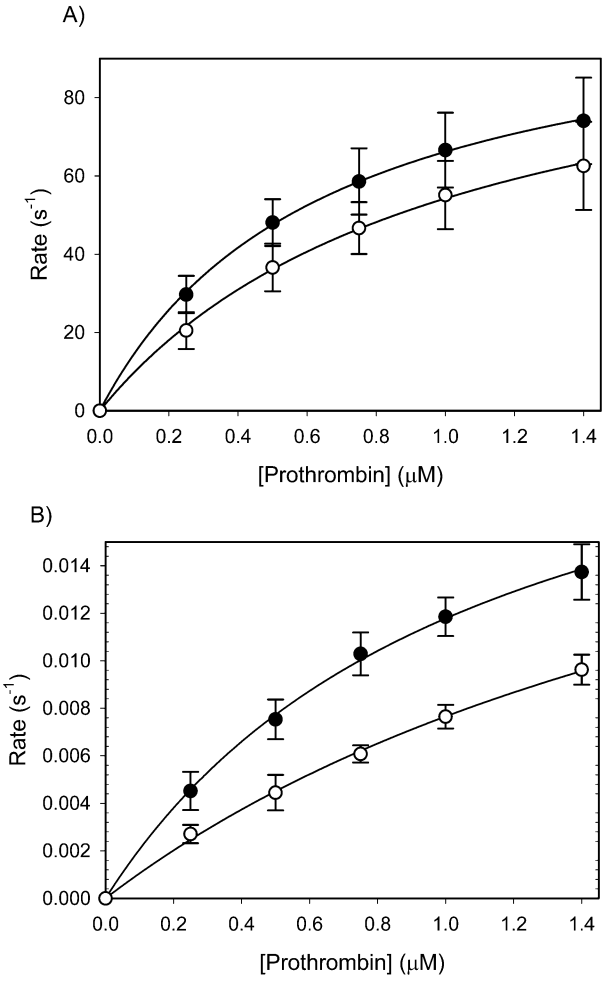

Figure 8 - Prothrombin activation by (A) prothrombinase or (B) without FVa.

Initial rates of thrombin generation were determined from the fluorescence profiles using Equation 1 at varying WTPT (●) and PT6 (○) concentrations and were plotted with respect to the initial prothrombin concentrations. Non-linear regression was performed to estimate the kinetic parameters k_{cat} and K_m, which were then used to calculate the catalytic efficiency (k_{cat}/K_m). Each point represents at least three independent trials, in duplicates. The error bars represent standard deviation.

performed to estimate the kinetic constants k_{cat} and K_m, which were used to calculate the catalytic efficiency (k_{cat}/K_m) (Table 1). When prothrombinase was the activator (Figure 8A), the catalytic efficiency of PT6 activation was 33% lower than that of WTPT ($p<0.005$), which was due to a 1.62-fold increase in the K_m as there were no significant differences in the k_{cat} values. In the absence of FVa (Figure 8B), the catalytic efficiency of PT6 activation was 51% lower than that of WTPT ($p<0.005$), which was due to a 2.31-fold increase in the K_m value while the k_{cat} values did not significantly change.

SDS-PAGE Time-Course Analysis of Prothrombin Activation

To visualize the potential differences in prothrombin consumption as well as intermediate and thrombin generation during activation of the prothrombin variants, SDS-PAGE was utilized to generate time course profiles of each species. When prothrombinase was the activator under limiting FXa conditions (Figure 9), there were clear differences in the band intensities between WTPT and PT6, especially in mIIa, which is inferred by the F1.2-A-chain band. Quantitative densitometry was performed on these bands to determine the time courses of prothrombin, mIIa, F1.2, and the B-chain of thrombin (Figure 10). Prothrombin cleavage (Figure 10A) was lower in PT6 compared with WTPT. The total mIIa generation (Figure 10B), which was quantified by calculating the area under the curves of each plot, was roughly 50% lower in PT6 than WTPT. Although the differences in the F1.2 time course was minimal (Figure 10C), there was a decrease in B-chain accumulation for PT6 compared with WTPT (Figure 10D).

Table 1 - Kinetic parameters obtained from DAPA fluorescence analysis of prothrombin activation.

Non-linear regression was used to estimate the k_{cat} and K_m values. Catalytic efficiency was calculated by dividing k_{cat} by K_m.

Prothrombin Activator		k_{cat} s^{-1}	K_m μM	Catalytic Efficiency M^{-1} s^{-1}
FXa, $CaCl_2$, FVa, PCPS	WTPT	109.9 ± 21.6	0.65 ± 0.16	(1.71 ± 0.27) x 10^8
	PT6	111.7 ± 27.6	1.05 ± 0.32	(1.14 ± 0.22) x 10^8
	p	0.5	< 0.01	< 0.005
FXa, $CaCl_2$, PCPS	WTPT	(25.1 ± 2.5) x 10^{-3}	1.14 ± 0.28	(2.26 ± 0.37) x 10^4
	PT6	(27.6 ± 8.5) x 10^{-3}	2.63 ± 1.12	(1.11 ± 0.20) x10^4
	p	0.5	0.01	< 0.005

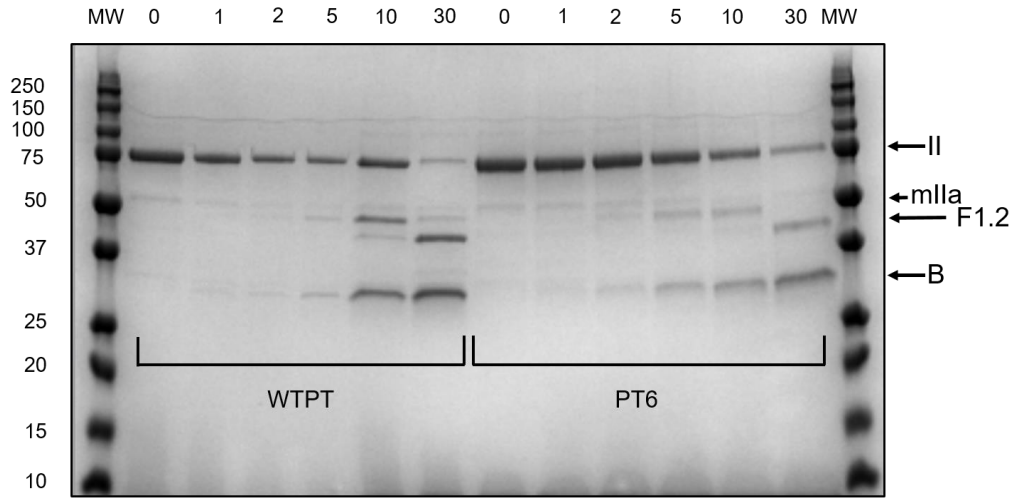

Figure 9 - SDS-PAGE time-course analysis of prothrombin activation by prothrombinase.

WTPT or PT6 (1.4 μM) was incubated with FVa (20 nM), PCPS (50 μM), $CaCl_2$ (5 mM), DAPA (10 μM) in HBST. Reactions were initiated by the addition of FXa (20 pM) at 25ºC. Samples were removed at indicated times by adding into 0.134 N acetic acid and the contents were resolved by SDS-PAGE under reducing conditions.

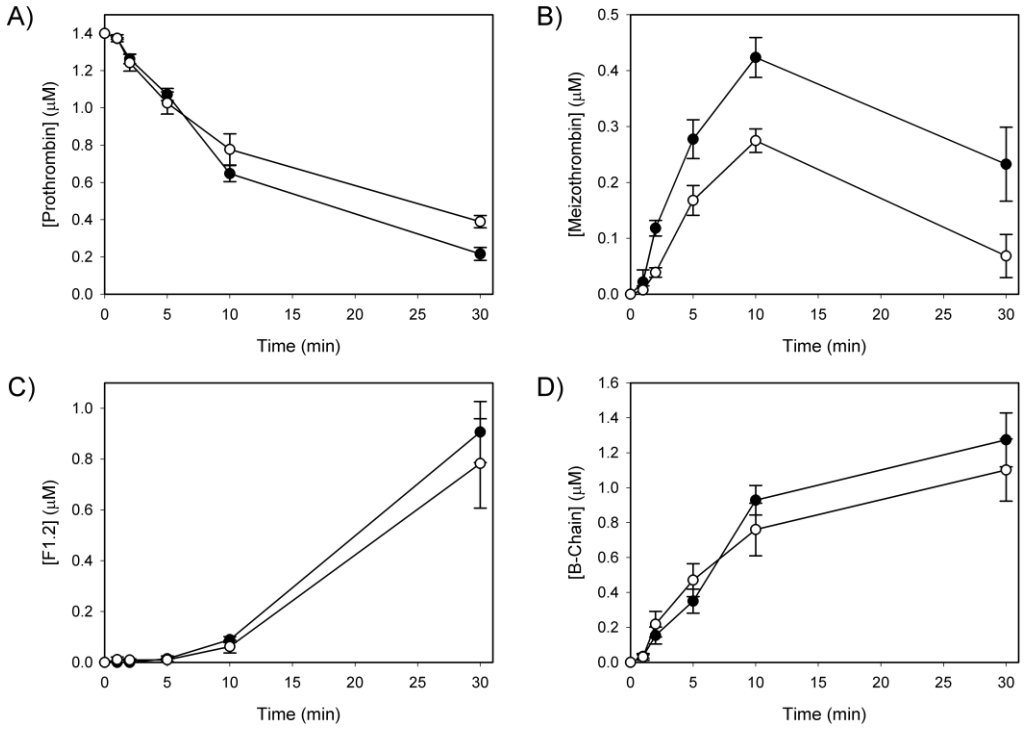

Figure 10 - Quantitation of time-course activation of WTPT and PT6 by prothrombinase.

Gels from SDS-PAGE time-course analysis of WTPT (●) and PT6 (○) activation by prothrombinase were quantified using densitometry to generate the time-course profiles of *(A)* prothrombin, *(B)* mIIa, *(C)* F1.2, and *(D)* B-chain of thrombin.

In the absence of FVa (Figure 11), the band patterns were quite different than with full prothrombinase (Figure 9). Upon quantitative densitometry, it was evident that prothrombin consumption of PT6 was delayed compared with WTPT (Figure 12A). Unlike with prothrombinase, however, there was a substantial accumulation of Pre1 in the absence of FVa (Figure 12B). The differences, however, were minimal, suggesting that the cleavage at Arg155 is not largely affected by the mutations imposed here. Pre2 accumulates at higher levels in the earlier time points of PT6 activation (5-10 min) than it does in WTPT (Figure 12C), despite the prothrombin band of PT6 showing less consumption than WTPT (Figure 12A). PT6 had a substantially lower B-chain accumulation compared with WTPT (Figure 12D). This decrease in B-chain formation in PT6 is accounted for by the decrease in PT6 prothrombin consumption, slightly higher formation of Pre1, as well as higher levels of Pre2 accumulation compared with WTPT. Cleavage of prothrombin to Pre1 by thrombin is inhibited by DAPA (Krishnaswamy et al. 1987a), but has been previously observed to occur by free FXa, in limiting concentrations of FVa (Kamath and Krishnaswamy 2008).

Activation Kinetics of WTPT and PT6 in Limiting FVa Conditions

Prothrombin activation was additionally carried out under limiting FVa conditions. Under these conditions FXa was in excess (1 nM) and FVa was the limiting factor for formation of prothrombinase (20 pM). The kinetic data obtained from these experiments are congruent with what was observed under limiting FXa conditions. The catalytic efficiency of prothrombin activation was 50% lower ($p<0.05$) in PT6 (1.24 ±

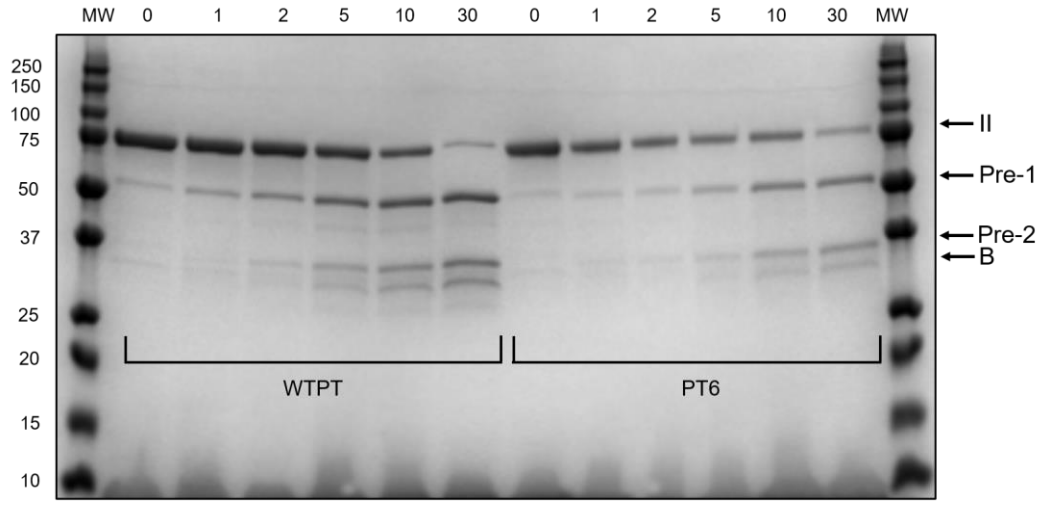

Figure 11 - SDS-PAGE time-course analysis of prothrombin activation by FXa, PCPS and Ca^{2+}.

WTPT or PT6 (1.4 μM) was incubated with PCPS (50 μM), $CaCl_2$ (5 mM), DAPA (10 μM) in HBST. Reactions were initiated by the addition of FXa (20 nM) at 25ºC. Samples were removed at indicated times by adding into 0.134 N acetic acid and the contents resolved by SDS-PAGE under reducing conditions.

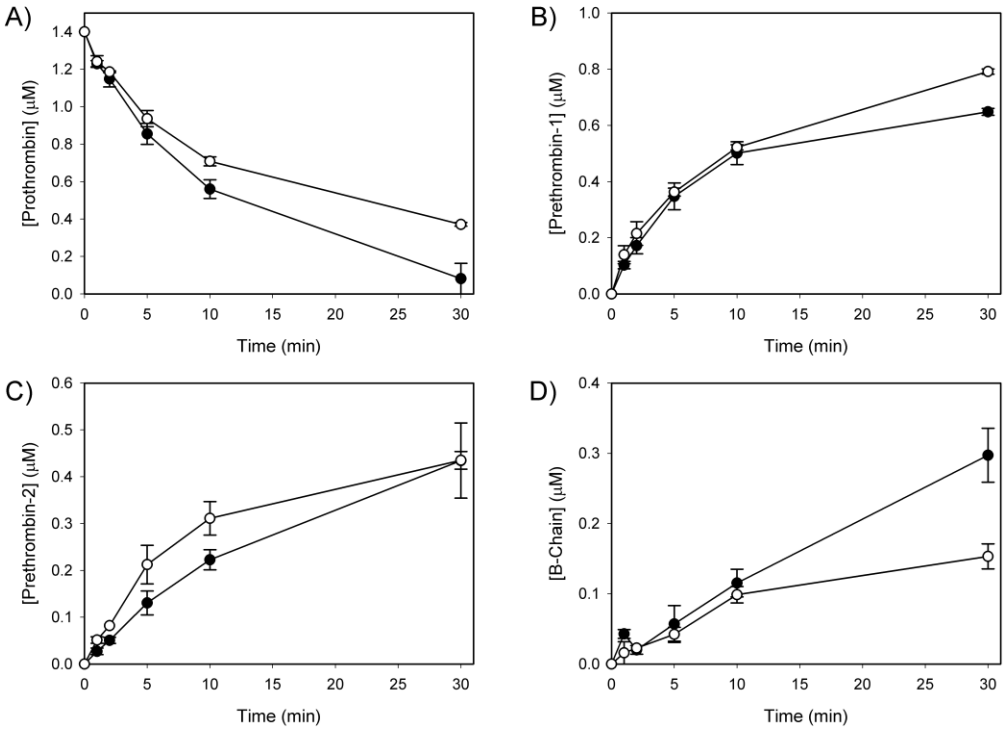

Figure 12 - Quantitation time-course activation of WTPT and PT6 by FXa, PCPS and Ca^{2+}.

Gels from SDS-PAGE time-course analysis of WTPT (●) and PT6 (○) activation by FXa, PCPS and Ca^{2+} were quantified using densitometry to generate the time-course profiles of *(A)* Prothrombin, *(B)* Pre1, *(C)* Pre2, and *(D)* B-chain of thrombin.

0.51 x 10^8 $M^{-1}s^{-1}$) compared with WTPT (2.49 ± 1.03 x 10^8 $M^{-1}s^{-1}$). This was due to a 2.39-fold increase in K_m values, as k_{cat} values were not significantly different (Table 2).

Activation Kinetics of WTPT and PT6 in the Absence of a Membrane Surface

WTPT and PT6 were activated by FXa in the absence of a membrane surface, both in the presence and absence of FVa. There were no significant differences in catalytic efficiency between WTPT and PT6 (Table 3). Overall, our experiments show that removal of FVa from the prothrombinase complex decreased the catalytic efficiency of thrombin generation by 4 orders of magnitude, and activation of prothrombin by FXa/FVa in the absence of a membrane surface, and by FXa alone decreased the catalytic efficiency by 5 orders of magnitude.

Analysis of Total Thrombin Generation

Prothrombin was activated by full prothrombinase (FXa/FVa/PCPS) with 20 pM FXa and 20 nM FVa (Figure 13). Activation by FXa/FVa (20 nM FVa), FXa/PCPS and FXa alone were carried out with 5 nM FXa (Figure 14). These experiments were carried out with 1 μM of WTPT or PT6. Significant decreases in initial rates were observed under all conditions without full prothrombinase (Table 4). From the DAPA fluorescence time course profiles, the rate of thrombin generation was determined with respect to time. This was done by initially smoothing the noisy fluorescence data using TableCurve 2D v.5.01 to generate a best-fit regression line, and the rate of change of thrombin concentration was calculated and plotted with respect to time. The area under the curve of these plots represents total amount of thrombin generated for each experiment (Table 5). PT6 generated 43% less thrombin than WTPT when activated by prothrombinase ($p<0.005$)

Table 2 - Kinetic parameters of prothrombin activation by prothrombinase under limiting FVa conditions.

Non-linear regression was used to estimate the k_{cat} and K_m values. Catalytic efficiency was calculated by dividing k_{cat} by K_m.

Prothrombin Activator		k_{cat} s^{-1}	K_m μM	Catalytic Efficiency $M^{-1}\ s^{-1}$
FXa/FVa/PCPS	PTWT	79.6 ± 18.6	0.35 ± 0.14	(2.49 ± 1.03) x 10^8
	PT6	98.0 ± 28.3	0.84 ± 0.22	(1.24 ± 0.51) x 10^8
	p	0.21	<0.005	< 0.05

Table 3 - Kinetic Parameters of WTPT and PT6 Without Membrane Surface by FXa/FVa or FXa Alone.

Non-linear regression was used to estimate the k_{cat} and K_m values. Due to the slow nature of these reactions, only catalytic efficiency values were compared.

Prothrombin Activator		Catalytic Efficiency M^{-1} s^{-1}
FXa, $CaCl_2$, FVa	PTWT	$(4.24 \pm 2.72) \times 10^3$
	PT6	$(3.78 \pm 3.04) \times 10^3$
	p	0.79
FXa, $CaCl_2$	PTWT	$(1.36 \pm 0.71) \times 10^3$
	PT6	$(0.98 \pm 0.26) \times 10^3$
	p	0.29

Table 4 - Initial Rates of Thrombin Generation.

Activation of WTPT or PT6 (1 μM) by prothrombinase (FXa/FVa/PCPS) was carried out with 20 pM FXa, 20 nM FVa, 5 mM $CaCl_2$ and 50 μM PCPS. Activation by FXa/FVa (20 nM FVa), FXa/PCPS and FXa alone were carried out with 5 nM FXa to minimize Pre1 formation, as observed in prior experiments with 20 nM FXa. Fold differences in initial rates are calculated separately for WTPT and PT6, relative to activation by FXa alone. Statistical significance was measured between WTPT and PT6 for each set of experiments using equal variance t-test.

	WTPT		PT6		*p*
	Rate (RFU·sec^{-1})	Fold Diff.	Rate (RFU·sec^{-1})	Fold Diff.	
FXa/FVa/PCPS	66.61	**6.92×10^4**	55.11	**7.84×10^4**	<0.05
FXa/FVa	2.71E-02	**2.82×10^1**	1.16E-02	**1.65×10^1**	<0.0005
FXa/PCPS	2.53E-02	**2.63×10^1**	1.74E-02	**2.48×10^1**	<0.0005
FXa	9.62E-04	**1**	7.03E-04	**1**	<0.05

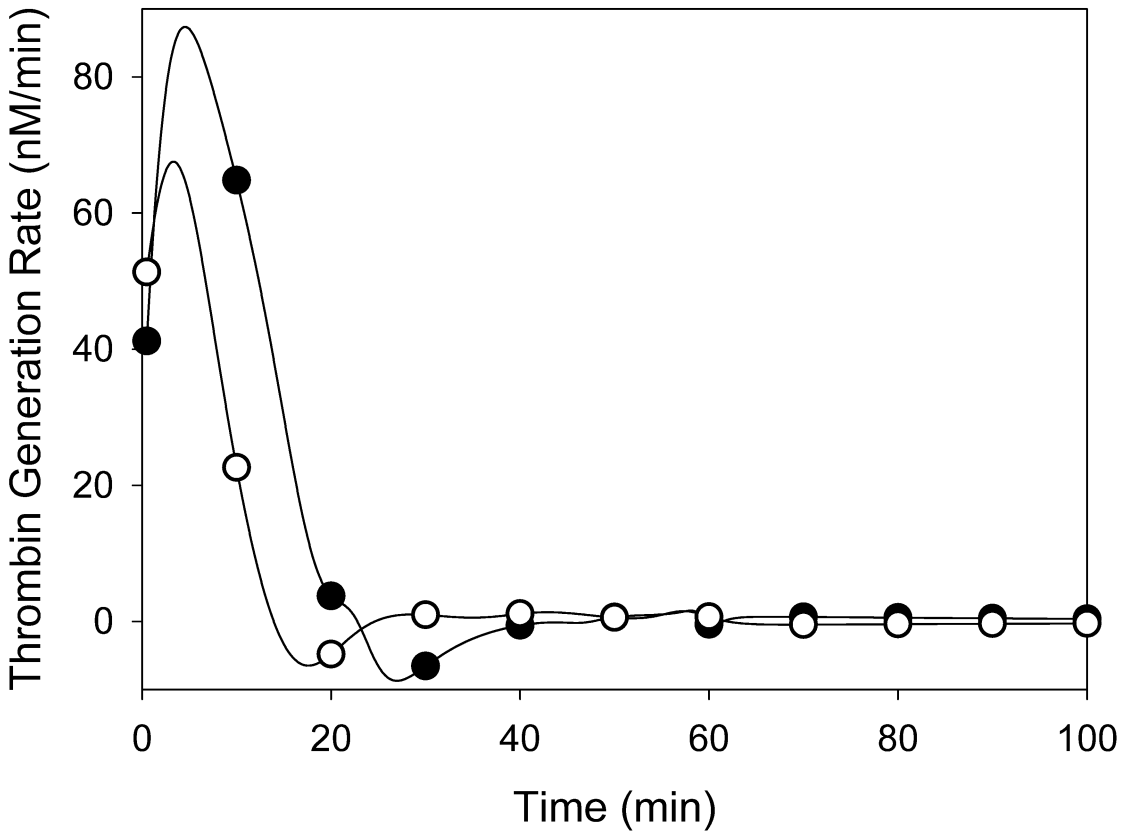

Figure 13 - Thrombin generation rates by upon activation by prothrombinase.

Lines represent rates of thrombin generation, calculated using 0.5 min intervals of DAPA time-course profiles of WTPT (●) and PT6 (○) activation. Data points are shown in 10 min intervals for the purpose of differentiating between line plots. Reactions were activated by 20 pM FXa in the presence of 20 nM FVa and 50 μM PCPS.

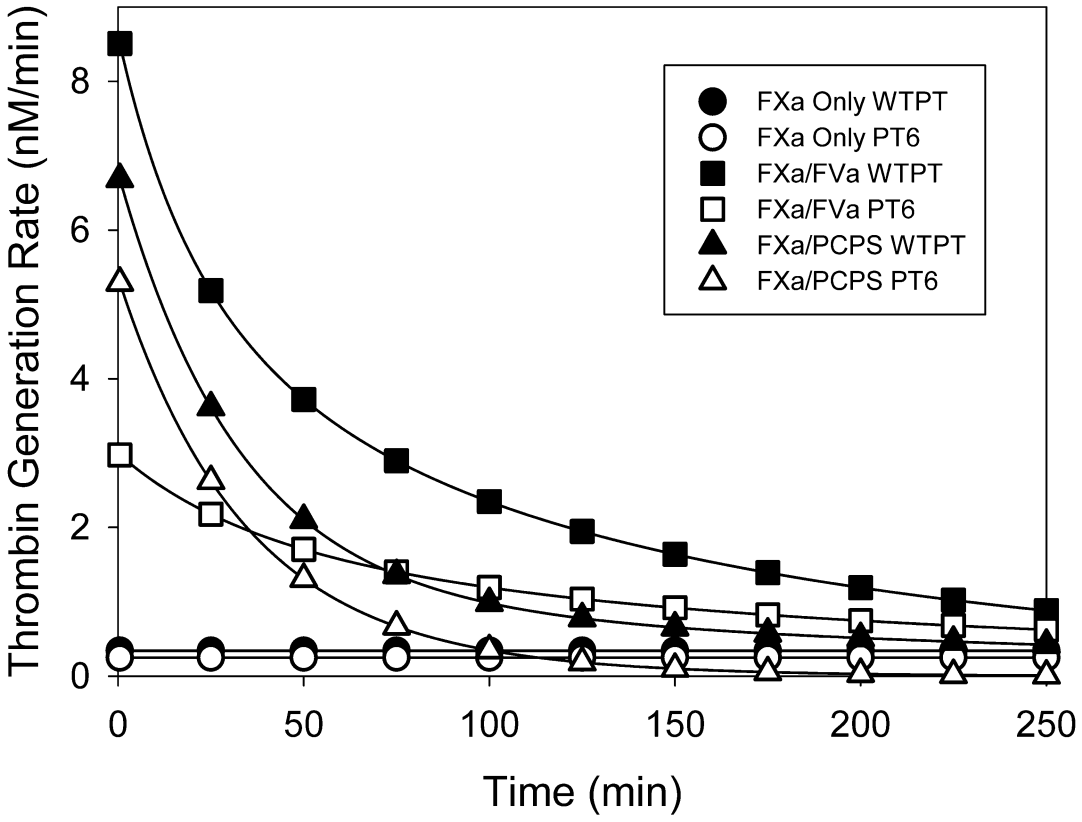

Figure 14 - Thrombin generation rates by upon activation by FXa, FXa/FVa and FXa/PCPS.

Lines represent rates of thrombin generation, calculated using 0.5 min intervals of DAPA time-course profiles of prothrombin activation. Data points are shown in 25 min intervals for the purpose of differentiating between line plots. WTPT is represented by closed symbols and PT6 by open symbols, as described in the figure legend. Reactions were activated by 5 nM FXa either alone (FXa only), with either 20 nM FVa (FXa/FVa) or with 50 μM PCPS (FXa/PCPS).

Table 5 – Quantitation of thrombin generation.

Area under curves were calculated in 0.5 min intervals for plots of thrombin generation rate against time shown above. Area under curves between 0-250 min were calculated. These values represent total thrombin generation (nM) shown in this table.

Prothrombin Activator		Thrombin Generation (nM)
FXa/FVa/PCPS	WTPT	953.2 ± 91.7
	PT6	545.4 ± 67.7
	p	<0.005
FXa/FVa	WTPT	563.7 ± 52.8
	PT6	278.0 ± 94.7
	p	<0.005
FXa/PCPS	WTPT	342.5 ± 120.9
	PT6	247.1 ± 40.1
	p	0.09
FXa	WTPT	71.7 ± 33.6
	PT6	58.2 ± 25.8
	p	0.2

and 51% less thrombin when activated by FXa/FVa ($p<0.005$). Thrombin generation was not significantly reduced in PT6 upon activation by FXa/PCPS or FXa alone.

WTPT and PT6 were also activated under limiting FVa conditions (Figure 15). WTPT or PT6 (1 μM) were activated by FXa/FVa/PCPS (1 nM FXa, 20 pM FVa). The total thrombin generation was significantly decreased in PT6 compared with WTPT (WTPT: 935.1 ± 35.9 nM, PT6: 665.6 ± 13.4 nM, $p<0.005$).

Activation of WTPT and PT6 on Thrombin-Activated Platelets

Activation of prothrombin species by the prothrombinase complex on thrombin-activated platelets (Figure 16, Table 6) showed similar kinetics to activation on PCPS vesicles (Table 1). The catalytic efficiency of PT6 activation was significantly lower than WTPT activation, by roughly 50%. This was due to a 1.45-fold increase in the K_m value of PT6 compared to WTPT. The overall k_{cat} and catalytic efficiency of prothrombin activation on platelets was roughly 10-fold lower than activation on PCPS vesicles. Overall, the kinetic differences between WTPT and PT6 activation is consistent with our previous findings on PCPS vesicles.

SDS-PAGE time-course analysis of prothrombin activation on platelets did not show much difference between WTPT and PT6 (Figure 17). Intermediates were not clearly visible except for a slight accumulation of Pre2 in the later time points of WTPT activation, which was not observed in PT6 activation. Meizothrombin was not able to be quantified from these analyses. PT6 showed a slightly reduced extent of activation, with less prothrombin consumption and less B-chain and F1.2 formation.

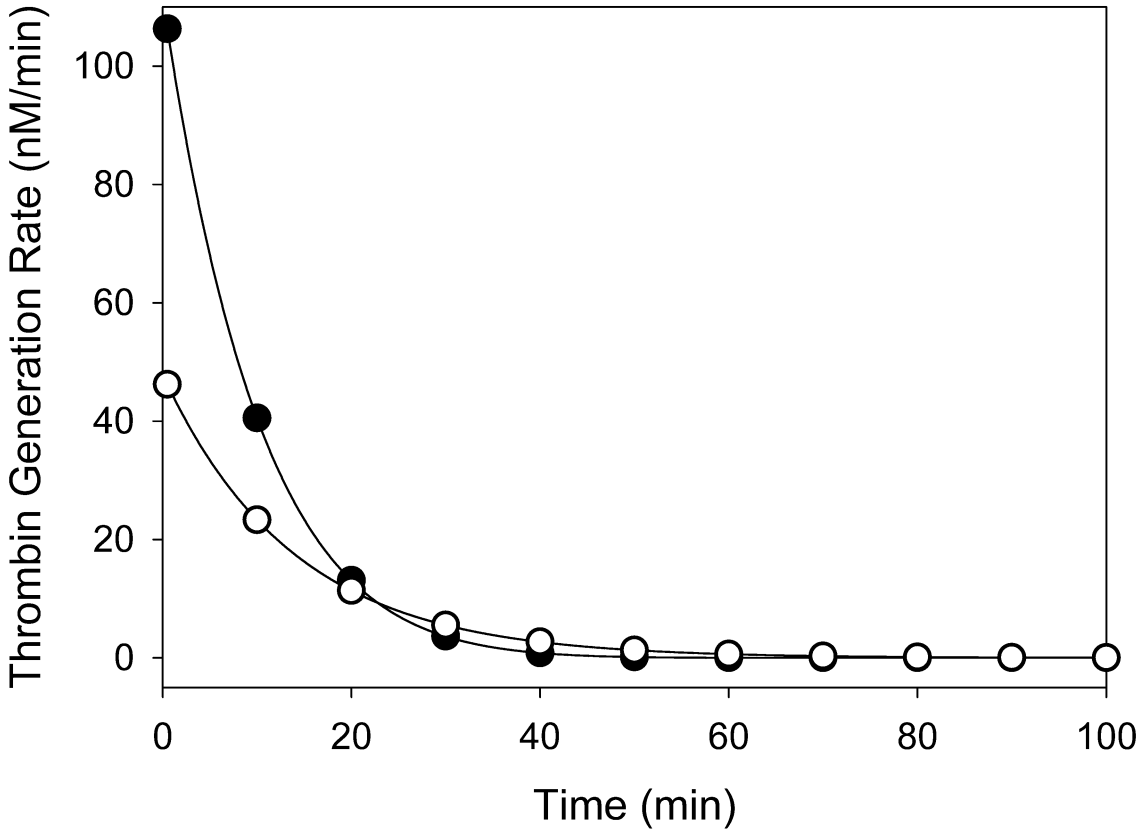

Figure 15 - Thrombin generation rates by upon activation by prothrombinase under limiting FVa conditions.

Lines represent rates of thrombin generation, calculated using 0.5 min intervals of DAPA time-course profiles of WTPT (●) and PT6 (○) activation. Data points are shown in 10 min intervals for the purpose of differentiating between line plots. Reactions were activated by 1 nM FXa in the presence of 20 pM FVa and 50 μM PCPS.

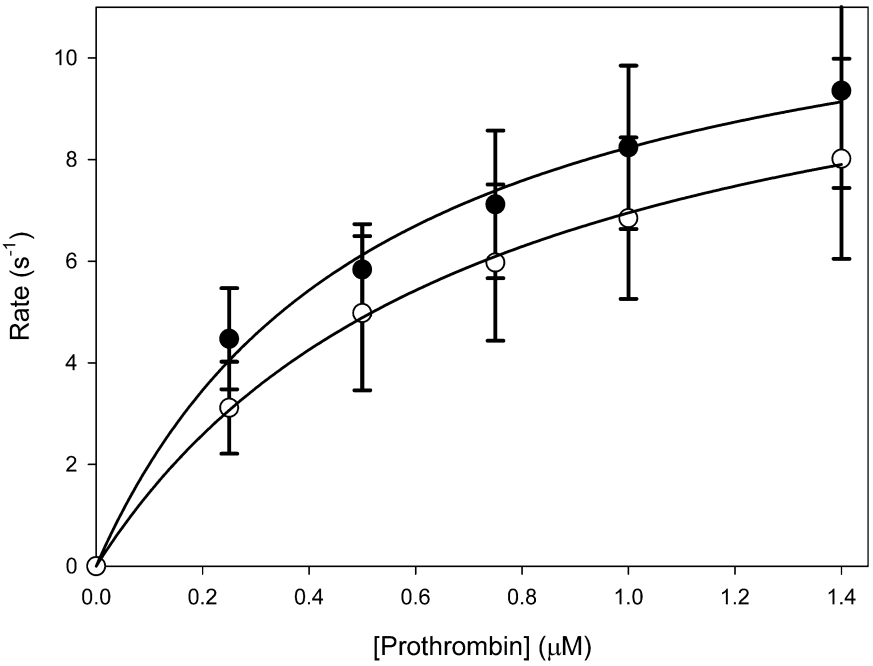

Figure 16 - Prothrombin activation by prothrombinase on the activated platelet surface.

Initial rates of thrombin generation were determined from the fluorescence profiles using Equation 1 at varying WTPT (●) and PT6 (○) concentrations and were plotted with respect to the initial prothrombin concentrations. Non-linear regression was performed to estimate the kinetic parameters k_{cat} and K_m, which were then used to calculate the catalytic efficiency (k_{cat}/K_m). Each point represents at four independent trials, in duplicates. The error bars represent standard deviation.

Table 6 - Kinetic Parameters of WTPT and PT6 Activation on Thrombin-Activated Platelets.

Non-linear regression was used to estimate the k_{cat} and K_m values. Catalytic efficiency was calculated by dividing k_{cat} by K_m.

Prothrombin Activator		k_{cat} s^{-1}	K_m μM	Catalytic Efficiency M^{-1} s^{-1}
FXa, $CaCl_2$, FVa, Thrombin-Activated Platelets	PTWT	12.48 ± 2.85	0.51 ± 0.17	(2.26 ± 0.37) x 10^7
	PT6	11.86 ± 3.02	0.74 ± 0.27	(1.11 ± 0.20) x10^7
	p	0.96	<0.05	< 0.05

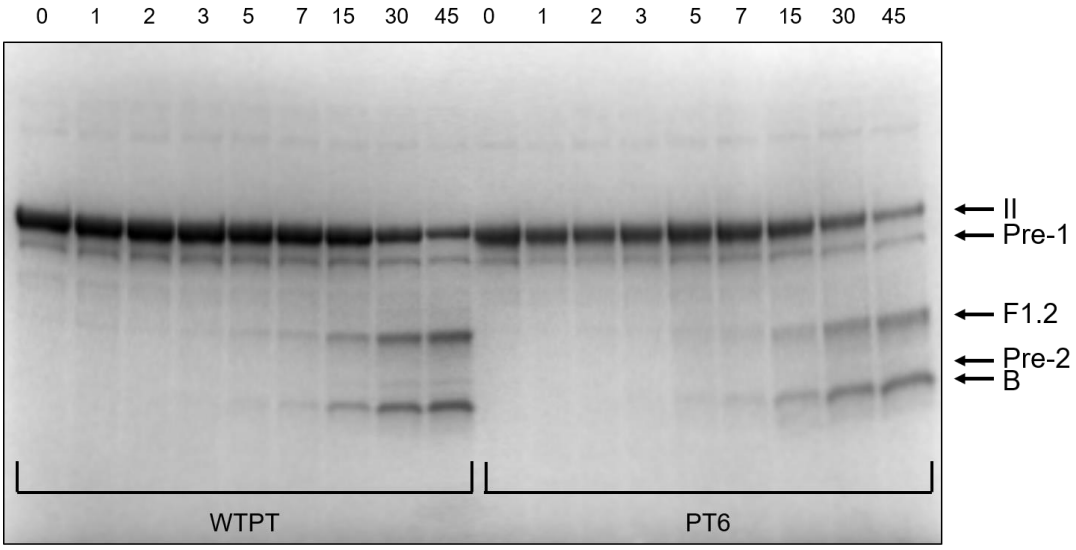

Figure 17 - SDS-PAGE time-course analysis of prothrombin activation by the prothrombinase complex on the activated platelet surface.

WTPT or PT6 (1.4 μM) was incubated with thrombin-activated platelets (5×10^{-7} mL^{-1}), $CaCl_2$ (5 mM), DAPA (10 μM) in HBST. Reactions were initiated by the addition of FXa (20 nM) at 25ºC. Samples were removed at indicated times by adding into 0.134 N acetic acid and centrifuged for 10 min at 10,000 RPM to remove platelets. Contents resolved by SDS-PAGE under reducing conditions.

VI. Discussion

Our experiments comparing the activation of WTPT to the mutant PT6 have given insight into the role that kringle domain 2 plays during prothrombin activation. Initial experiments showed significantly altered kinetic parameters in both the presence and absence of FVa. We observed a reduction in mIIa formation in PT6 compared with WTPT upon activation by prothrombinase, showing that cleavage at Arg^{320} appears to be less efficient in PT6 compared with WTPT. This notion is strengthened by the greater accumulation of Pre2, despite lower prothrombin cleavage in PT6 upon activation in the absence of FVa.

Membrane-dependent effects were considered, as the decreases in the overall catalytic efficiencies were due to increases in K_m, a parameter thought to be most affected by the protein-membrane interaction within prothrombinase (Rosing, Zwaal, and Tans 1986). To test the possible effect of this mutation on membrane binding, prothrombin DAPA fluorescence activation assays were carried out using FXa/FVa without a membrane surface, and by FXa alone as the activator (Table 3). The differences in catalytic efficiency between PT6 and WTPT were not significantly different in these reactions. Loss of the membrane surface resulted in loss of significant differences in catalytic efficiency between WTPT and PT6, thus part of the altered activation potential of PT6 can be attributed to altered membrane interactions. One possible explanation for this involves the binding of prothrombin fragment 2 to Pre2. After cleavage at Arg^{271} Pre2 has been shown to bind fragment 2 (and therefore F1.2) with high affinity, bridging Pre2 to the membrane surface via the GLA domain in fragment 1. This occurs through the

anionic binding exosite II region of thrombin (Kamath and Krishnaswamy 2008). This is a key binding interaction as the membrane binding plays an important role in the ability of prothrombinase to cleave substrates (Kamath and Krishnaswamy 2008). The binding of F1.2 to Pre2 substantially increases cleavage at Arg^{320} by prothrombinase (Kamath and Krishnaswamy 2008), so the loss of this binding interaction in PT6 is a plausible explanation for the decreased prothrombin cleavage and buildup of Pre2 observed in PT6 activation without FVa (Figure 11, Figure 12).

Initial experiments were likely affected by substantial accumulation of Pre1. Figure 12 shows that in the absence of FVa, more prothrombin is actually converted to Pre1 than is converted to B-chain and F1.2. Cleavage at Arg^{155} forms Pre1 and results in release of the GLA-containing fragment 1. Blostein *et al.* showed that a fragment spanning the first 46 residues of prothrombin, representing the GLA domain, was able to bind FVa and inhibit prothrombin activation. (Blostein et al. 2000). Under initial experimental conditions where activation was not carried out by prothrombinase, 20 nM FXa was used as the activator, with 20 nM FVa for prothrombin activation by the FXa/FVa complex. Under these conditions where FVa was not in excess, increasing free FXa levels has been shown to increase FXa-mediated cleavage at Arg^{155} (Kamath and Krishnaswamy 2008). In our experiments conducted without membrane surface, fragment 1 may have interfered with FVa, leading to inhibition of prothrombin activation. Another issue with the formation of Pre1 is that it is a poor substrate for cleavage by prothrombinase (Nesheim et al. 1988). Therefore, allowing free FXa to accumulate in our system interferes with data interpretation in two ways: fragment 1 may interfere with FVa

cofactor activity during prothrombin activation, while prothrombin is cleaved to the less suitable substrate Pre1. This would likely cause reaction rates with activation by FXa/FVa to appear lower than expected and may explain why these rates were similar to activation by FXa alone. This introduces uncertainty to prior data obtained from experiments with FXa/FVa, FXa/PCPS and FXa, and was the rationale for conducting these experiments using lowered FXa concentrations.

Experiments without the full prothrombinase complex (i.e. FXa/FVa, FXa/PCPS, FXa only) were carried out in a separate set of experiments using 5 nM FXa and 20 nM FVa to saturate FXa with FVa molecules. When compared with WTPT, initial rates of PT6 activation were decreased by 57% when activated by FXa/FVa ($p<0.005$), 31% when activated by FXa/PCPS ($p<0.005$) and 27% when activated by FXa alone ($p<0.05$). To further investigate these reactions, rates of thrombin generation were plotted against time. Area under the curve was calculated to compare the total thrombin generated under each set of conditions. Values in Table 5 represent total thrombin generation, in nM. Compared with WTPT, PT6 thrombin generation was 43% lower when activated by prothrombinase ($p<0.005$) and 51% lower when activated by FXa/FVa ($p<0.005$). Thrombin generation was not significantly decreased when activated by FXa/PCPS or FXa alone. This data demonstrates that the residues mutated in PT6 inhibit thrombin generation in a FVa-dependent manner. Experiments carried out under limiting FVa conditions yielded consistent results as those under limiting FXa conditions. Absolute values and relative difference of catalytic efficiency between WTPT and PT6 were similar, as was total thrombin generation.

Our findings of decreased catalytic efficiency in the presence and absence of FVa, but not in the absence of PCPS indicate that there is likely a membrane-dependent effect of the mutations in PT6 independent of FVa. When looking at total thrombin generation, however, a FVa-dependent effect is apparent, which is contradictory to the initial rate kinetic analyses. The differences in these findings are likely due to the complexity of prothrombin activation. Prothrombin requires two separate cleavages to form thrombin, allowing two pathways of formation. Cleavage at Arg^{271} has a V_{max} that is 30-fold lower than cleavage at Arg^{320}. However prior cleavage of prothrombin at Arg^{320} drives a conformational change in the substrate, increasing the V_{max} for Arg^{271} cleavage 30-fold (Bianchini et al. 2005). Cleavage of Arg^{320} is believed to be the first step in prothrombin activation by prothrombinase. In the absence of FVa, however, the initial cleavage occurs at Arg^{271}. Channeling has also been observed, where thrombin is generated without the release of intermediates (Boskovic, Bajzar, and Nesheim 2001). Furthermore, prothrombinase as a complex has been postulated to exist in two-equilibrating forms that exhibit a ping-pong-like mechanism in activating prothrombin (Brufatto and Nesheim 2003; Kim and Nesheim 2007). The complexities of prothrombin activation and the cleavage pathway variations that exist make it difficult to rely solely on initial rate analysis to study reactions. Total thrombin generation is a facet less affected by different cleavage pathways and is important to consider when studying prothrombin activation.

An interesting observation was the relative changes in catalytic efficiency between different combinations of prothrombinase constituents. It is heavily entrenched in literature that within the prothrombinase complex, the membrane surface and FVa are

responsible for increasing the efficiency of thrombin formation by 2 and 3 orders of magnitude, respectfully (Rosing, Zwaal, and Tans 1986; Pozzi and Di Cera 2014). Our data is not consistent with these findings. Our initial experiments have shown that activation of prothrombin in the absence of FVa reduces the catalytic efficiency by 4 orders of magnitude, and the absence of a membrane surface reduces the catalytic efficiency by 5 orders of magnitude. Activation by FXa alone also reduced the catalytic efficiency by 5 orders of magnitude compared to prothrombinase. Initial rates of thrombin generation with reduced FXa concentrations (with activation by FXa/FVa, FXa/PCPS, FXa) were compared with the initial rates of thrombin generation by prothrombinase (Table 4). In these experiments, FVa and PCPS appear to have a similar effect on reaction rates, where loss of either causes a decrease in rate by 3 orders of magnitude. Rates of activation by FXa alone are 4 orders of magnitude lower compared to prothrombinase. Overall, our experiments have shown the effect of the full prothrombinase complex compared with FXa alone to be comparable with literature but not the individual effects of FVa and the lipid surface. The concept of membrane and FVa individually increasing thrombin generation efficiency by 2 and 3 orders of magnitude has been criticized by experts in this field of work (Krishnaswamy, personal communication, 2017), who claim that these individual effects are not observed experimentally, only the overall increase upon full assembly of prothrombinase. We are therefore confident that our data trends are not due to artifact and reasonably support our conclusions.

There has been some dispute as to whether the use of PCPS vesicles accurately represents prothrombin activation *in vivo,* as the traditional notion has been that thrombin

generation occurs on activated platelets (Ayombil et al. 2013). However, prothrombin activation experiments carried out on both thrombin-activated platelets and PCPS showed no significant differences as reported by Church *et al*., who concluded that upon assembly of prothrombinase, orientation and presentation of kringle 2 was identical on PCPS and activated platelets (Church, Ouellette, and Messier 1991).

Work done by Tracy and coworkers has shown that activation of prothrombin by prothrombinase on the activated platelet surface may proceed solely through the Pre2 pathway, with an absence of mIIa formation (Haynes et al. 2012; Wood et al. 2011). However, a number of studies have demonstrated that other activated cell surfaces likely play a much larger role than previously thought (Ivanciu, Krishnaswamy, and Camire 2014; Atkinson et al. 2010; Cooley and Herrera 2013).

Laser-induced vessel wall injury leads to rapid activation of the endothelium, preceding platelet accumulation *in vivo*. Laser injury of endothelial cells *in vitro* also leads to rapid activation of injured cells and of those immediately surrounding the site of injury (Atkinson et al. 2010). Work published by the Krishnaswamy and Camire research groups showed localization of FXa and FVa to occur on activated endothelium upon laser-induced vascular injury, independent of activated platelets. They found the majority of platelet-bound FXa and FVa to be located at the core injury site and that dramatic reduction in platelet adhesion led to only minor decreases in FXa/FVa binding (Ivanciu, Krishnaswamy, and Camire 2014). Krishnaswamy and Camire therefore concluded that non-platelet membrane surfaces, such as activated endothelial cells, microparticles and

neutrophils likely play a much larger role than previously thought (Ivanciu, Krishnaswamy, and Camire 2014).

Platelets are still believed to play the most significant role in providing a procoagulant surface, facilitating prothrombinase assembly *in vivo* (Ivanciu, Krishnaswamy, and Camire 2014). We therefore conducted experiments using thrombin-activated platelets to test for differences in activation kinetics compared to PCPS vesicles. Similar trends were observed in these experiments, where catalytic efficiency of PT6 activation was half that of WTPT, with no significant change in k_{cat} and a 1.45-fold increase in K_m. Intermediates were not able to be quantified by SDS-PAGE analysis.

Our experiments demonstrate that mutation of residues Gln^{177}, Gln^{179}, Arg^{181}, Leu^{182}, Val^{184} and Thr^{185} on prothrombin leads to significantly decreased catalytic efficiency, due to increases in K_m and was found to be consistent on both synthetic PCPS vesicles and thrombin-activated platelets. Part of this effect is likely due to changes in the membrane-binding capability of prothrombin, possibly through its ability to find F1.2 upon initial cleavage at Arg^{271}. It appears that the ability of FXa to cleave Arg^{320} on prothrombinase is decreased in PT6 compared with WTPT. Measuring total thrombin formation during various reaction conditions has shown that mutation of these residues causes a substantial FVa-dependent reduction in thrombin generation. Taking our findings together with previous peptidyl mimicry and NMR data from Nicole Brufatto and Michael Nesheim, we conclude that prothrombin residues Gln^{177}, Gln^{179}, Arg^{181}, Leu^{182}, Val^{184} and Thr^{185}on fragment 2 are important in expressing FVa cofactor activity during prothrombin activation by FXa.

VII. Future Directions

1. Replicate time-course activation with activated platelets using radiolabeled prothrombin with phosphorimaging of SDS-PAGE gels. This has been used in literature to more accurately quantify intermediates via SDS-PAGE analysis.
2. Generate two new mutants of prothrombin using PT6/155 as a template, substituting individually, Arg^{271} or Arg^{320} with alanine.
    - Test the ability of PT6 to be cleaved at the individual activation cleavage sites.
3. Replicate experiments with activated platelets in the presence of APC to test for FVa-independent effects of mutations on the activated platelet surface.

References

Adams, Ty E., and James A. Huntington. 2015. "Structural Transitions during Prothrombin Activation: On the Importance of Fragment 2." *Biochimie*. Elsevier B.V, 4–11. doi:10.1016/j.biochi.2015.09.013.

Atkinson, Ben T, Reema Jasuja, Vivien M Chen, Prathima Nandivada, Bruce Furie, and Barbara C Furie. 2010. "Laser-Induced Endothelial Cell Activation Supports Fibrin Formation." *Blood* 116 (22): 4675–83. doi:10.1182/blood-2010-05-283986.

Ayombil, F., S. Abdalla, P. B. Tracy, and B. A. Bouchard. 2013. "Proteolysis of Plasma-Derived Factor V Following Its Endocytosis by Megakaryocytes Forms the Platelet-Derived Factor V/Va Pool." *Journal of Thrombosis and Haemostasis* 11 (8). NIH Public Access: 1532–39. doi:10.1111/jth.12307.

Baroni, M, G Pavani, M Pinotti, A Branchini, F Bernardi, and R M Camire. 2015. "Asymmetric Processing of Mutant Factor X Arg386Cys Reveals Differences between Intrinsic and Extrinsic Pathway Activation." *Biochimica et Biophysica Acta* 1854 (10 Pt A): 1351–56. doi:10.1016/j.bbapap.2015.05.012.

Bianchini, Elsa P, Virginie B Louvain, Pierre-Emmanuel Marque, Maria A Juliano, Luiz Juliano, and Bernard F Le Bonniec. 2002. "Mapping of the Catalytic Groove Preferences of Factor Xa Reveals an Inadequate Selectivity for Its Macromolecule Substrates." *The Journal of Biological Chemistry* 277 (23): 20527–34. doi:10.1074/jbc.M201139200.

Bianchini, Elsa P, Steven J Orcutt, Peter Panizzi, Paul E Bock, and Sriram Krishnaswamy. 2005. "Ratcheting of the Substrate from the Zymogen to Proteinase Conformations Directs the Sequential Cleavage of Prothrombin by Prothrombinase." *Proceedings of the National Academy of Sciences of the United States of America* 102 (29): 10099–104. doi:10.1073/pnas.0504704102.

Bloom, J W, M.E. Nesheim, and Kenneth G. Mann. 1979. "Phospholipid-Binding Properties of Bovine Factor V and Factor Va." *Biochemistry* 18 (20): 4419–25. http://www.ncbi.nlm.nih.gov/pubmed/486430.

Blostein, M D, A C Rigby, M Jacobs, B Furie, and B C Furie. 2000. "The Gla Domain of Human Prothrombin Has a Binding Site for Factor Va." *The Journal of Biological Chemistry* 275 (48): 38120–26. doi:10.1074/jbc.M007174200.

Boskovic, D S, L S Bajzar, and M.E. Nesheim. 2001. "Channeling during Prothrombin Activation." *The Journal of Biological Chemistry* 276 (31): 28686–93. doi:10.1074/jbc.M101813200.

Bradford, Harlan N, Steven J Orcutt, and Sriram Krishnaswamy. 2013. "Membrane Binding by Prothrombin Mediates Its Constrained Presentation to Prothrombinase for Cleavage." *The Journal of Biological Chemistry* 288 (39): 27789–800. doi:10.1074/jbc.M113.502005.

Brufatto, Nicole, and M.E. Nesheim. 2003. “Analysis of the Kinetics of Prothrombin Activation and Evidence That Two Equilibrating Forms of Prothrombinase Are Involved in the Process.” *The Journal of Biological Chemistry* 278 (9): 6755–64. doi:10.1074/jbc.M206413200.

Buddai, Sai K, Larisa Toulokhonova, Peter W Bergum, George P Vlasuk, and Sriram Krishnaswamy. 2002. “Nematode Anticoagulant Protein c2 Reveals a Site on Factor Xa That Is Important for Macromolecular Substrate Binding to Human Prothrombinase.” *The Journal of Biological Chemistry* 277 (29). American Society for Biochemistry and Molecular Biology: 26689–98. doi:10.1074/jbc.M202507200.

Butenas, Saulius, and Kenneth G. Mann. 2002. “Blood Coagulation.” *Biochemistry (Moscow)* 67 (1). Kluwer Academic Publishers-Plenum Publishers: 3–12. doi:10.1023/A:1013985911759.

Butenas, Saulius, Thomas Orfeo, Kathleen E. Brummel-Ziedins, and Kenneth G. Mann. 2007. “Tissue Factor in Thrombosis and Hemorrhage.” *Surgery* 142 (4). Elsevier: S2–14. doi:10.1016/j.surg.2007.06.032.

Chen, Lin, Likui Yang, and Alireza R Rezaie. 2003. “Proexosite-1 on Prothrombin Is a Factor Va-Dependent Recognition Site for the Prothrombinase Complex.” *The Journal of Biological Chemistry* 278 (30): 27564–69. doi:10.1074/jbc.M302707200.

Chevreux, Guillaume, Nolwenn Tilly, Valegh Faid, and Nicolas Bihoreau. 2015. “Mass Spectrometry Based Analysis of Human Plasma-Derived Factor X Revealed Novel Post-Translational Modifications.” *Protein Science* 24 (10). Wiley-Blackwell: 1640–48. doi:10.1002/pro.2756.

Church, W R, L a Ouellette, and T L Messier. 1991. “Modulation of Human Prothrombin Activation on Phospholipid Vesicles and Platelets Using Monoclonal Antibodies to Prothrombin Fragment 2.” *The Journal of Biological Chemistry* 266 (13): 8384–91.

Cooley, Brian C, and Anthony J Herrera. 2013. “Cross-Modulatory Effects of Clopidogrel and Heparin on Platelet and Fibrin Incorporation in Thrombosis.” *Blood Coagulation & Fibrinolysis : An International Journal in Haemostasis and Thrombosis* 24 (6): 593–98. doi:10.1097/MBC.0b013e3283602a03.

Danckwardt, Sven, Matthias W. Hentze, and Andreas E. Kulozik. 2013. “Pathologies at the Nexus of Blood Coagulation and Inflammation: Thrombin in Hemostasis, Cancer, and beyond.” *Journal of Molecular Medicine* 91 (11): 1257–71. doi:10.1007/s00109-013-1074-5.

Das, R, and E F Plow. 2011. “Phosphatidylserine as an Anchor for Plasminogen and Its Plasminogen Receptor, Histone H2B, to the Macrophage Surface.” *Journal of Thrombosis and Haemostasis : JTH* 9 (2). NIH Public Access: 339–49. doi:10.1111/j.1538-7836.2010.04132.x.

Degen, Sandra J. Friezner, and Earl W. Davie. 1987. “Nucleotide Sequence of the Gene

for Human Prothrombin." *Biochemistry* 26 (19). American Chemical Society: 6165–77. doi:10.1021/bi00393a033.

Deguchi, H, H Takeya, E C Gabazza, J Nishioka, and K Suzuki. 1997. "Prothrombin Kringle 1 Domain Interacts with Factor Va during the Assembly of Prothrombinase Complex." *The Biochemical Journal* 321 (3): 729–35. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1218129&tool=pmcentrez&rendertype=abstract.

Dharmawardana, K. R., S. T. Olson, and P. E. Bock. 1999. "Role of Regulatory Exosite I in Binding of Thrombin to Human Factor V, Factor Va, Factor Va Subunits, and Activation Fragments." *Journal of Biological Chemistry* 274 (26): 18635–43. doi:10.1074/jbc.274.26.18635.

Di Scipio, R G, M A Hermodson, S G Yates, and E W Davie. 1977. "A Comparison of Human Prothrombin, Factor IX (Christmas Factor), Factor X (Stuart Factor), and Protein S." *Biochemistry* 16 (4): 698–706. http://www.ncbi.nlm.nih.gov/pubmed/836809.

Dombrose, F A, S N Gitel, K Zawalich, and C M Jackson. 1979. "The Association of Bovine Prothrombin Fragment 1 with Phospholipid. Quantitative Characterization of the Ca2+ Ion-Mediated Binding of Prothrombin Fragment 1 to Phospholipid Vesicles and a Molecular Model for Its Association with Phospholipids." *The Journal of Biological Chemistry* 254 (12): 5027–40. http://www.ncbi.nlm.nih.gov/pubmed/447632.

Guinto, E R, and C T Esmon. 1984. "Loss of Prothrombin and of Factor Xa-Factor Va Interactions upon Inactivation of Factor Va by Activated Protein C." *J. Biol. Chem.* 259 (22): 13986–92. http://www.jbc.org/content/259/22/13986.short.

Gunasekaran, Prasad, and Deepak K Parashara. 2015. "Periprocedural Management of Non-Vitamin K Oral Anticoagulants in Chronic Kidney Disease: A Review of Existing Heterogeneity and Contemporary Evidence." *Journal of Atrial Fibrillation* 8 (4): 1230. doi:10.4022/jafib.1230.

Halliez, Maxime, Marc Fouassier, Nelly Robillard, Catherine Ternisien, Marianne Sigaud, Marc Trossaert, and Marie-Christine Bene. 2015. "Detection of Phosphatidyl Serine on Activated Platelets' Surface by Flow Cytometry in Whole Blood: A Simpler Test for the Diagnosis of Scott Syndrome." *British Journal of Haematology*, March. doi:10.1111/bjh.13391.

Hankins, Hannah M, Ryan D Baldridge, Peng Xu, and Todd R Graham. 2015. "Role of Flippases, Scramblases and Transfer Proteins in Phosphatidylserine Subcellular Distribution." *Traffic* 16 (1). NIH Public Access: 35–47. doi:10.1111/tra.12233.

Haynes, L. M., B. A. Bouchard, P. B. Tracy, and Kenneth G. Mann. 2012. "Prothrombin Activation by Platelet-Associated Prothrombinase Proceeds through the Prethrombin-2 Pathway via a Concerted Mechanism." *Journal of Biological*

Chemistry 287 (46): 38647–55. doi:10.1074/jbc.M112.407791.

Hertzberg, M. 1994. "Biochemistry of Factor X." *Blood Reviews* 8 (1): 56–62. http://www.ncbi.nlm.nih.gov/pubmed/8205010.

Hoffman, M, and D M Monroe. 2001. "A Cell-Based Model of Hemostasis." *Thrombosis and Haemostasis* 85 (6): 958–65. http://europepmc.org/abstract/med/11434702.

Hortin, G L. 1990. "Sulfation of Tyrosine Residues in Coagulation Factor V." *Blood* 76 (5). American Society of Hematology: 946–52. http://www.bloodjournal.org/content/76/5/946.abstract.

Hugenholtz, Greg G C, Robert J Porte, and Ton Lisman. 2009. "The Platelet and Platelet Function Testing in Liver Disease." *Clinics in Liver Disease* 13 (1): 11–20. doi:10.1016/j.cld.2008.09.010.

Ivanciu, Lacramioara, Sriram Krishnaswamy, and Rodney M Camire. 2014. "New Insights into the Spatiotemporal Localization of Prothrombinase in Vivo." *Blood* 124 (11): 1705–14. doi:10.1182/blood-2014-03-565010.

Kalafatis, Michael, and Daniel O Beck. 2002. "Identification of a Binding Site for Blood Coagulation Factor Xa on the Heavy Chain of Factor Va. Amino Acid Residues 323-331 of Factor V Represent an Interactive Site for Activated Factor X." *Biochemistry* 41 (42): 12715–28. http://www.ncbi.nlm.nih.gov/pubmed/12379114.

Kamath, Parvathi, and Sriram Krishnaswamy. 2008. "Fate of Membrane-Bound Reactants and Products during the Activation of Human Prothrombin by Prothrombinase." *The Journal of Biological Chemistry* 283 (44): 30164–73. doi:10.1074/jbc.M806158200.

Kim, Paul Y, and M.E. Nesheim. 2007. "Further Evidence for Two Functional Forms of Prothrombinase Each Specific for Either of the Two Prothrombin Activation Cleavages." *The Journal of Biological Chemistry* 282 (45): 32568–81. doi:10.1074/jbc.M701781200.

Koppaka, V, W F Talbot, X Zhai, and B R Lentz. 1997. "Roles of Factor Va Heavy and Light Chains in Protein and Lipid Rearrangements Associated with the Formation of a Bovine Factor Va-Membrane Complex." *Biophysical Journal* 73 (5): 2638–52. doi:10.1016/S0006-3495(97)78293-6.

Kotkow, K J, S R Deitcher, B Furie, and B C Furie. 1995. "The Second Kringle Domain of Prothrombin Promotes Factor Va-Mediated Prothrombin Activation by Prothrombinase." *The Journal of Biological Chemistry* 270: 4551–57. doi:10.1074/jbc.270.9.4551.

Kotkow, K J, B Furie, and B C Furie. 1993. "The Interaction of Prothrombin with Phospholipid Membranes Is Independent of Either Kringle Domain." *J. Biol. Chem.* 268 (21): 15633–39. http://www.jbc.org/content/268/21/15633.

Krishnaswamy, Sriram. 1990. “Prothrombinase Complex Assembly. Contributions of Protein-Protein and Protein-Membrane Interactions toward Complex Formation.” *The Journal of Biological Chemistry* 265 (7): 3708–18. http://www.ncbi.nlm.nih.gov/pubmed/2303476.

———. 2013. “The Transition of Prothrombin to Thrombin.” *Journal of Thrombosis and Haemostasis* 11 (June): 265–76. doi:10.1111/jth.12217.

Krishnaswamy, Sriram, William R Church, M.E. Nesheim, and Kenneth G. Mann. 1987a. “Activation of Human Prothrombin by Human Prothrombinase. Influence of Factor Va on the Reaction Mechanism.” *The Journal of Biological Chemistry* 262 (7): 3291–99.

Krishnaswamy, Sriram, WR Church, M.E. Nesheim, and Kenneth G. Mann. 1987b. “Activation of Human Prothrombin by Human Prothrombinase. Influence of Factor Va on the Reaction Mechanism.” *J. Biol. Chem.* 262 (7): 3291–99. http://www.jbc.org/content/262/7/3291.short.

Krishnaswamy, Sriram, K C Jones, and Kenneth G. Mann. 1988. “Prothrombinase Complex Assembly. Kinetic Mechanism of Enzyme Assembly on Phospholipid Vesicles.” *The Journal of Biological Chemistry* 263 (8): 3823–34. http://www.ncbi.nlm.nih.gov/pubmed/3346225.

Lancellotti, Stefano, Maria Basso, and Raimondo De Cristofaro. 2013. “Congenital Prothrombin Deficiency: An Update.” *Seminars in Thrombosis and Hemostasis* 39 (6): 596–606. doi:10.1055/s-0033-1348948.

Leytus, S P, D W Chung, W Kisiel, K Kurachi, and E W Davie. 1984. “Characterization of a cDNA Coding for Human Factor X.” *Proceedings of the National Academy of Sciences of the United States of America* 81 (12): 3699–3702. http://www.ncbi.nlm.nih.gov/pubmed/6587384.

Lollar, P. 1996. “Involvement of Thrombin Anion-Binding Exosites 1and 2in the Activation of Factor V and Factor VIII.” *Journal of Biological Chemistry* 271 (23): 13882–87. doi:10.1074/jbc.271.23.13882.

Lottenberg, R, J A Hall, E Pautler, A Zupan, U Christensen, and C M Jackson. 1986. “The Action of Factor Xa on Peptide P-Nitroanilide Substrates: Substrate Selectivity and Examination of Hydrolysis with Different Reaction Conditions.” *Biochimica et Biophysica Acta* 874 (3): 326–36. http://www.ncbi.nlm.nih.gov/pubmed/3790574.

Macedo-Ribeiro, S, W Bode, R Huber, M A Quinn-Allen, S W Kim, T L Ortel, G P Bourenkov, et al. 1999. “Crystal Structures of the Membrane-Binding C2 Domain of Human Coagulation Factor V.” *Nature* 402 (6760). Nature Publishing Group: 434–39. doi:10.1038/46594.

Majumder, Rinku, Mary Ann Quinn-Allen, William H Kane, and Barry R Lentz. 2008. “A Phosphatidylserine Binding Site in Factor Va C1 Domain Regulates Both

Assembly and Activity of the Prothrombinase Complex." *Blood* 112 (7). American Society of Hematology: 2795–2802. doi:10.1182/blood-2008-02-138941.

Malhotra, O P, M.E. Nesheim, and Kenneth G. Mann. 1985. "The Kinetics of Activation of Normal and Gamma-Carboxyglutamic Acid-Deficient Prothrombins." *The Journal of Biological Chemistry* 260 (1): 279–87. http://www.ncbi.nlm.nih.gov/pubmed/2578125.

Mann, Kenneth G., Richard J. Jenny, and Sriram Krishnaswamy. 1988. "Cofactor Proteins in the Assembly and Expression of Blood Clotting Enzyme Complexes." *Annual Review of Biochemistry* 57 (1): 915–56. doi:10.1146/annurev.bi.57.070188.004411.

Mann, Kenneth G., Sriram Krishnaswamy, and J H Lawson. 1992. "Surface-Dependent Hemostasis." *Seminars in Hematology* 29 (3): 213–26. http://www.ncbi.nlm.nih.gov/pubmed/1641667.

Miao, C H, S P Leytus, D W Chung, and E W Davie. 1992. "Liver-Specific Expression of the Gene Coding for Human Factor X, a Blood Coagulation Factor." *J. Biol. Chem.* 267 (11): 7395–7401. http://www.jbc.org.libaccess.lib.mcmaster.ca/content/267/11/7395.

Myles, Timothy, Thomas H. Yun, Scott W. Hall, and Lawrence L K Leung. 2001. "An Extensive Interaction Interface between Thrombin and Factor V Is Required for Factor V Activation." *Journal of Biological Chemistry* 276 (27): 25143–49. doi:10.1074/jbc.M011324200.

Myles, Timothy, Thomas H Yun, and Lawrence L K Leung. 2002. "Structural Requirements for the Activation of Human Factor VIII by Thrombin." *Blood* 100 (8). American Society of Hematology: 2820–26. doi:10.1182/blood-2002-03-0843.

Nesheim, M.E., T Abbott, R Jenny, and Kenneth G. Mann. 1988. "Evidence That the Thrombin-Catalyzed Feedback Cleavage of Fragment 1.2 at Arg154-Ser155 Promotes the Release of Thrombin from the Catalytic Surface during the Activation of Bovine Prothrombin." *The Journal of Biological Chemistry* 263 (2): 1037–44. http://www.ncbi.nlm.nih.gov/pubmed/3422076.

Nesheim, M.E., L.S. Hibbard, P.B. Tracy, J.W. Bloom, K.H Myrmel, and Kenneth G. Mann. 1980. *The Regulation of Coagulation*. New York: Elsevier.

Nesheim, M.E., Jerry A. Katzmann, Paula B. Tracy, and Kenneth G. Mann. 1981. "Factor V." *Methods in Enzymology* 80: 249–74. doi:10.1016/S0076-6879(81)80023-7.

Nesheim, M.E., C Kettner, E Shaw, and Kenneth G. Mann. 1981. "Cofactor Dependence of Factor Xa Incorporation into the Prothrombinase Complex." *J. Biol. Chem.* 256 (13): 6537–40. http://www.jbc.org/content/256/13/6537.short.

Nesheim, M.E., F G Prendergast, and Kenneth G. Mann. 1979. "Interactions of a Fluorescent Active-Site-Directed Inhibitor of Thrombin: Dansylarginine N-(3-Ethyl-

1,5-Pentanediyl)amide." *Biochemistry* 18 (6): 996–1003. http://www.ncbi.nlm.nih.gov/pubmed/427102.

Neuenschwander, P F, M M Fiore, and J H Morrissey. 1993. "Factor VII Autoactivation Proceeds via Interaction of Distinct Protease-Cofactor and Zymogen-Cofactor Complexes. Implications of a Two-Dimensional Enzyme Kinetic Mechanism." *The Journal of Biological Chemistry* 268 (29): 21489–92. http://www.ncbi.nlm.nih.gov/pubmed/8407997.

Peña, E., G. Arderiu, and L. Badimon. 2012. "Subcellular Localization of Tissue Factor and Human Coronary Artery Smooth Muscle Cell Migration." *Journal of Thrombosis and Haemostasis* 10 (11). Blackwell Publishing Ltd: 2373–82. doi:10.1111/j.1538-7836.2012.04910.x.

Petrovan, R J, and W Ruf. 2001. "Residue Met(156) Contributes to the Labile Enzyme Conformation of Coagulation Factor VIIa." *The Journal of Biological Chemistry* 276 (9): 6616–20. doi:10.1074/jbc.M004726200.

Pinotti, M, G Marchetti, M Baroni, F Cinotti, M Morfini, and F Bernardi. 2002. "Reduced Activation of the Gla19Ala FX Variant via the Extrinsic Coagulation Pathway Results in Symptomatic CRMred FX Deficiency." *Thrombosis and Haemostasis* 88 (2): 236–41. http://www.ncbi.nlm.nih.gov/pubmed/12195695.

Pittman, Debra D., Kathleen N. Tomkinson, Donna Michnick, Uri Selighsohn, and Randal J. Kaufman. 1994. "Posttranslational Sulfation of Factor V Is Required for Efficient Thrombin Cleavage and Activation and for Full Procoagulant Activity." *Biochemistry* 33 (22). American Chemical Society: 6952–59. doi:10.1021/bi00188a026.

Pozzi, Nicola, Z. Chen, D. W. Gohara, W. Niu, T. Heyduk, and E. Di Cera. 2013. "Crystal Structure of Prothrombin Reveals Conformational Flexibility and Mechanism of Activation." *Journal of Biological Chemistry* 288 (31): 22734–44. doi:10.1074/jbc.M113.466946.

Pozzi, Nicola, Zhiwei Chen, Leslie A Pelc, Daniel B Shropshire, and Enrico Di Cera. 2014. "The Linker Connecting the Two Kringles Plays a Key Role in Prothrombin Activation." *Proceedings of the National Academy of Sciences of the United States of America* 111 (21): 7630–35. doi:10.1073/pnas.1403779111.

Pozzi, Nicola, and Enrico Di Cera. 2014. "Prothrombin Structure: Unanticipated Features and Opportunities." *Expert Review of Proteomics* 11 (6): 653–55. doi:10.1586/14789450.2014.971763.

Qureshi, Shabir H, Likui Yang, Chandrashekhara Manithody, and Alireza R Rezaie. 2009. "Membrane-Dependent Interaction of Factor Xa and Prothrombin with Factor Va in the Prothrombinase Complex." *Biochemistry* 48 (22): 5034–41. doi:10.1021/bi900240g.

Raskob, G. E., P. Angchaisuksiri, A. N. Blanco, H. Buller, A. Gallus, B. J. Hunt, E. M. Hylek, et al. 2014. “Thrombosis: A Major Contributor to Global Disease Burden.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 34 (11): 2363–71. doi:10.1161/ATVBAHA.114.304488.

Renné, Thomas, and David Gailani. 2007. “Role of Factor XII in Hemostasis and Thrombosis: Clinical Implications.” *Expert Review of Cardiovascular Therapy* 5 (4). Expert Reviews Ltd.: 733–41. doi:10.1586/14779072.5.4.733.

Rodgers, G M, and M A Shuman. 1983. “Prothrombin Is Activated on Vascular Endothelial Cells by Factor Xa and Calcium.” *Proceedings of the National Academy of Sciences of the United States of America* 80 (22): 7001–5. http://www.ncbi.nlm.nih.gov/pubmed/6580622.

Rosing, J, EM Bevers, P Comfurius, HC Hemker, G van Dieijen, HJ Weiss, and RF Zwaal. 1985. “Impaired Factor X and Prothrombin Activation Associated with Decreased Phospholipid Exposure in Platelets from a Patient with a Bleeding Disorder.” *Blood* 65 (6). http://www.bloodjournal.org/content/65/6/1557.short?sso-checked=true.

Rosing, J, R F Zwaal, and G Tans. 1986. “Formation of Meizothrombin as Intermediate in Factor Xa-Catalyzed Prothrombin Activation.” *The Journal of Biological Chemistry* 261 (9): 4224–28. http://www.ncbi.nlm.nih.gov/pubmed/3753979.

Rudolph, A E, M P Mullane, R Porche-Sorbet, S Tsuda, and J P Miletich. 1996. “Factor XSt. Louis II. Identification of a Glycine Substitution at Residue 7 and Characterization of the Recombinant Protein.” *The Journal of Biological Chemistry* 271 (45): 28601–6. http://www.ncbi.nlm.nih.gov/pubmed/8910490.

Scambler, P J, and R Williamson. 1985. “The Structural Gene for Human Coagulation Factor X Is Located on Chromosome 13q34.” *Cytogenetics and Cell Genetics* 39 (3): 231–33. http://www.ncbi.nlm.nih.gov/pubmed/4042693.

Schuijt, T. J., K. Bakhtiari, S. Daffre, K. DePonte, S. J. H. Wielders, J. A. Marquart, J. W. Hovius, et al. 2013. “Factor Xa Activation of Factor V Is of Paramount Importance in Initiating the Coagulation System: Lessons From a Tick Salivary Protein.” *Circulation* 128 (3): 254–66. doi:10.1161/CIRCULATIONAHA.113.003191.

Skogen, W F, C T Esmon, and A C Cox. 1984. “Comparison of Coagulation Factor Xa and Des-(1-44)factor Xa in the Assembly of Prothrombinase.” *The Journal of Biological Chemistry* 259 (4): 2306–10. http://www.ncbi.nlm.nih.gov/pubmed/6698966.

Soriano-Garcia, M, C H Park, A Tulinsky, K G Ravichandran, and E Skrzypczak-Jankun. 1989. “Structure of Ca2+ Prothrombin Fragment 1 Including the Conformation of the Gla Domain.” *Biochemistry* 28 (17): 6805–10. http://www.ncbi.nlm.nih.gov/pubmed/2819034.

Steen, Mårten. 2002. “Factor Va-Factor Xa Interactions: Molecular Sites Involved in Enzyme:cofactor Assembly.” *Scandinavian Journal of Clinical and Laboratory Investigation. Supplementum* 237 (January): 5–11. http://www.ncbi.nlm.nih.gov/pubmed/12570161.

Steen, Mårten, Sinh Tran, Ludovic Autin, Bruno O Villoutreix, Ann-Louise Tholander, and Björn Dahlbäck. 2008. “Mapping of the Factor Xa Binding Site on Factor Va by Site-Directed Mutagenesis.” *The Journal of Biological Chemistry* 283 (30): 20805–12. doi:10.1074/jbc.M802703200.

Steen, Mårten, Bruno O Villoutreix, Eva A Norstrøm, Tomio Yamazaki, and Björn Dahlbäck. 2002. “Defining the Factor Xa-Binding Site on Factor Va by Site-Directed Glycosylation.” *Journal of Biological Chemistry* 277 (51): 50022–29. doi:10.1074/jbc.M205609200.

Steiner, Thorsten, Jeffrey I. Weitz, and Roland Veltkamp. 2017. “Anticoagulant-Associated Intracranial Hemorrhage in the Era of Reversal Agents.” *Stroke* 48 (5): 1432–37. doi:10.1161/STROKEAHA.116.013343.

Teitel, J M, and R D Rosenberg. 1983. “Protection of Factor Xa from Neutralization by the Heparin-Antithrombin Complex.” *The Journal of Clinical Investigation* 71 (5): 1383–91. http://www.ncbi.nlm.nih.gov/pubmed/6222069.

Thorelli, E, R J Kaufman, and B Dahlbäck. 1998. “Cleavage Requirements of Factor V in Tissue-Factor Induced Thrombin Generation.” *Thrombosis and Haemostasis* 80 (1): 92–98. http://www.ncbi.nlm.nih.gov/pubmed/9684792.

Thorelli, Elisabeth, Randal J. Kaufman, and Bjorn Dahlback. 1997. “Cleavage Requirements for Activation of Factor V by Factor Xa.” *European Journal of Biochemistry* 247 (1): 12–20. doi:10.1111/j.1432-1033.1997.00012.x.

Tracy, P B, L L Eide, and Kenneth G. Mann. 1985. “Human Prothrombinase Complex Assembly and Function on Isolated Peripheral Blood Cell Populations.” *The Journal of Biological Chemistry* 260 (4): 2119–24. http://www.ncbi.nlm.nih.gov/pubmed/3972783.

Vanden Hoek, A. L., K. Talbot, I. S. R. Carter, L. Vickars, C. J. Carter, S. C. Jackson, R. T. A. MacGillivray, and E. L. G. Pryzdial. 2012. “Coagulation Factor X Arg386 Specifically Affects Activation by the Intrinsic Pathway: A Novel Patient Mutation.” *Journal of Thrombosis and Haemostasis* 10 (12): 2613–15. doi:10.1111/jth.12021.

Versteeg, H. H., J. W. M. Heemskerk, M. Levi, and P. H. Reitsma. 2013. “New Fundamentals in Hemostasis.” *Physiological Reviews* 93 (1): 327–58. doi:10.1152/physrev.00016.2011.

Walker, RK, and Sriram Krishnaswamy. 1993. “The Influence of Factor Va on the Active Site of Factor Xa.” *J. Biol. Chem.* 268 (19): 13920–29. http://www.jbc.org.libaccess.lib.mcmaster.ca/content/268/19/13920.

Walsh, Peter N, and Syed S Ahmad. 2002. “Proteases in Blood Clotting.” *Essays in Biochemistry* 38 (January): 95–111. http://www.ncbi.nlm.nih.gov/pubmed/12463164.

Wang, H, D C Riddell, E R Guinto, R T MacGillivray, and J L Hamerton. 1988. “Localization of the Gene Encoding Human Factor V to Chromosome 1q21-25.” *Genomics* 2 (4): 324–28. http://www.ncbi.nlm.nih.gov/pubmed/3220473.

Wang, Yiming, Adili Reheman, Christopher M Spring, Jalil Kalantari, Alexandra H Marshall, Alisa S Wolberg, Peter L Gross, et al. 2014. “Plasma Fibronectin Supports Hemostasis and Regulates Thrombosis.” *The Journal of Clinical Investigation* 124 (10): 4281–93. doi:10.1172/JCI74630.

Wiencek, Joesph R, Mahesheema Na, Jamila Hirbawi, and Michael Kalafatis. 2013. “Amino Acid Region 1000-1008 of Factor V Is a Dynamic Regulator for the Emergence of Procoagulant Activity.” *The Journal of Biological Chemistry* 288 (52): 37026–38. doi:10.1074/jbc.M113.462374.

Wood, Jeremy P, Jay R Silveira, Nicole M Maille, Laura M Haynes, and Paula B Tracy. 2011. “Prothrombin Activation on the Activated Platelet Surface Optimizes Expression of Procoagulant Activity.” *Blood* 117 (5): 1710–18. doi:10.1182/blood-2010-09-311035.

Wu, Q. Y., J. P. Sheehan, M. Tsiang, S. R. Lentz, J. J. Birktoft, and J. E. Sadler. 1991. “Single Amino Acid Substitutions Dissociate Fibrinogen-Clotting and Thrombomodulin-Binding Activities of Human Thrombin.” *Proceedings of the National Academy of Sciences* 88 (15): 6775–79. doi:10.1073/pnas.88.15.6775.

Xiong, Qinmei, Yee Cheng Lau, Keitaro Senoo, Deirdre A. Lane, Kui Hong, and Gregory Y.H. Lip. 2015. “Non-Vitamin K Antagonist Oral Anticoagulants (NOACs) in Patients with Concomitant Atrial Fibrillation and Heart Failure: A Systemic Review and Meta-Analysis of Randomized Trials.” *European Journal of Heart Failure* 17 (11): 1192–1200. doi:10.1002/ejhf.343.

Yegneswaran, Subramanian, J A Fernandez, J H Griffin, and P E Dawson. 2002. “Factor Va Increases the Affinity of Factor Xa for Prothrombin: A Binding Study Using a Novel Photoactivable Thiol-Specific Fluorescent Probe.” *Chem Biol* 9 (4): 485–94. doi:10.1016/s1074-5521(02)00132-1.

Yegneswaran, Subramanian, Rolf M Mesters, José A Fernández, and John H Griffin. 2004. “Prothrombin Residues 473-487 Contribute to Factor Va Binding in the Prothrombinase Complex.” *The Journal of Biological Chemistry* 279 (47): 49019–25. doi:10.1074/jbc.M406645200.

Zwaal, R F A, P Comfurius, and E M Bevers. 2005. “Surface Exposure of Phosphatidylserine in Pathological Cells.” *Cellular and Molecular Life Sciences : CMLS* 62 (9). Birkhäuser-Verlag: 971–88. doi:10.1007/s00018-005-4527-3.