NOVEL MECHANISMS OF FIBRINOLYSIS IN HEALTH AND DISEASE

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Abstract

This is a two-part thesis, focusing first on a clinical and then on a biochemical aspect of fibrinolysis, the process that dissolves blood clots.

Hyperfibrinolysis: An explanation for reduced cardiovascular disease in hemophilia patients

Hemophilia is a coagulation disorder where factor (F) VIII or FIX deficiency results in prolonged bleeding. Therapeutic FVIII or FIX replacement has lengthened hemophilia patient life expectancy. Interestingly, retrospective studies have demonstrated a lower standard mortality risk from cardiovascular disease (CVD) compared to the normal population and enhanced fibrinolytic capability has been proposed as an explanation for this relative protection from CVD. Through the analysis of tissue-type plasminogen activator (tPA), the initiator of fibrinolysis and two inhibitors of fibrinolysis: plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI), the current study showed that some hemophilia patients have reduced inhibition of the fibrinolytic pathway compared to age and cardiovascular risk matched controls. A trend of hyperfibrinolysis was also seen in hemophilia patients through accelerated plasma clot lysis with 50% of the patients having at least a 2-fold enhancement, and 22% with at least a 10-fold enhancement.

Regulation of Clotting Factor Xa Auxiliary Cofactor Function in Fibrinolysis through β-Peptide Excision

We have shown that clotting factor Xa (FXa) cleaved by the fibrinolysis protease, plasmin (Pn), produces consecutive fragments, called FXaβ and Xa33/13. These both have newly exposed C-terminal lysines (Lys) that accelerate tPA in purified clot lysis assays. However, in plasma Xa33/13 rapidly loses this fibrinolytic function due to degradation. It is therefore important to define the role

of four possible basic amino acid residues in generating FXaβ; Lys(K)427, Arg(R)429, K433 and K435. Using site directed mutagenesis, K435 was defined as the preferred cleavage site, while R429 was unfavourable. K433 and K435 were found to be important for RVV-X activation and β-peptide cleavage facilitates the production of Xa33/13. Interestingly, when all four basic residues were mutated to Gln (Q), preventing production of FXaβ, an unexpected distal cleavage site was demonstrated to also enhance Pn generation. Replacing the R429 with lysine generated a hyperfibrinolytic species suggesting a potential novel therapeutic approach.

Preface

The fibrinolysis in hemophilia component of this dissertation (Chapter 3) is a part of a larger study: "Risk of Ischemic Heart Disease in Hemophiliacs" approved by the University of Calgary Ethics ID: E-21650 and UBC Ethics ID: H09-02426.

Plasma clot lysis assays in section 2.2.1 were carried out by Kim Talbot, a Senior Research Assistant in the Pryzdial lab, and subsequent analysis of these data was conducted by Kathleen De Asis.

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List of Symbols and Abbreviations

^{125}I-Pg: Radiolabelled plasminogen with Iodine-125

Ab: antibody

Ag: antigen

APC: Activated protein C

aPL: Anionic phospholipid

AT: Antithrombin

BSA: Bovine serum albumin

Ca^{2+}: Calcium ion

$CaCl_2$: Calcium chloride

CO2: carbon dioxide

CVD: Cardiovascular disease

DMEM/F-12: Dulbecco's modified Eagle's medium supplemented with F-12 nutrient mixture

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediamine tetraacetic acid

EGF: Epidermal growth factor

FBS: Fetal bovine serum

FII: Prothrombin

FIIa: Thrombin

FIX: Factor IX

FIXa: Activated factor IX

FV: Factor V

FVa: Activated factor V

FVII: Factor VII

FVIIa: Activated factor VII

FVIII: Factor VIII

FVIIIa: Activated factor VIII

FX: Factor X

FXa: Activated factor X

FXaα: Intact activated factor X

FXaβ: Activated factor X with a short C-terminal peptide removed

FXI: Factor XI

FXIa: Activated factor XI

FXIII: Factor XIII

FXIIIa: Activated factor XIII

Gla: γ-carboxylated glutamic acid residue; aPL binding domain

H_2O_2: Hydrogen peroxide

H_2SO_4: Sulfuric acid

HBS: 20 mM HEPES, 150 mM NaCl, pH 7.4

HEK: Human embryonic kidney

HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

HK: High molecular weight kininogen

HRP: Horseradish peroxidase

ITS: Insulin-transferrin-selenium supplement

K330: Lys 330; residue in autolysis loop of factor X

K3βQ: Triple-point factor X mutant; Lys427Gln/Lys433Gln/Lys435Gln

K427: Lys 427; residue in factor Xa C-terminal β-peptide region

K427Q: Lys 427 mutated to Gln in factor Xa

K433: Lys 433; residue in FXa C-terminal β-peptide region

K433Q: Lys 433 mutated to Gln in factor Xa

K435: Lys 435; residue in FXa C-terminal β-peptide region

K435Q: Lys 435 mutated to Gln in factor Xa

KK: Kallikrein

KR4βQ: Quadruple point factor X mutant; Lys427Gln/Arg429Gln/Lys433Gln/Lys435Gln

LB: Luria-Bertani

LMV: Large multilamellar vesicles (phospholipids)

nFX: Commercial plasma derived factor X

NO: Nitric Oxide

OPD: O-phenylenediamine dihydrochloride

Opti-MEM: Modified Eagle's minimum essential medium (reduced serum)

PAI-1: Plasminogen activator inhibitor 1

PAP: inactive plasmin/α-2-antiplasmin

PC: Phosphatidylcholine

PCR: Polymerase chain reaction

PDB: Protein database

PEG: Polyethylene glycol

PK: Prekallikrein

Pg: Plasminogen

PGI_2: Prostacyclin, vasodilator

Pn: Plasmin

Pro-TAFI: Zymogen thrombin activatable fibrinolysis inhibitor

PS: Phosphatidylserine

PT: Prothrombin time

PVDF: Polyvinylidine difluoride

PZ: Protein Z

R429: Arg 429; residue in FXa C-terminal β-peptide region

R429K: Arg 429 mutated to Lys in factor Xa

R429Q: Arg 429 mutated to Gln in factor Xa

rFX: recombinant factor X

rpm: Revolutions per minute

RVV-X: Russell's viper venom factor X activator

S-2251: Chromogenic substrate for plasmin

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Serpin: Serine protease inhibitor

SUV: Small unilamellar vesicles (phospholipids)

TAFIa: Activated thrombin activatable fibrinolysis inhibitor

TAFIai: Inactivated thrombin activatable fibrinolysis inhibitor

TBS: Tris-buffered saline

TBST: TBS with 0.1% Tween-20

TF: Tissue factor

TFPI: Tissue factor pathway inhibitor

TMB: Tetramethylbenzidine

tPA: Tissue-type plasminogen activator

vWF: von Willebrand factor

WTFX: Wild-type factor X (recombinant)

Xa33/13: Factor Xa fragment containing a 33 kDa fragment non-covalently linked to a 13kDa fragment

Xa40: 40 kDa factor Xa fragment lacking the Gla domain

α2AP: α -2-antiplasmin

εACA: ε-aminocaproic acid

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1. Introduction

1.1 Hemostasis overview

Hemostasis is the physiological process of controlling bleeding and is made up of four parts: constitutive anticoagulation, primary hemostasis, coagulation, and fibrinolysis (1). Plasma components such as antithrombin (AT) maintain constitutive anticoagulation in order to prevent clotting from occurring when it is not needed (2). Upon vascular injury, blood flow is slowed by constriction of the blood vessels allowing platelets to adhere to the sub-endothelium and aggregate, forming a temporary platelet plug to quickly stop the bleeding. Coagulation is initiated on TF bearing cells upon exposure to blood, amplified as platelets become activated, and propagates on the activated platelet surface to form fibrin and stabilize the thrombus (3-5). Once damage to the blood vessel has been repaired, the process can be reversed and the clot is broken down through the fibrinolysis pathway. Hemostatic balance is once again shifted towards anticoagulation. When the delicate balance between coagulation, anticoagulation and/or fibrinolysis is dysregulated, a number of health problems can arise. Cardiovascular disease (CVD) is caused by undesired coagulation occurring and forming thrombi which can obstruct blood flow. On the other side of the balance, hemophilia A and B is caused by a lack of coagulation factors VIII or IX, respectively, and results in prolonged and excessive bleeding.

Cardiovascular disease (CVD) is the cause of an estimated 17.1 million deaths annually worldwide and is the number one cause of death globally according to The World Health Organization (6). One of the main causes of this disease is a thrombus, which cuts off the flow of blood and results in ischemic tissue damage, especially in the heart and in the brain. This gives rise to ischemic events such as myocardial infarctions and strokes. One form of treatment is to restore the blood flow by breaking up the thrombus and to prevent further thrombus formation

by treatment with anti-platelet and anticoagulant agents. Currently, the drug of choice administered to achieve this clot-dissolving has been tissue plasminogen activator (tPA), and it has saved many lives (7,8). However, the time frame to administer tPA from onset of symptoms is only 3-4.5 hours to lessen neurological damage. Furthermore, a side-effect of tPA is hemorrhage and some thrombi are resistant to tPA for poorly understood reasons (8-10). For all of these reasons, there has been significant research over many years into creating a better recombinant tPA and possible alternative treatments for dissolving thrombi. In order to accomplish this, one must have a biochemical understanding of hemostasis.

1.1.1 Coagulation

The coagulation cascade ultimately leads to fibrin deposition and thrombus formation. The pathway can be loosely described as Y-shaped with two upstream pathways, extrinsic and intrinsic. These meet at the common pathway and result in the production of the protease thrombin and the formation of the clot (Figure 1). First is the extrinsic pathway, or the "initiating pathway" that begins with vascular damage exposing subendothelial tissue factor (TF). This damage also results in the exposure of anionic phospholipid (aPL), initially due to recruitment and activation of platelets to the subendothelium. This localizes the coagulation factors to the site of injury and also allows for the interaction between factor VII (FVII) from the circulation and TF. FVIIa and its cofactor TF form the extrinsic tenase complex with aPL and a source of calcium ion (Ca^{2+}), and activates the substrate factor X (FX), to factor Xa (FXa) (11,12). TF-FVIIa can also activate FIX to IXa, although comparatively poorly. FXa combines with FVa (also activated by thrombin), aPL and Ca^{2+} to form the prothrombinase complex, which activates prothrombin (FII) to thrombin (FIIa). The initial FIIa that is generated feeds back into the amplifying intrinsic pathway by activating FXI to FXIa, which then activates FIX to FIXa (13). Factor VIII (FVIII) is efficiently activated by thrombin to FVIIIa, which then serves as a cofactor for

FIXa and forms the intrinsic tenase complex with aPL and Ca^{2+}, enhancing the activation of FX by FIXa by 57,000-fold in humans (14,15). Deficiencies of FVIII or FIX lead to bleeding disorders respectively called hemophilia A or B, an aspect of my thesis work. The resulting thrombin also activates FXIII to FXIIIa, which is a transglutaminase that crosslinks the Lys and Gln side chains of fibrin α- and γ-chains to form an irreversible amide bond that stabilizes the insoluble fibrin hemostatic plug.

In pathological circumstances, such as inherited thrombosis or trauma, recent evidence has suggested that the upstream contact phase of the coagulation cascade may be involved in amplifying clot formation, where high-molecular-weight kininogen (HK), prekallikrein (PK), and factor XII (FXII) form a FXIa-activating complex. In this complex both prekallikrein and FXII become activated to kallikrein (KK) and FXIIa respectively. FXIIa from this upstream pathway can activate factor XI (FXI) to FXIa which then continues downstream as the intrinsic pathway. Deficiencies of HK, PK, and FXII are not associated with excessive bleeding, and FXI deficiencies are mild (16). Interestingly, Renné et al demonstrated that FXII knockout mice had normal hemostasis, but defective thrombus formation (17). Thus, the contact phase is likely not important for physiological hemostasis, but rather pathological thrombosis in vivo (18).

The role of both the extrinsic and intrinsic pathways is the activation of FX to FXa. Furthermore, FXa is the essential protease of the common pathway enzyme complex, prothrombinase. Thus, regulation of FX activation is central to coagulation physiology and pathology, emphasizing the importance of FX in its clotting role. In addition to a critical function in hemostasis, FX also plays a role in the opposing fibrinolysis pathway, which is an important focus of the current thesis.

The cascade model simplifies the enzymatic steps and is useful for understanding coagulation of plasma in vitro; however it does not fully describe the process in vivo. The newer

cell-based model of coagulation includes the role of two-types of cells: TF-bearing cells, such as fibroblasts and monocytes, and platelets (3-5). In this model there are three main phases: initiation, amplification, and propagation. Initiation occurs upon vascular injury as in the cascade's extrinsic pathway where blood flow becomes exposed to a TF-bearing cell. Circulating FVIIa binds to the TF and this complex catalyzes the activation of both FX and FIX. Any FXa that dissociates from the TF bearing cell is inhibited by tissue factor pathway inhibitor (TFPI) or AT, ensuring that FXa activity remains localized to the cell. FIXa is free to dissociate and bind to a nearby platelet (5). On the TF-bearing cell, FXa binds to cofactor FVa forming the prothrombinase complex and generates a small amount of FIIa that can dissociate and bind to a nearby platelet. This leads to the amplification phase, where FIIa: i) activates platelets exposing procoagulant aPL surface, ii) releases vWF from FVIII causing platelet adhesion and aggregation, and iii) activates FV, FVIII and FXI as in the cascade's contact phase and intrinsic pathway. In the propagation phase, the activated coagulation factors generated in the previous phases localize on the procoagulant surface of activated platelets and form the intrinsic tenase, generating FXa which binds with FVa to form prothrombinase and generates large amounts of FIIa directly on the platelet. This burst of FIIa cleaves fibrinogen to fibrin polymerizing into strands and forming the hemostatic plug.

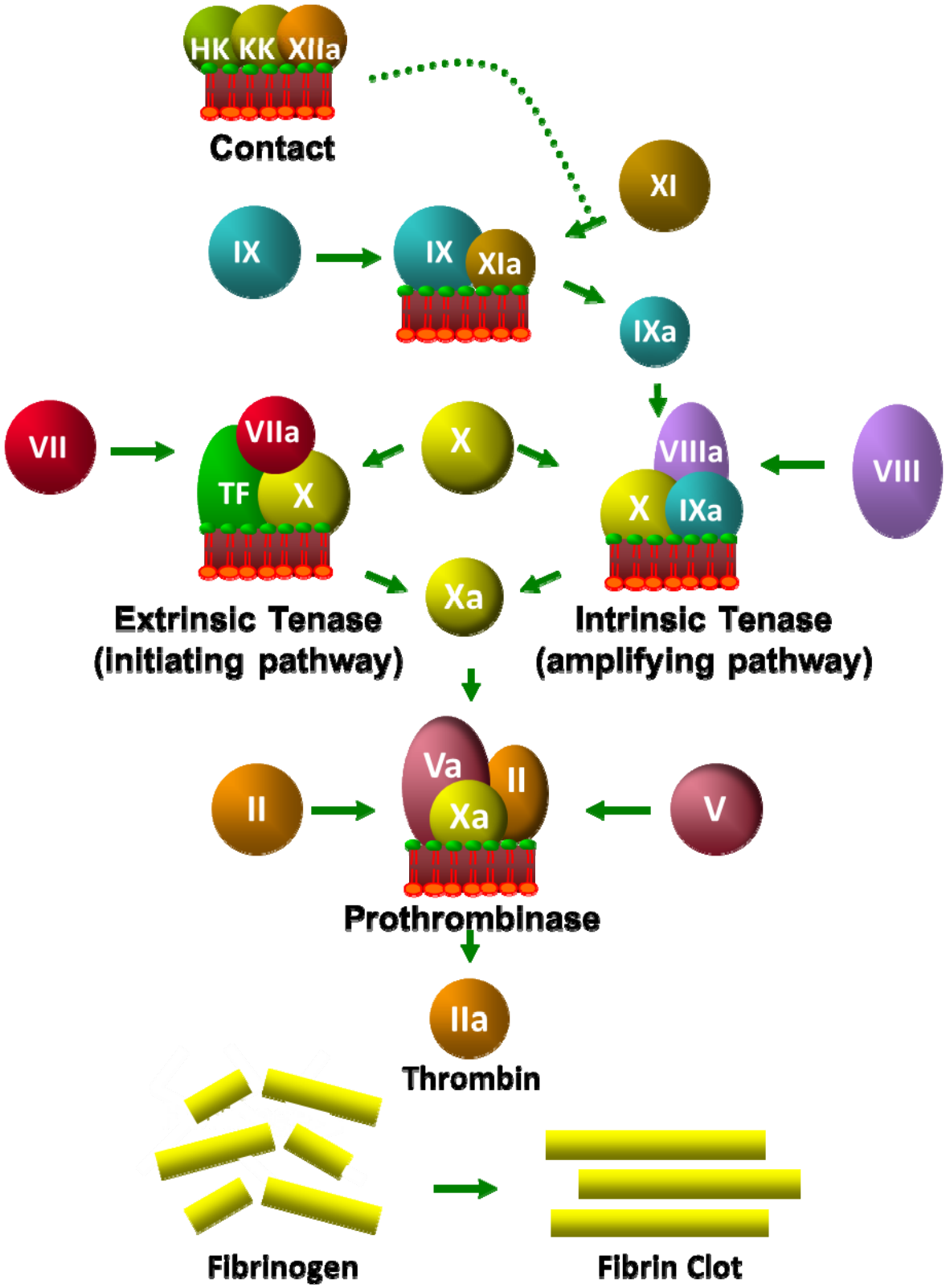

HK KK XIIa
Contact
XI
IX
IX XIa
IXa
VII
VIIa
TF X
X
VIIIa
X IXa
VIII
Xa
Extrinsic Tenase
(initiating pathway)
Intrinsic Tenase
(amplifying pathway)
II
Va
Xa
II
V
Prothrombinase
IIa
Thrombin
Fibrinogen
Fibrin Clot

Figure 1: The coagulation cascade

The coagulation cascade is a series of enzymatic activations by which the body forms clots in order to stop bleeding. Two interconnected pathways meet at a final common pathway resulting in the deposition of fibrin and clot formation. The extrinsic pathway, or "initiating" pathway, begins with vascular damage exposing anionic phospholipid (green phospholipids), which localizes the coagulation factors needed to form extrinsic tenase: tissue factor (TF), FX, and FVIIa. This extrinsic tenase activates FX to FXa that then goes on to form prothrombinase with FVa and Prothrombin (FII). Prothrombinase complex activates prothrombin to thrombin (FIIa) which then feeds back into the intrinsic pathway or "amplifying" pathway by activating FXI to FXIa, which activates FIX to FIXa. In this second pathway, the intrinsic tenase is formed with FVIIIa (also activated by FIIa), FIXa and FX, activating it to FXa similar to the extrinsic tenase. Further upstream with contact phase, FXI can also be activated by the complex of FXIIa, high-molecular-weight kininogen (HK), and kallikrein (KK).

1.1.1 Physiological anticoagulation

The balance between coagulation and fibrinolysis must be maintained in order to ensure clots form only when and where they are required. Constitutive anticoagulation is in place to maintain blood fluidity and prevent thrombus formation. The endothelium plays a major role in physiological anticoagulation. The vascular lumen synthesizes heparan sulfate, a heparin-like glycosaminoglycan that can bind AT. AT is a serine protease inhibitor (serpin) that binds and neutralizes the serine proteases thrombin, FIXa, FXa, FXIa, FXIIa (2). Platelet activation and aggregation is also inhibited by the endothelium by the release of ADPase, and vasodilators prostacyclin (PGI_2) and nitric oxide (NO) (1). The endothelial cell surface also expresses thrombomodulin which serves as a cofactor for thrombin activation of Protein C to activated protein C (APC), a Vitamin K-dependent serine protease (19). With protein S, endothelial protein C receptor, calcium and anionic phospholipid as cofactors, APC proteolytically inactivates FVa and FVIIIa. Tissue factor pathway inhibitor (TFPI) reversibly inhibits the extrinsic tenase by forming a complex with FXa. This Xa-TFPI complex can subsequently inhibit the FVIIa-TF complex within the same tenase by forming a quaternary TF/VIIa/Xa/TFPI complex (20).

1.1.2 Fibrinolysis

The fibrinolysis pathway ultimately leads to the breakdown of fibrin clots into soluble fragments (Figure 2). This is primarily done through cleavage by plasmin (Pn), which is the active form of plasminogen (Pg) (21). The dogma of fibrinolysis has been as follows: 1) Fibrin is the only required cofactor to enhance tPA-mediated Pg activation to Pn. 2) Intact fibrin has co-localizing binding sites for tPA and the substrate Pg (described in 1.1.4.1) to initiate low levels of Pn generation. 3) Limited cleavage of fibrin by Pn occurs that exposes C-terminal lysines (Lys), which “primes” the fibrin clot. These C-terminal Lys form integral components of new binding

sites for tPA and Pg. 4) More tPA and Pg binding, and faster Pn generation are enabled on "primed" fibrin, ultimately allowing the Pn concentration to exceed the antifibrinolytic threshold for apparent "all-or-nothing" degradation of fibrin.

tPA is an enzyme that cleaves Pg at Arg 561 to two chains linked by a disulfide bond, activating it to Pn. During the solubilisation process, the clot is systematically cleaved by Pn into well-documented fibrin degradation products (FDP): Fragment X, Y, D, E and D-Dimer:E complexes. Serpins such as plasminogen activator inhibitor-1 (PAI-1) and α-2-antiplasmin (α2AP), form irreversible 1:1 complexes with tPA and Pn respectively. Thus, PAI-1 inhibits the conversion of Pg to active form Pn by forming a complex with tPA and blocking interaction with Pg. Similarly, α2AP inhibits plasmin activity by forming the inactive plasmin/α-2-antiplasmin (PAP) complex. Thrombin activatable fibrinolysis inhibitor (pro-TAFI) is a proenzyme that when activated with proteolytic cleavage by the thrombin/thrombomodulin complex to TAFIa, cleaves C-terminal lysine and arginine residues on fibrin. The removal of these C-terminal amino acids inhibits the binding and activation of Pg by tPA and decreases fibrinolytic activity.

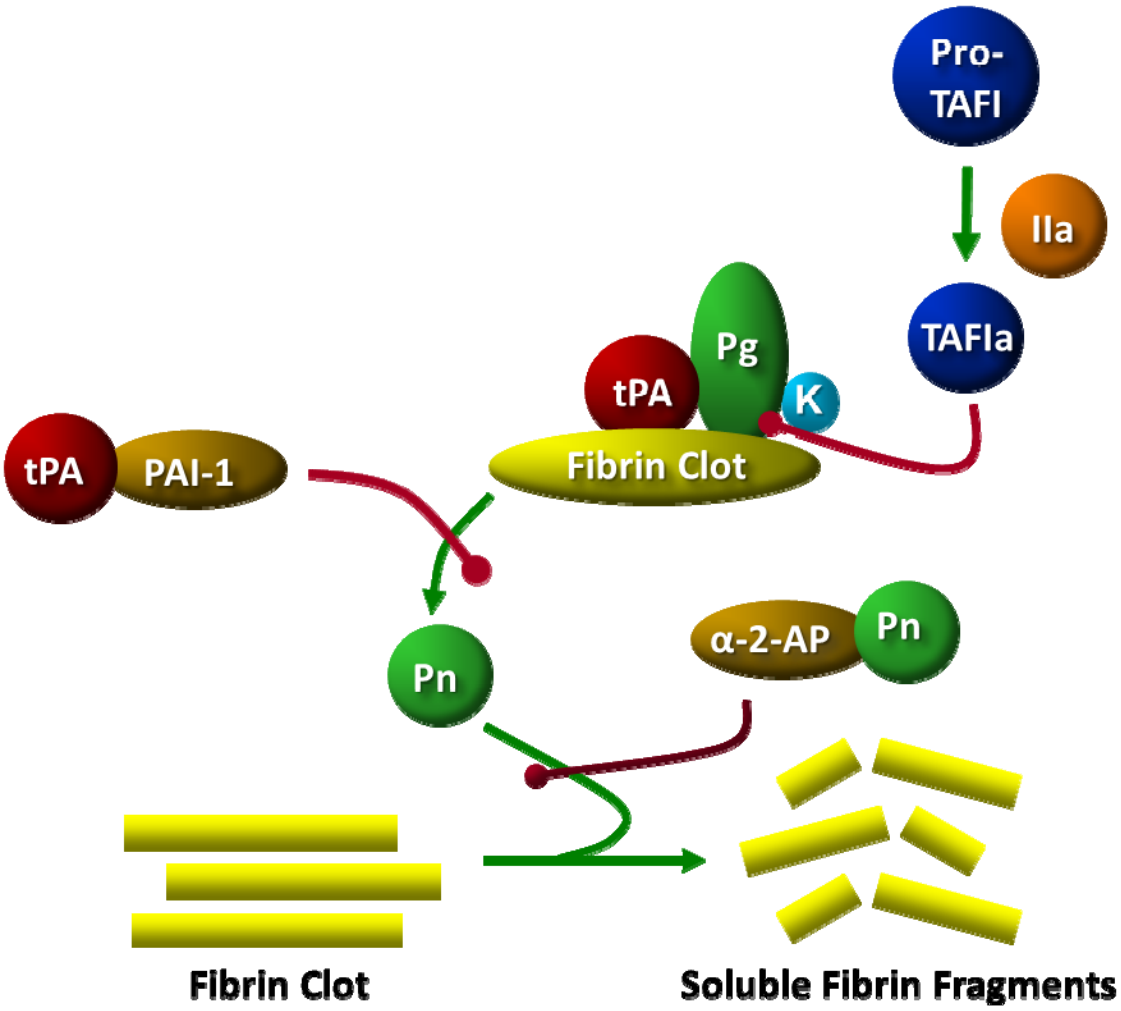


Figure 2: The fibrinolysis pathway

Fibrinolysis is the break-down of fibrin clots into soluble fragments. This is primarily done through cleavage by Pn, which is the active form of Pg. Pg binds to fibrin or other cofactors, where it is activated to Pn predominantly through cleavage by tPA. Serpins, PAI-1 and α2AP form inhibition complexes tPA and Pn respectively. Pro-TAFI is a proenzyme that is proteolytically activated by FIIa to TAFIa, which inhibits the activation of Pg by cleaving the C-terminal lysine (K) constituent of the Pg and tPA binding sites on fibrin and other cofactors.

1.1.3 Auxiliary cofactor model of fibrinolysis

Previous work on the prothrombinase components FV and FX demonstrated that cleavage by Pn generates fragments that can serve as cofactors for tPA, and accelerate Pn generation (22). Pn cleavage of FXa yields fragments FXaβ and Xa33/13, which contain new C-terminal lysine residues that allow them to act as Pg receptors and accelerate tPA activity (22-27). Both FXaβ and Xa33/13 have been shown to enhance tPA-mediated Pn generation as well as the dissolution of purified fibrin clots at physiological concentrations of tPA (24). Pryzdial et al. demonstrated that Xa33/13 bound more Pg than FXaβ (23). However, FXaβ accelerated purified fibrin clot lysis to a greater extent compared to Xa33/13, and in unpublished plasma lysis experiments (in Dr. Amanda Vanden Hoek's PhD thesis (28)) Xa33/13 did not enhance tPA activity (24,28).

As suggested by work from our lab, a revised fibrinolysis model is postulated that includes auxiliary cofactors: 1. Proteins in the vicinity of the clot provide C-terminal Lys before those exposed on fibrin. 2. Pn cleaves FXa approximately 2-orders of magnitude more effectively than fibrin to expose C-terminal Lys. 3. The FXaβ functions as an auxiliary cofactor to enhance the initial Pn generation at the site of a clot that ultimately primes the fibrin for fast Pn generation and clot solubilisation (Figure 3).

This model combines clotting and fibrinolysis proteins in a new way, with modulated clotting factors working towards the opposite goal, which is profibrinolysis. FV and FX along with aPL and Ca^{2+} form the prothrombinase complex, which is responsible for activating the substrate prothrombin into the important coagulation protein thrombin. It is rational that these two main components of the complex, which are localized to the site of the clot, are eventually modulated to enhance fibrinolysis as tPA cofactors and generate Pn.

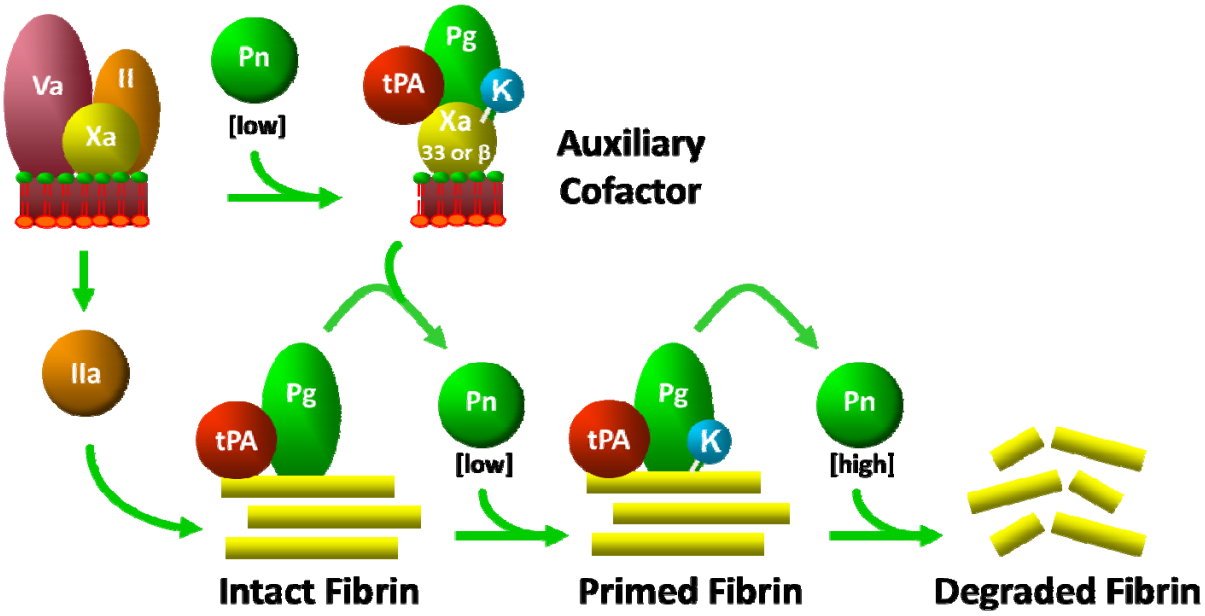


Figure 3: Auxiliary cofactor fibrinolysis model

Prothrombinase generates FIIa, which produces fibrin through proteolytic activation of fibrinogen. Intact fibrin serves as a "slow" cofactor for tPA. Pg binds and is cleaved to generate a small amount of initiating Pn. FXa fragments, such as Xa33/13 or FXaβ can act as auxiliary cofactors for tPA and provide additional sites localized to the clot, for Pg and tPA to bind and generate a low level of Pn. This Pn goes on to cleave fibrin, exposing C-terminal Lys (K) to more readily activate Pg for a high level of Pn generation, which in turn results in faster fibrin degradation.

1.1.3.1 Plasmin-mediated factor Xa fragmentation

In addition to the well-established central role of FX in coagulation, our lab has shown that FX and FXa have fibrinolytic function by serving as an auxiliary cofactor for tPA. To acquire this activity, C-terminal lysines must be exposed on FX or FXa, by proteolysis, which form Pg and tPA binding sites (Figure 4). The domain structure of intact FX can be divided into an activation peptide, anionic phospholipid (aPL) binding or Gla domain, protease domain, and a β peptide (Figure 5). Upon activation to FXa, the activation peptide is excised leaving full-length FXaα. This "α" form undergoes proteolytic excision of the C-terminal β-peptide from the heavy-chain, mediated by Pn or by autoproteolysis, leaving a form of FXa called FXaβ. The precise cleavage site rendering this "β" form is unknown and therefore may have four possible different C-termini: Lys 427 (K427), Arg 429 (R429), Lys 433 (K433), and Lys 435 (K435). After FXaβ is produced, further proteolysis can occur through one of two pathways. In the presence of calcium ($CaCl_2$) and aPL, which favours binding of FXaα and FXaβ to the aPL, FXaβ is cleaved by Pn at Lys 330 (K330) to yield Xa33/13. The latter has negligible plasma clotting activity. Under conditions that do not favour binding to aPL (i.e. In the absence of aPL or in the presence of the Ca^{2+}-chelator, ethylenediamine tetraacetic acid (EDTA)), FXaβ is cleaved by Pn at Lys 43 (K43), which excises the Gla domain. Since the resulting cleavage product, Xa40 is non-functional as a tPA cofactor (Figure 5), it is likely that FXaβ is first generated by cleavage at Arg 429 rather than one of the nearby Lys residues. Thus, in the auxiliary cofactor model of fibrinolysis, FXa function is proteolytically modulated from procoagulant to profibrinolytic by Pn and this conversion is localized by the accessibility of aPL.

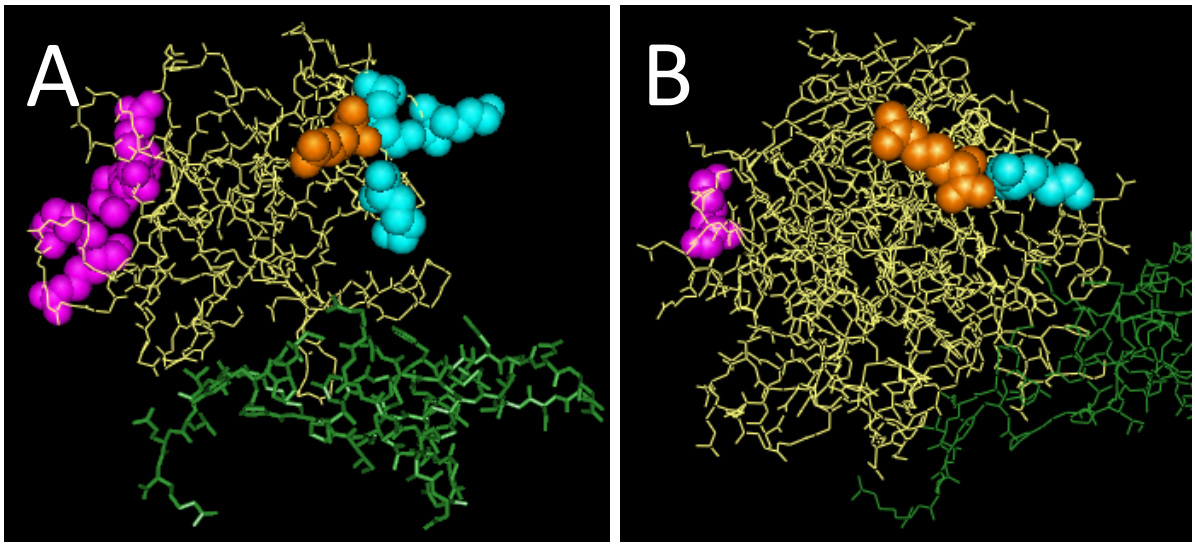


Figure 4: Three dimensional models of FXa highlighting plasminogen binding sites exposed by plasmin cleavage

Two partial crystal structures of FXa (A: Protein Database (PDB) accession code 1HCG (29); B: PDB accession code 1XKA (30) including the heavy chain (yellow) and part of the light chain (green) were rastorized using Pymol software (www.pymol.org). Plasmin cleavage of FXa reveals potential plasminogen binding sites: the β-peptide (Lys in cyan, and Arg in orange) and the autolysis loop (magenta) where Lys330 is cleaved to generate Xa33/13. Two models are shown here to demonstrate the variation of plasminogen binding site orientation in different crystals (29).

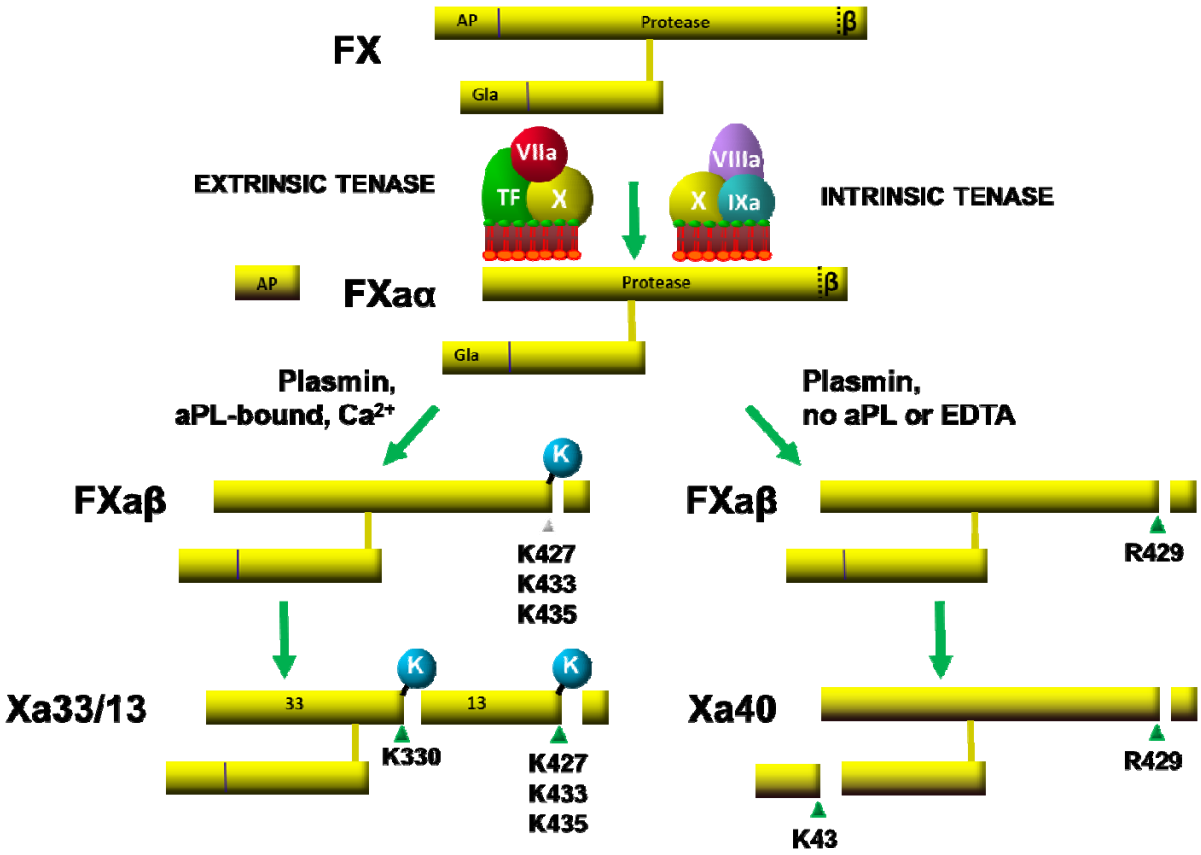


Figure 5: Plasmin cleavage of Factor Xa

FX is activated by either the extrinsic or intrinsic tenase into FXaα. C-Terminal Lys are fundamental for expression of fibrinolytic activity. It is unknown which basic amino acid is cleaved to convert FXaα into FXaβ: K427, K433, K435, or R429. Furthermore, it is unknown if there is preferential cleavage by plasmin or whether β-peptide cleavage is necessary prior to Xa33/13 generation. FXaα is cleaved by two pathways depending on whether it is bound to aPL. When bound to aPL, C-terminal Lys are exposed, which enables fibrinolytic activity. The first cleavage product gives FXaβ, which could be at any of three nearby Lys. The second cleavage, when bound to aPL gives Xa33/13, a fibrinolytic species. When not on aPL, cleavage is likely at R429 to produce FXaβ and then Xa40, which cannot facilitate fibrinolysis.

1.1.4 Communication and crosstalk between coagulation and fibrinolysis

To simplify the interpretation of already complicated data, coagulation and fibrinolysis are typically studied separately. However there must be ample communication between these opposing processes to ensure sequential coordination and indeed numerous lines of evidence have revealed that they are connected. The pathways are similar in that they involve enzymes, cofactors, and inhibitors, and they both follow a sequential cascade design with many protein interactions. Both pathways can be regulated through inhibition by serpins and other inhibitors or amplification using positive feedback mechanisms. The main difference is that they have opposing functions, but work together to maintain hemostatic balance by preventing excessive bleeding as well as clotting. There are two well-documented proteins in particular that are common between the two different processes: fibrin and thrombin.

1.1.4.1 Fibrinogen activation and fibrin degradation

Circulating fibrinogen has an overall dumbbell-shaped quaternary structure consisting of two flanking D-domains and a central E-domain made up of three types of peptide chains: Aα, Bβ and γ. Monomeric fibrinogen is converted to insoluble fibrin through thrombin-mediated cleavage that releases N-terminal fibrinopeptides A and B from the Aα and Bβ chains (respectively), which exposes sites "A & B knobs" that allow the fibrin to polymerize with adjacent fibrin molecules at matching "a & b holes" in the D domain (31) (Figure 6). Fibrin feedback regulates fibrinolysis and is thus strategically positioned in hemostasis. The hemostatic equilibrium is shifted towards coagulation, forming a fibrin network that serves as the hemostatic seal to stop bleeding due to vascular damage. Once the damage has been repaired,

the balance shifts in favour of fibrinolysis to dissolve the clot and restore blood flow. Fibrin facilitates Pn activation and thus fibrin degradation by binding Pg and tPA (32,33).

The conversion of fibrinogen to fibrin exposes two sites that bind Pg & tPA. Kringle domains are loops stabilized by 3 disulfide linkages that are key for C-terminal binding interaction. The Aα chain 154–159 site of fibrin can bind to Kringle domains located in either Pg (Kringles 1-3 of 5 total), or in tPA (Kringle 2 of 2 total), but preferentially binds Pg in vivo because of a higher circulating concentration (31,34). The γ chain 312–324 binding site only binds tPA at the tPA finger domain (31,34) (Figure 6). Fibrin has also been shown to bind FXa at the Gla domain on Aα chain 82-123 (35) (Figure 6).

In soluble fibrinogen or monomeric fibrin, plasmin cleaves Aα chains at the D domain terminal end to make Fragment X (D-E-D). Fragment X is cleaved through all three Aα, Bβ and γ chains in between the D & E domains to yield fragments Y (D-E) & D. Fragment Y then becomes further degraded into Fragment E and a second Fragment D (36,37). Cross-linked fibrin is cleaved by plasmin in a similar way, however it also results in a unique fragment called the D-dimer (Figure 7). The D-dimer usually remains complexed with an E domain because of FXIIIa covalent crosslinking. Fibrinogen has minimal to no effect on tPA mediated Pn generation, however fibrinogen still has both a high affinity Pg binding site and a tPA binding site in the D domain (38).

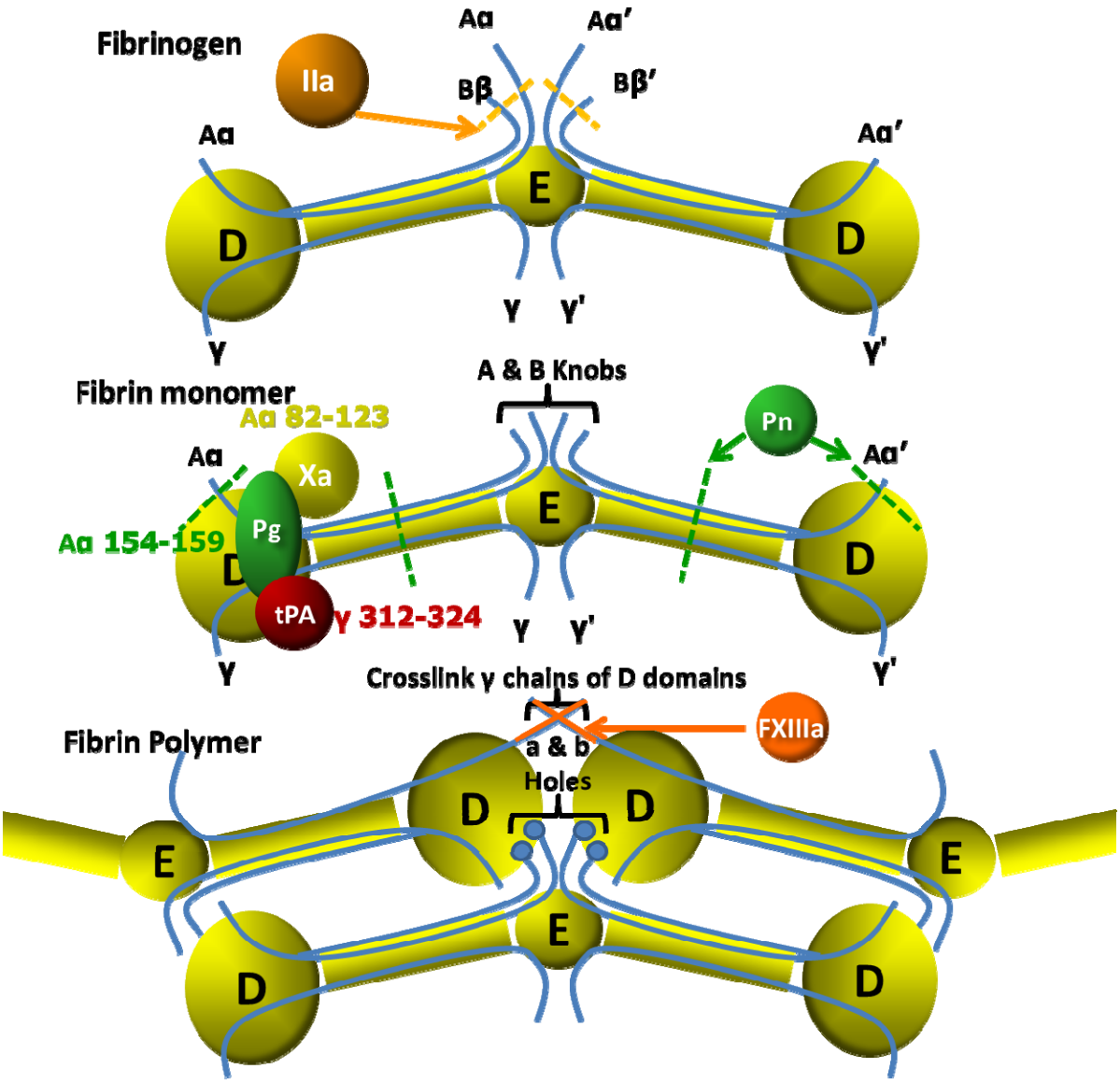


Figure 6: Fibrinogen and fibrin structure

Fibrinogen has a dumbbell shape of two D domains and a central E domain made of three peptide chains: Aα, Bβ and γ. FIIa activates fibrinogen to fibrin by cleaving and releasing N-terminal fibrinopeptides A and B exposing A & B knobs that fit into a & b holes of the D domain of adjacent fibrin molecules. The activation of fibrinogen to fibrin exposes two sites that bind Pg and tPA:

- Aα 154-159 (D region) sequence that can bind either tPA (via the Kringle 2 domain) or Pg (via Kringles 1-3). Aα 154-159 can bind both Pg & tPA, but preferentially binds Pg in vivo because of a higher circulating concentration.
- γ312–324 (D region) only binds tPA, and binds at the tPA finger domain.

FXa has been shown to also bind Fibrin at Aα 82-123 (coiled region between D & E).

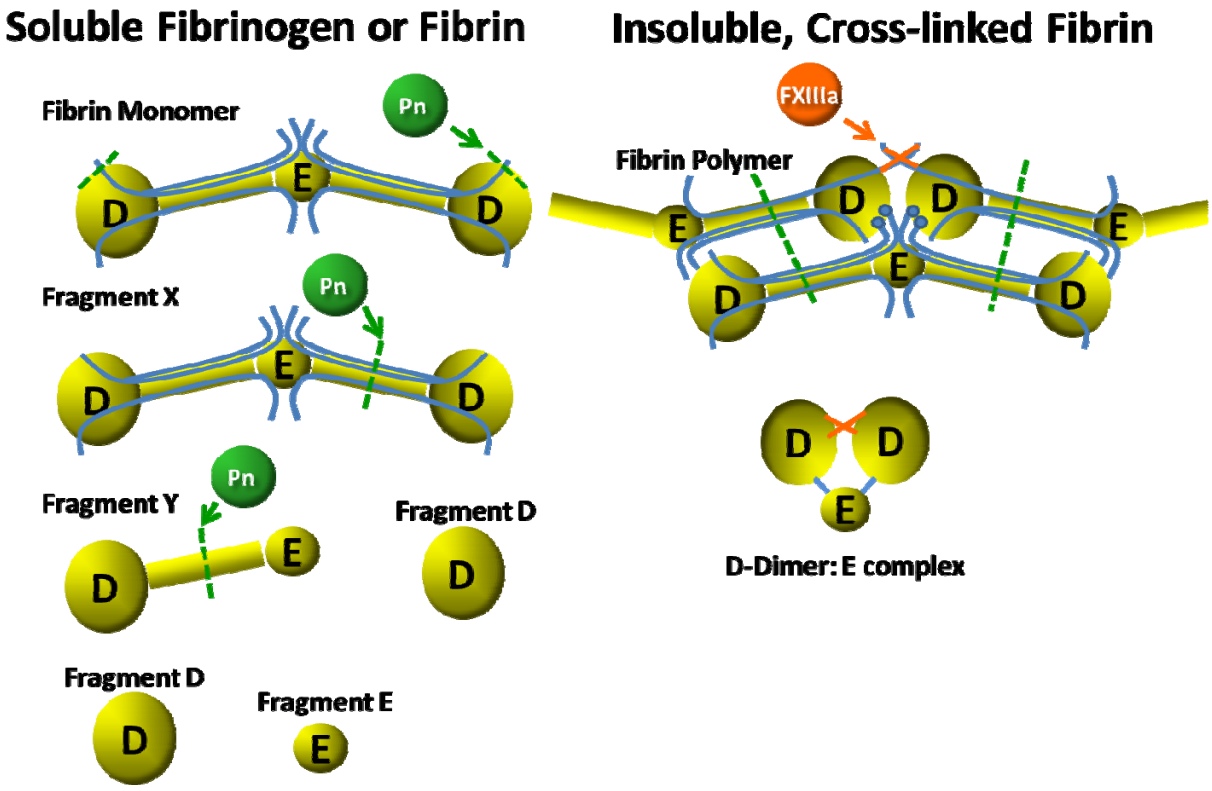


Figure 7: Fibrin degradation

In soluble fibrinogen or fibrin, Pn cleaves α chains at D terminals to make fragment X. Fragment X is then cleaved into Fragments Y & D. From this point, Fragment Y is further cleaved by Pn into Fragment E and a second Fragment D. Fragments X, Y, D, and E are measured clinically as fibrinogen degradation products (FDP). Pn cleaves insoluble, cross-linked fibrin in a similar way as soluble fibrin, however results in a unique fragment called the D-Dimer. The D-dimer, usually remaining complexed with an E domain, is the crosslinked D domains from two distinct fibrin monomers, preserved together because of FXIIIa crosslinking

1.1.4.2 Thrombin

The prothrombinase complex consists of the protease FXa, cofactor FVa, and the precursor prothrombin. While the complex is bound to aPL in the presence of calcium, prothrombin is cleaved twice by FXa: first at Arg 320 to meizothrombin, then at Arg 271 into the active form thrombin (11). Thrombin is one of the most important protease in the entire coagulation pathway as it converts fibrinogen to fibrin and activates upstream clotting factors creating a positive feedback to create even more thrombin and thus more fibrin for the clot. Thrombin is also linked to fibrinolysis through thrombin/thrombomodulin-mediated activation of TAFI, which inhibits fibrinolysis by preventing Pg binding and activation (12-14).

1.1.4.3 Thrombin activatable fibrinolysis inhibitor

Through TAFI, thrombin protects the fibrin clot from premature degradation, giving it an antifibrinolytic property in addition to its procoagulant features. TAFI, also known as carboxypeptidase U or B2, is a 401 amino acid proenzyme secreted by the liver and circulates at approximately 5.0µg/ml or 100nM (1). The proenzyme, pro-TAFI (39), is activated through cleavage at Arg-92 by trypsin-like enzymes, such as thrombin and plasmin (40). Recombinant meizothrombin has also been shown to be a potent activator of TAFI when complexed with thrombomodulin, however only 10% as effective as recombinant thrombin (41). The activated form TAFIa, has a binding pocket (Asp257, Gly244, Ser207) within the catalytic domain for C-terminal basic amino acids. With this pocket, TAFIa binds to the C-terminal Lys exposed on fibrin and other cofactors and removes the Lys required for the amplification of Pg and tPA binding, thus inhibiting fibrinolysis (42).

However, in addition to activation of pro-TAFI, thrombin can also rapidly inactivate TAFIa. Through mutagenesis experiments, Boffa et al have shown that Arg-302 is the thrombin cleavage site in TAFIa that results in its inactivation to TAFIai (43) (Figure 8). This inactivation

suggests that significant antifibrinolysis may not be achieved through TAFIa due to the short half-life of 10 mins at the physiological temperature of 37°C, with insufficient time to induce a physiologically relevant antifibrinolytic effect (44). However, there have been studies supporting a physiological role for TAFIa as an inhibitor of fibrinolysis. Reditz et al. showed that in a canine model of electrically induced thrombosis in the coronary artery, TAFIa activity was increased in plasma samples during thrombosis and prolonged the time for reperfusion during thrombolytic therapy with tPA infusion (45). Rabbit models showed that using an inhibitor of TAFIa enhanced thrombolysis or reduced the amount of tPA needed to achieve lysis (46,47). Nesheim and Bajzar studied the activation of TAFI in vivo using a baboon E. coli-induced sepsis model. In this sepsis model, different activators of TAFI, such as thrombin, thrombin/thrombomodulin and plasmin, were elevated. They found that the increase in TAFIa levels correlated with E. coli in a dose-dependent manner (from 10^6 to 10^8 CFU/kg) and with the use of a monoclonal antibody inhibiting specific activators of TAFI showed that thrombin/thrombomodulin was the main activator of TAFI (48,49). In humans, high total TAFI concentrations have been correlated with a 2-fold increase in risk for DVT (50). Antovic et al. found that in hemophilia there was no difference in levels of total TAFI antigen, however Pro-TAFI was significantly reduced in haemophilia patients compared to controls (39). In severe hemophilia, significantly higher concentrations of pro-TAFI were found in more severe hemorrhagic phenotypes (6.2 μg/ml) compared to mild hemorrhagic (3.0 μg/ml) and non-hemophilia groups (3.2 μg/ml) (51). Foley et al. found a correlation between TAFIa and thrombin generation, suggesting that both could be used to evaluate bleeding diathesis in hemophilia patients (52).

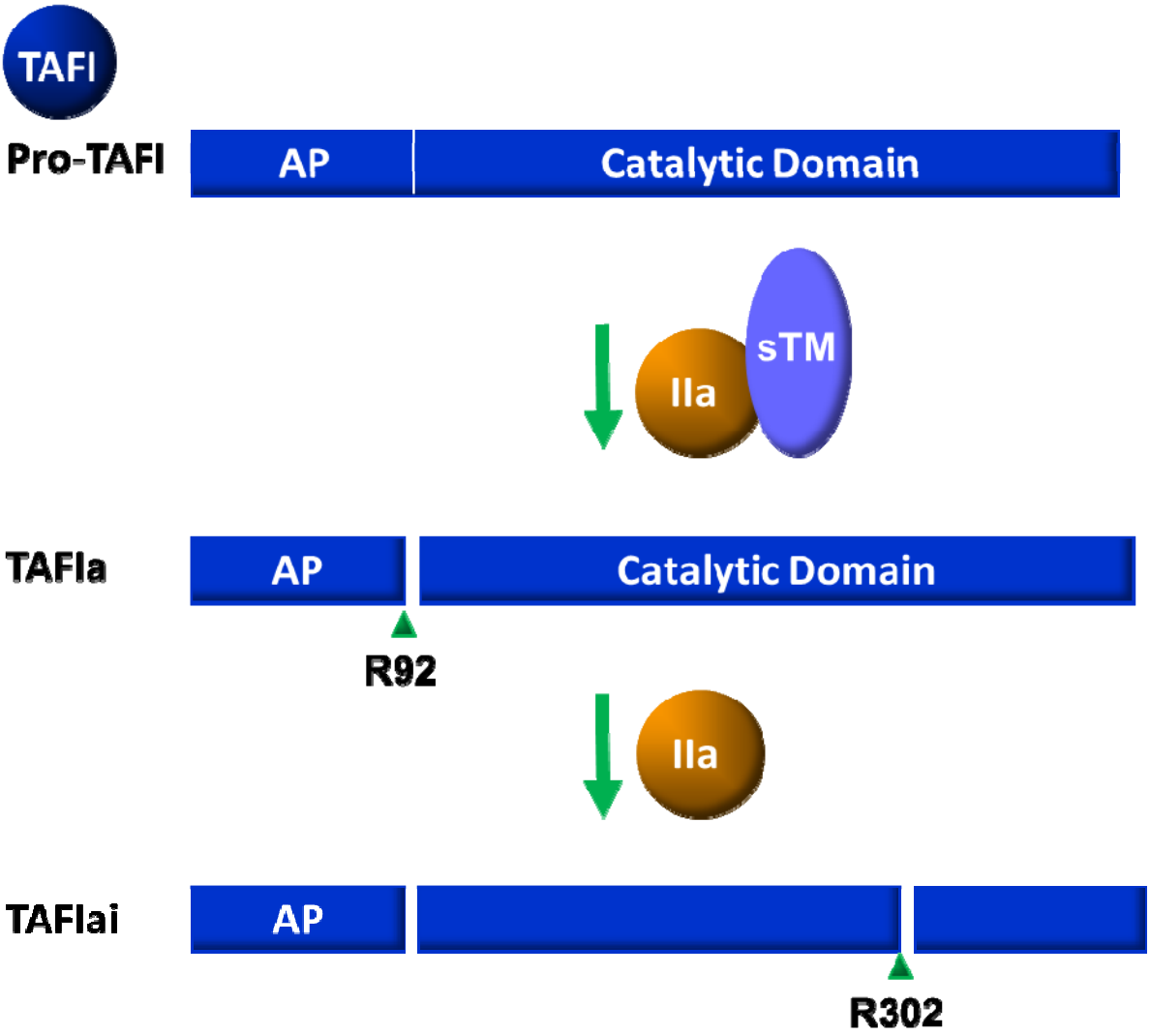


Figure 8: TAFI activation and inactivation

TAFI is a carboxypeptidase B, an inhibitor of fibrinolysis acting to remove C-terminal Lys sites that bind plasminogen and tPA. It primarily circulates in the zymogen form (pro-TAFI), which then is activated through cleavage at R92 by trypsin-like enzymes such as FIIa and plasmin. In this project a recombinant thrombin/soluble thrombomodulin (IIa/sTM) complex was used to convert TAFI to TAFIa. TAFIa has a short half-life of only 10mins at 37°C, and becomes inactivated through thrombin cleavage at R302.

1.1.4.4 Platelets and PAI-1

Platelets are anucleate cells derived from megakaryocytes. Vascular damage causes subendothelial exposure of TF, and the cell adhesion ligands, collagen and von Willebrand factor (vWF), to blood, whereupon they come into contact with platelets. Platelets are consequently activated, resulting in shape change, pseudopod extension, secretion of granule contents, and aggregation. The latter step involves fibrinogen and vWF as bridging molecules between cells. Through granule release, platelets regulate fibrinolysis. They release the tPA inhibitor PAI-1, to inactivate the ability to convert Pg to Pn. Only 10% of PAI-1 from platelets is in the active form. Under physiological conditions the active form spontaneously converts to the latent form, which has a half-life of approximately 2 hours (53). The nonreactive form has been shown to be reactivated with denaturants, causing refolding (54). PAI-1 is also secreted from endothelial cells into plasma, mostly in the active form, but the main physiologic source is platelets (53). The normal range of PAI-1 in (reference) plasma is ~26-47ng/ml (55-57) and baseline PAI-1 activity is ~24 IU/ml (58). PAI-1 levels were found to increase with age in women (59). Increased levels of PAI-1 activity have been implicated in cardiovascular diseases such as myocardial infarction, (60) coronary artery disease, (61) and deep vein thrombosis (62). The opposite could be true for hemophilia patients, in that decreased levels of PAI-1 activity may enhance fibrinolysis and explain how patients with hemophiliac are protected against vascular disease.

1.1.4.5 Plasminogen and plasmin

The inactive precursor of Pn, Pg, circulates as a 90kDa, 791 amino acid glycoprotein with: an N-terminal signal peptide, activation peptide, five Kringle domains, and a serine protease domain (Figure 9). Plasminogen is activated to plasmin by tPA through cleavage at Arg 561 (63). The activation peptide can be released through plasmin cleavage at Lys 78 before or after activation yielding Lys-Pg or Lys-Pn, respectively. However, Lys-Pg is activated at least 10-fold

more efficiently making physiological Glu-Pn unlikely. Plasminogen binds to fibrin at Kringle domains 1-3 (31) and the Lys-plasminogen form has been shown to both bind fibrin and becomes activated more readily than the Glu-plasminogen form (64).

Plasmin is the most important protease in the fibrinolysis pathway, as it is the main protease in fibrin degradation. It is a link to coagulation as it has been found to regulate the activation of clotting factors such as FV and FX through cleavage at specific amino acid residues (22,65). Previous studies in the Pryzdial lab and by collaborators have shown that FV and FX fragments from Pn digestion can act as auxiliary cofactors in fibrinolysis and enhance tPA activity.

In addition to TAFI and PAI-1, fibrinolysis can also be inhibited through Pg and plasmin. The main physiological inhibitor of plasmin is α2AP, a 452 amino acid serine protease inhibitor synthesized in the liver that can irreversibly bind plasmin and form a PAP complex (66,67). α2AP can also effectively bind the inactive form, Pg, preventing it from binding to fibrin (68).

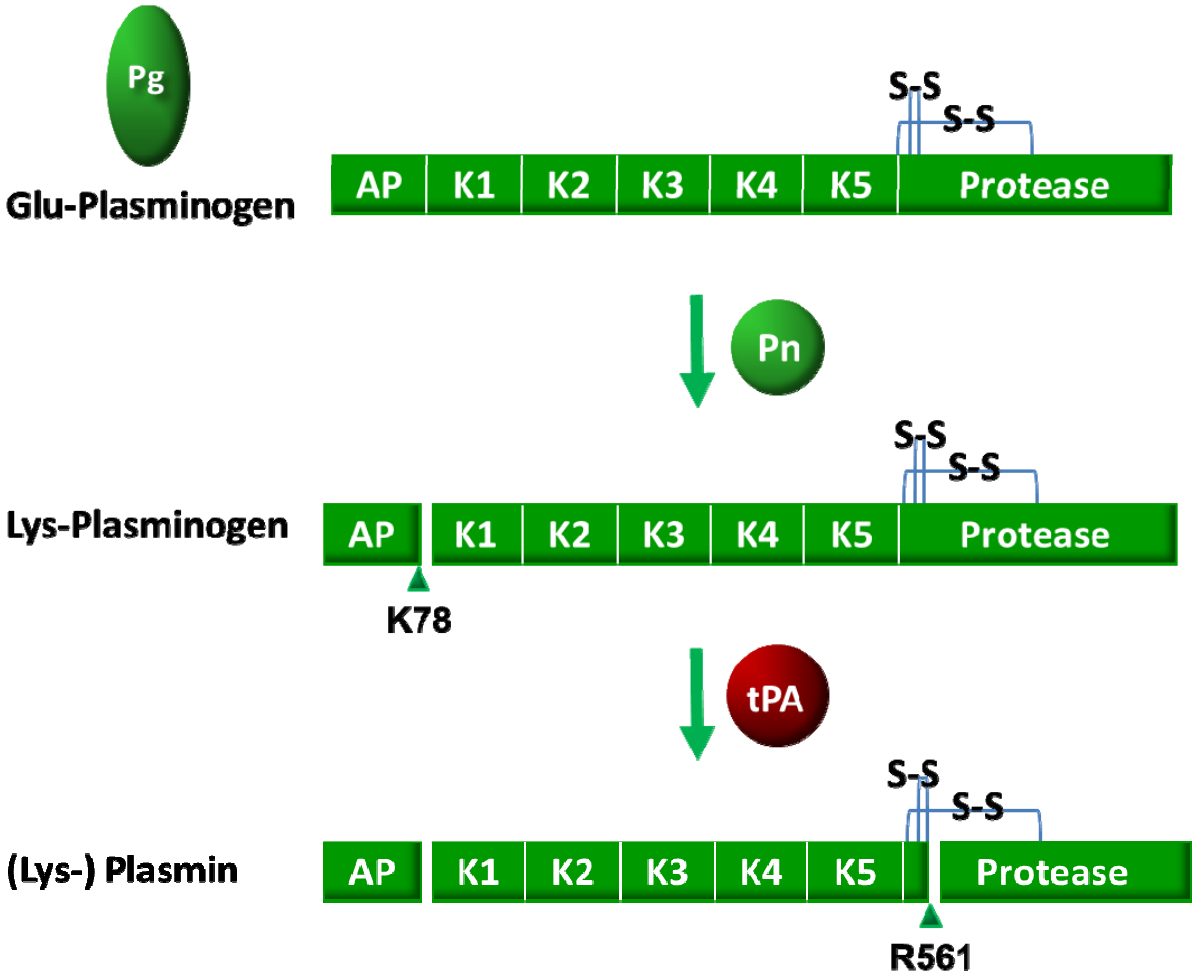


Figure 9: Plasminogen activation

The circulating form of Pg, Glu-Pg has an activation peptide (AP), five Kringle domains (K1-K5), and a serine protease domain. The AP is released by plasmin cleavage at K78. This can happen either before or after activation by tPA yielding Lys-Pg or Lys-Pn. Both Glu-and Lys-Pg are activated by tPA cleavage at R561, yielding a two-chain disulfide-linked enzyme plasmin.

1.1.4.6 Tissue-type plasminogen activator

tPA, as the name suggests, is responsible for converting Pg to the active form plasmin. It is secreted by endothelial cells and circulates as a single chain 72 kDa, 572 amino acid glycoprotein in the bloodstream at ~ 5μg/ml (1). It consists of an N-terminal finger-like domain, epidermal growth factor-like domain, two Kringle domains, and a serine protease domain. Plasmin can cleave single chain tPA at Arg 275 to yield a two-chain form, which is approximately 10-fold more active (69) (Figure 10). tPA Ag levels have been found to increase with age in both men and women, from approximately 3 μg/L in adults ~30 years old to 10 μg/L for those >60 years old (70). Binding of tPA to fibrin involves Kringle 2 and the finger domains (31,34).

Recombinant tPA is the main thrombolytic treatment for myocardial infarctions and strokes, and has saved numerous lives. However, it is not a perfect therapeutic. There is only a small window of opportunity, 3-4.5 hrs from the onset of symptoms, in which tPA can been administered with a greater chance for effective results (71-73). Some patient clots are resistant to tPA, possibly due to increased levels of fibrinolysis inhibitors such as PAI-1 (TAFI was found to have no correlation) (74). Another theory on the cause of a clot resistance to tPA was investigated using clot turbidity and tPA fused to green fluorescent protein (GFP) to follow fibrin localization. These investigators found that the presence of DNA and histones caused fibrin fibers to thicken, less tPA-GFP to bind, and lysis to proceed more slowly (75). Furthermore, a supra-physiological dosage of tPA is given due to a rapid clearance rate with tPA half-life of ~3mins, (1) which can result in systemic plasmin generation and possibly lead to hemorrhage (76,77).

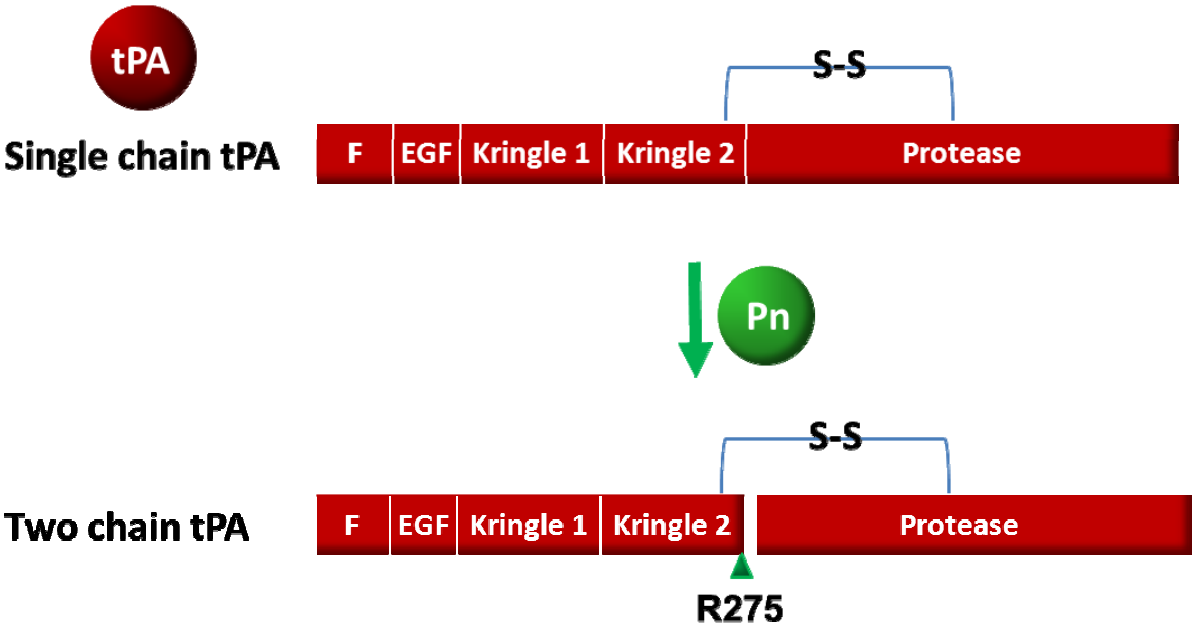


Figure 10: Single chain and two chain forms of tPA

tPA circulates in the single chain form containing an N-terminal finger-like domain (F), epidermal growth factor-like domain (EGF), two Kringle domains, and a serine protease domain. It can be cleaved into a two-chain disulfide-linked enzyme that is approximately 10-fold more active than the single chain form.

1.2 Hemophilia and cardiovascular disease

Hemophilia A and B are bleeding disorders with affected individuals harboring a mutation in either Factor VIII (FVIII) or IX (FIX) genes, respectively. This results in a dysfunctional or absent FVIII or FIX coagulation protein that causes variable degrees of bleeding. The severity of hemophilia is classified according to baseline FVIII or IX activity: mild (5-40%), moderate (1-5%) and severe (<1%). Current replacement therapies have decreased the bleeding-related death rate enabling the hemophilia population to dramatically increase life expectancy to that equivalent to, or approaching, the non-hemophilia population. Age-related disorders such as cardiovascular disease (CVD) are now being increasingly reported in this population. Retrospective epidemiological studies have suggested that hemophilia patients are relatively protected from CVD as they have a lower risk of mortality from CVD compared to the non-hemophiliac population (78-81). The standard mortality ratio (SMR) is the ratio of deaths observed in a group to the expected deaths in the normal population and can be shown as a percentage. The largest cohort consisting of 6018 people (HIV positive individuals excluded) in the UK from 1976-1998 reported a 38% reduction in SMR with no difference between the severities of hemophilia (82). Sramek et al observed 1012 mothers of known hemophilia patients in the Netherlands and found that even female carriers of hemophilia, who usually demonstrate modestly reduced or normal coagulation factor activity, demonstrated a 36% reduction in SMR, suggesting that this decrease in mortality may not only be due to coagulation factor level (83). Biere-Rafi et al. looked at cardiovascular risk assessment (body mass index, blood pressure, cholesterol levels, and fasting glucose levels) in hemophilia patients to determine if the protection was due to fewer CVD risk factors (84). They found that there was no difference in the prevalence of CVD risk factors in hemophilia patients compared to controls, suggesting that hypocoagulability or hyperfibrinolysis may be reducing the cardiovascular

mortality rates in hemophilia patients. Mosnier et al. showed that plasma lysis times in hemophilia patients were significantly faster and suggest that the severe bleeding in hemophilia is a "triple defect" involving reduced thrombin generation in both initiating extrinsic and amplifying intrinsic pathways, as well as decreased inhibition of fibrinolysis due to reduced TAFI activation (85). A study in Germany also found hyperfibrinolysis was the cause for more severe hemorrhagic phenotypes among patients with hemophilia, with no difference in endogenous thrombin potential, (51) and this hyperfibrinolysis may also provide an explanation for the protection from CVD in the same population.

1.3 Thesis rationale and hypotheses

Hyperfibrinolysis: An explanation for reduced cardiovascular disease in patients with hemophilia

With the advent of FVIII and FIX replacement therapy, hemophilia patients are aging and acquiring cardiovascular disease CVD. However, an as yet unknown characteristic appears to be protecting this population from CVD-related mortality compared to the non-hemophilia population (78-84). The work in this thesis addresses this phenomenon at a molecular level and is exploratory in this poorly understood area of hemophilia. Decreased thrombin generation in various severities of hemophilia demonstrates a close relationship to baseline FVIII & FIX levels. In hemophilia A patients, thrombin generation has been shown to correlate with severity classified by FVIII activity levels, but not to *clinical* severity based on frequency of hemorrhage (86). This suggests that other factors contribute to the bleeding tendency, such as fibrinolysis, platelet activity, protein C and S, TF, and properties of the endothelial wall. Since TAFI is activated by thrombin/thrombomodulin, it is anticipated that hemophilia patients will have

lower levels of TAFIa compared to controls. This would be consistent with a previous study that observed lower levels of Pro-TAFI in hemophilia patients (39). With less available Pro-TAFI and decreased thrombin generation there is consequently less activation of Pro-TAFI and thus less TAFIa produced. In addition to decreased thrombin generation, fibrinolytic inhibitors such as TAFI and PAI-1 also have an effect on bleeding tendency. Grunewald et al. studied a cohort of severe hemophilia patients with varying bleeding diatheses. In severe hemophilia, significantly higher concentrations of pro-TAFI were found in more severe hemorrhagic phenotypes (6.2 µg/ml) compared to mild hemorrhagic (3.0 µg/ml) and non-hemophilia groups (3.2 µg/ml). The increased levels of pro-TAFI in severe hemophilia suggests the production of TAFI is in response to the increased demand from incomplete clot formation and associated bleeding from increased fibrinolytic activity; however with decreased levels of thrombin in severe hemophilia there is less activation of TAFI leading to the loss of fibrinolytic inhibition (51). The same group also found that a higher concentration of PAI-1 was found in more severe hemorrhagic phenotypes (16.9 AU/L) compared to milder hemorrhagic (8.9 AU/L) and non-hemophilia groups (6.5 AU/L). Grunewald et al. explain these counterintuitive PAI-1 data as a result of co-stimulation of the fibrinolysis pathway alongside the coagulation pathway that is enhanced in response to the severe hemophilia patient's deficiency in producing stable clots (51). They suggested that hemophilia patients have increased levels of tPA (4.2ng/ml) compared to the normal population (2.9ng/ml) and discovered that there was a further increase in tPA found in the intensely hemorrhagic phenotype (7.5 ng/ml) (51). However, it is important to note that this group used an arbitrary method of assigning the "intensely hemorrhagic phenotype" based on the number of joint bleeds (>3 joints affected), and 19/21 patients had hepatitis C, and two patients were HIV positive. This means that they might just be measuring the effects of inflammation caused by the arthropathy and viral infections which has been associated with

increased levels of tPA,PAI-1 (87) and TAFI (88). Their study was limited to a small group of patients in one clinic and has not been confirmed or re-assessed in another setting. This project will build upon their initial findings concerning tPA, PAI-1 and TAFI.

This research project is aimed at addressing several questions:

1. Do hemophilia patients have specific fibrinolysis protein antigen and activity levels that correlate with enhanced fibrinolysis? This might include: i) increased tPA, ii) decreased PAI-1, and/or iii) decreased TAFI.
2. Do hemophilia patients exhibit evidence of enhanced fibrinolysis activity in comparison to age, gender and CVD risk factor matched controls?

Hypothesis: To explain the lower propensity for cardiovascular disease, hemophilia patients have enhanced fibrinolysis compared to non-hemophiliacs matched for age, gender, and CVD risk factor. Hyperfibrinolysis may include shorter plasma clot lysis times, due to increased tPA, decreased PAI-1 or decreased TAFI levels.

Regulation of Clotting Factor Xa Auxiliary Cofactor Function in Fibrinolysis through β-Peptide Excision

Previous work in our lab has suggested that the proteolytic fragment of FXa, Xa33/13, enhances purified fibrin lysis as an auxiliary cofactor for tPA, which enhances plasmin generation for dissolving the clot (24). This is likely due to the two C-terminal Lys that are exposed on this species that facilitate Pg binding and activation. However, fibrinolysis experiments in plasma have shown that there is no effect of Xa33/13 (28), although FXa is effective along with the XaAT complex (89), which is the likely source of Xa33/13 in plasma. Unpublished data have suggested that Xa33/13 is more susceptible to inactivation than FXa or XaAT by a mechanism in plasma

that does not exist in the purified fibrinolysis assay. These data furthermore suggest that the fibrinolytic activity of FXaβ is more stable than that of Xa33/13 and enhances fibrinolysis in plasma. Interestingly, a mixture of purified FXaα and FXaβ is more active in purified fibrinolysis experiments than Xa33/13 for poorly understood reasons, further complicated by the latter associating more avidly with Pg (24). It is not yet known whether the C-terminal arginine (R429) FXaβ would have the same enhancing effect, although this has been reasoned to be the form of FXaβ that is incapable of binding to Pg when generated in the absence of aPL-binding (24) .

This research project is aimed at addressing several questions:

1. Is FXaα cleaved preferentially at K427 R429, K433 or K435 to produce FXaβ and is this affected by the presence or absence of $CaCl_2$ and aPL?
2. Is the production of FXaβ obligate for subsequent K330 cleavage resulting in Xa33/13 generation?
3. Can FXaβ with a C-terminal Arg429 be cleaved by Pn at K330 to generate the Xa33/13 fragment?

Hypothesis: Pn-mediated conversion of FXaα to profibrinolytic FXaβ will be facilitated by preferential cleavage at one of three lysines under conditions that favour aPL-binding and switched to predominantly cleave at the nearby Arg when not bound to aPL. β-peptide excision will facilitate subsequent Pn-mediated cleavage of K330 to generate Xa33/13.

2. Materials and Methods

2.1 Materials

Dr. Rodney Camire generously provided the pCMV4-ss-pro-II-FX and pcDNA3.1 plasmids (90). The Stratagene Quikchange II XL site-directed mutagenesis kit was purchased from Agilent Technologies (California, USA) and primers were ordered from Integrated DNA Technologies (Iowa, USA). Ampicillin, Opti-Mem, DMEM/F-12, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (pen/strep), trypsin, geneticin, and sodium chloride (NaCl) were bought from Gibco (Invitrogen; California, USA). QIAprep spin miniprep kit was acquired from Qiagen (Ontario, Canada). Lipofectamine LTX and PLUS reagent were obtained from Invitrogen (California, USA). Glycerol, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulphoxide (DMSO), polyethylene glycol 8000 (PEG 8000), bovine serum albumin (BSA), phosphatidylserine (PS), and phosphatidylcholine (PC) were all purchased from Sigma-Aldrich (Missouri, USA). Vitamin K1 was acquired from Baxter (Ontario, Canada). 6-well plates, 96-well plates, T-75, T-150, and Hyperflasks (10 layer) cell culture flasks were purchased from Corning (Massachusetts, USA) and triple flasks from Nalge Nunc International (New York, USA). Insulin-transferrin-sodium (ITS) was obtained from Roche (Indiana, USA). Polyvinylidene fluoride (PVDF) membranes, Amicon and Microcon centrifugal filtration devices, and regenerated cellulose ultrafiltration membranes were bought from Millipore (Massachusetts, USA). The ECL-Plus detection kit was purchased from GE Healthcare (New Jersey, USA). Innovin was obtained from Siemens Healthcare (Marburg, Germany). The PageBlue PVDF stain was from Fermentas (Ontario, Canada). Pooled normal human plasma was obtained from both Affinity Biologicals Inc. (Ontario, Canada) and George King Bio-medical, Inc. (Kansas, USA). FX-immunodepleted plasma was obtained from Biopool International (California,

USA). Chromogenic substrates S-2251 was from Diapharma Group Inc (Ohio, USA). Small unilamellar vesicles (SUV) and large multilamellar vesicles (LMV) consisting of 75:25 PC:PS were prepared by extrusion and quantified through the release of phosphorus as described previously (26). Iodogen used for labeling was obtained from Thermo Scientific, Rockford IL, USA.

2.2 Hemophilia patient study

Plasma was collected from 25 Calgary consenting hemophilia subjects with variable degrees of either FVIII or FIX deficiency invited to participate in a prospective study evaluating endothelial function and the risk of ischemic heart disease under ethics approval U of C Ethics ID E-21650, and in Vancouver, UBC Ethics ID: H09-02426. These samples were collected by nurses at Foothills Hospital in Calgary as part of a larger study on the risk of ischemic heart disease in hemophilia evaluating endothelial dysfunction and the development of CVD (91). For the control group, 75 banked plasmas were generously shared from the Firefighters and Their Endothelium study by Todd Anderson (92). The controls were matched 3:1 to patients according to age, gender, and cardiovascular risk profile (diabetes, hypertension, cholesterol, smoking habits, family history of CVD). Neither the patient nor control plasma samples were collected specifically for this study, and were not collected at a specific time of day nor according to venipuncture protocol specific for tPA, PAI-1, or TAFI analysis. Plasma was drawn before factor replacement to obtain therapeutic trough levels and then stored at -80°C. Samples were thawed and aliquoted in order to minimize freeze-thaw cycles that can cause an increase in PAI-1. TAFIa is labile and plasma collection is preferentially collected in tubes containing anticoagulant (14.4 µg/mL Heparin, 15.2 µM ε-aminocaproic acid (εACA); American Diagnostica catalogue ref:

452SCD3) to maintain stability. This was not done for the plasma samples evaluated in the current study and therefore endogenous TAFIa activity may be somewhat underestimated.

IMUBIND® tPA, IMUBIND® Plasma PAI-1, and IMUCLONE® TAFI ELISA kits and SPECTROLYSE® PAI Activity and ACTICHROME® TAFI Activity Assay kits were purchased from American Diagnostica (Stanford, USA) and assays were conducted according to the manufacturers' protocols.

In each IMUBIND® or IMUCLONE® ELISA kit a 96-well plate is coated with a polyclonal primary antibody (Ab), which captures the antigen (Ag) of interest from the plasma samples. A second polyclonal primary Ab (same as the well-coating Ab) conjugated to HRP binds to the captured Ag and reports on the antigen of interest according to the manufacturer's instructions.

The SPECTROLYSE® PAI Activity kit is a two-stage indirect assay for PAI-1 activity that measures residual tPA activity through Pn generation. In the first stage, a fixed amount of tPA (40IU/ml) is added to react with the PAI-1 present in the plasma sample. The sample is acidified with acetate buffer to destroy any α2AP present that might interfere with the Pn that will be generated. In the second stage, residual tPA activity is measured through the addition of excess Pg. A chromogenic substrate for Pn activity is used to measure the extent of Pg conversion by tPA. 1 IU PAI-1 Activity = the amount of PAI-1 that inhibits 1 IU of tPA (~1.45ng)

The ACTICHROME® TAFI Activity Assay kit was purchased from American Diagnostica (Stanford, USA) with the intent to measure TAFIa activity as the name of the kit might suggest, but was found to not be designed specifically for measuring TAFIa activity, but rather for thrombin/thrombomodulin activatable zymogen TAFI (pro-TAFI) (39). Two sets of samples are assayed simultaneously; one set is activated by adding a recombinant thrombin/thrombomodulin complex, and the other is not preactivated. Carboxypeptidase

activity including TAFIa is measured using a TAFI substrate. Intrinsic carboxypeptidase N-like activity was measured using the unactivated samples, and the difference between the activated and unactivated measurements corresponded to the pro-TAFI that is activatable by the recombinant thrombin/thrombomodulin complex.

2.2.1 Plasma clot lysis

To measure plasma fibrinolysis, clot formation in hemophilia and control plasma samples was induced using Innovin as a source of TF and aPL at a 1:16,000 dilution to allow participation of both branches of coagulation or at a 1:4000 dilution to exclude the FVIII-dependent intrinsic pathway. A low concentration of tPA (75 pM) was added to be within the physiologically relevant range. The final $CaCl_2$ concentration was made 15 mM to overcome the citrate in plasma. Patient and control samples were run in duplicate at 37 °C in 96-well-plates, sealed with a transparent adhesive plastic to prevent evaporation. Clot dissolution was followed by measuring Raleigh light scattering at 405nm using a SpectraMax microplate reader (Molecular Devices) over several days. Half-lysis times, the time it takes to reach 50% clot dissolution, were calculated using Graphpad Prism 4 software, and fitting the fibrinolysis data to a simple inverse sigmoidal curve.

2.3 Proteins

Single chain tPA was purchased from Genentech (California, USA). Fibrinogen and Lys-Pg were bought from Enzyme Research Laboratories (Indiana, USA). Human plasma-derived proteins including FX, FXa, thrombin, and plasmin, and Russell's viper venom-derived FX activator (RVV-X) were purchased from Haematologic Technologies Inc. (Vermont, USA). The monoclonal antibody specific for human FX(a) heavy chain was obtained from Green Mountain

Antibodies (Vermont, USA). Peroxidase-conjugated goat anti-mouse IgG used for detection of FX(a) and various fragments by Western blot was purchased from Jackson ImmunoResearch Laboratories (Pennsylvania, USA). Radiolabelled ^{125}I-Pg was prepared as described previously by the Pryzdial lab and did not exceed 200,000 dpm/µg (23).

2.4 Molecular biology of factor X

2.4.1 Site-directed mutagenesis

Seven factor X mutants were generated using the Quikchange site-directed mutagenesis kit according to manufacturer's protocol. Single-point mutants were made by replacing Lys427, Arg429, Lys433, and Lys435 with Gln and Arg429 with Lys. Gln was chosen instead of Ala to preserve the size of the side chain while still neutralizing the positive charge. A triple point mutant was made by mutating the three Lys 427, 433, and 435, to Gln, and a quadruple point mutant was generated mutating all four basic residues to Gln. Primers were designed using Oligo solftware under the guidance of Valerie Smith from Dr. Ross MacGillivray's lab, and then primers were ordered from Integrated DNA Technologies (Table 1).

Mutant	Forward Primer (5'-3')	Reverse Primer (5'-3')
Lys427Gln (K427Q)	CGACAGGTCCATGCAAACCAGGGGC	GCCCCTGGTTTGCATGGACCTGTCG
Arg429Gln (R429Q)	GGATCGACAGGTCCATGAAAACCCAGGGCTTGCCC	CGGCAAGCCCTGGGTTTTCATGGACCTGTCGATCC
Lys433Gln (K433Q)	GGCTTGCCCCAGGCCAAGAGCCATGCC	GGCATGGCTCTTGGCCTGGGGCAAGCC
Lys435Gln (K435Q)	GGCTTGCCCAAGGCCCAGAGCCATGCC	GGCATGGCTCTGGGCCTTGGGCAAGCC
Lys427Gln/Lys433Gln/ Lys435Gln (K3βQ)	AGGTCCATGCAAACCAGGGGATTGCCCCAGGCCCAGAGCCATGCC	GGCATGGCTGGCCTGGGGCAATCCCCTGGTTGCATGGACCT
Lys427Gln/Arg429Gln/ Lys433Gln/Lys435Gln (KR4βQ)	GACAGGTCCATGCAGACCCAGGGCTTGCCCCAGGCCCAGAGCCATGCCC	GGGCATGGCTCTGGGCCTGGGGCAAGCCCTGGGTCTGCATGGACCTGTC
Arg429Lys (R429K)	GACAGGTCCATGAAAACCAAGGGCTTGCCC	GGGCAAGCCCTTGGTTTTCATGGACCTGTC

Table 1: Primers used for mutagenesis of *F10* gene

Five single-point and two multi-point mutants of FX were created through site-directed mutagenesis. Oligo software was used to identify optimal forward and reverse primers that were obtained commercially from Integrated DNA Technologies (IDT). The altered codons are highlighted in yellow (Table 1), with the nucleic acid that was changed in red. The silent mutation for Gly (GCC → GG**A**) in the K3βQ mutant was included to prevent primer loop formation.

The mutations were inserted into a plasmid containing the FX gene (F10), pCMV4-ss-pro-II-FX gifted by Dr. Rodney Camire. The signal sequence and propeptide of FX in the plasmid were replaced with that of prothrombin to increase expression (90). For each mutant, plasmid and primers were amplified using polymerase chain reaction (PCR) and then restriction enzyme Dpn I was used to digest parental DNA. The PCR products were transformed into XL10-Gold ultracompetent cells with β-mercaptoethanol. Ampicillin (10 µg/mL)-Luria Bertani (LB) agar plates, prepared the day before, were used to plate and colonize the cells. For each mutant, six colonies were selected and grown in ampicillin-LB media. Using the Qiagen mini-prep kit, DNA was extracted and sequenced to confirm that mutation was successful and the F10 gene was complete. Aliquots of cells for each mutant were stored in ampicillin-LB media with 15% glycerol at -80°C.

Initial DNA work for the four single-point mutations to Gln was done by Dr. Mitra Panahi, a former postdoctoral fellow in our laboratory, and the triple-point mutant was done by Dr. Amanda Vanden Hoek, former PhD student from our laboratory.

2.4.2 Stable expression of FX mutants

Plasmid pcDNA3.1 was used as a selectable marker and co-transfected with each of the mutant F10-containing plasmids into human embryonic kidney (HEK) 293 cells using Lipofectamine LTX with and without PLUS reagent according to the manufacturer's protocol. Transfection was first attempted with Lipofectamine 2000, however there were no successful transfected cells due to high toxicity of this transfection reagent. HEK 293 cells were grown to ~80% confluence in 6-well plates and then incubated with a mixture of mutant plasmid, Lipofectamine LTX (with and without PLUS reagent) and Opti-Mem. Cells were allowed to grow overnight at 37 °C with 5 % carbon dioxide (CO_2), and then replaced with growth media: DMEM/F-12 supplemented with 5% FBS, 1% L-Glu, and 1% pen/strep and allowed to grow overnight again under the same incubation conditions. Dead suspended cells were aspirated and discarded. The viable adherent cells were lifted using 0.25% trypsin & 1mM EDTA and then reseeded at various cell dilutions in new 6-well plates containing selection media: DMEM/F-12 supplemented with 5% FBS, 1% L-Glu, and 1% pen/strep, 6ug/ml Vit K1 and 0.9% geneticin. Geneticin is the selector used to destroy cells that do not have the selectable marker pcDNA3.1 plasmid. The cells were allowed to grow for 2-4 weeks until colonies formed. Several colonies were selected for each mutant and each were seeded into T150 flasks for expansion to ~80-90% confluency. These selected clones were reseeded into triple flasks for expansion and a portion of these cells for each clone were frozen in selection media with 5% DMSO for later use. Once the transfected cells reached 80-90% confluency in the triple flasks, selection media was replaced with serum-deprived expression media: DMEM/F-12 supplemented with insulin-transferrin-selenium (ITS), 1% L-Glu, and 1% pen/strep, 6ug/ml Vit K1, and 0.9% geneticin. This conditioned media was collected and replaced daily for up to 2 weeks and stored at -80 °C for use in Western blots and activity assays.

2.4.3 Clone selection

2.4.3.1 Factor X antigen quantification

Conditioned media collected from each clone were thawed at 37 °C and analyzed by Western blot in quadruplicate to quantify FX antigen expressed for each clone using a standard curve of known purified FX (15ng–100ng) run on each gel. Compared to a conventional ELISA, this method is preferable because it also enabled evaluation of FX fragmentation. Media samples were diluted in sample buffer (Laemmli: 63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.01% Bromophenol blue) and boiled at 95 °C for 5 mins. The samples were then run on 10% acrylamide gels by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidine difluoride (PVDF) membranes. The PVDF membranes were blocked using 5% skim milk in Tris-buffered saline with Tween (TBST; Tris-HCl (50 mM), NaCl (150 mM), 0.05% Tween-20) for 1 hour, and then incubated with primary antibody (20ng/ml mouse anti-human FX heavy chain monoclonal antibody) in 5% skim milk in TBST for another hour. The excess antibody and milk were washed with TBST thrice for 5 mins and then membranes were incubated with secondary antibody (20 ng/ml horseradish peroxidise (HRP)-conjugated goat anti-mouse antibody) for 1 hour. The excess antibody was again washed with TBST three times for 5 mins and then ECL-Plus detection kit was used to visualize bands by chemiluminescence using a ChemiGenius imaging system and GeneTools software (PerkinElmer).

2.4.3.2 Factor Xa activity by prothrombin time

Factor Xa clotting activity in collected conditioned media for each clone was determined using a prothrombin time (PT) assay. Aliquots were rapidly thawed at 37 °C and then diluted into 50µL HEPES-buffered saline (HBS; HEPES (20 mM), NaCl (150 mM), at pH 7.4) and incubated for 1 min with 50 µL of FX-immunodepleted plasma (BioPool) at 37 °C. 100 µL of Innovin, pre-

warmed to 37 °C, was added to initiate clotting and clotting time was measured by the halt in motion of a metal ball in an ST4 coagulation analyzer (Diagnostica Stago). A standard curve was made with a range of dilutions of normal plasma as well as a second standard curve using a range of dilutions of normal plasma-derived FX with known specific activity (HTI).

2.4.3.3 Factor X specific activity

The specific activity (units/mg) was calculated for each clone using the antigen quantification by Western blot and activity determined by PT. The FX activity in normal pooled reference plasma was used to define FX units per mL. The activity of each clone in units/ml was calculated from the normal FX standard curve and then divided by the amount of FX antigen to obtain a specific activity in units/mg. Several clones for each mutant with the highest specific activities and antigen production were selected for large scale expression and purification for experiments.

2.4.4 Large-scale expression and purification of FX mutants by binding to anionic phospholipid vesicles

The stably transfected clones were thawed at 37°C and seeded into T-150 flasks and allowed to grow to ~80-90% confluency. The adherent cells were trypsinized and then reseeded into triple flasks and hyperflasks for expansion. A portion of these cells were frozen in selection media with 5% DMSO. The cells were then serum-deprived with expression media as before and the conditioned media was collected daily for up to 3 weeks (until cells were dying) and stored at -80°C. Immediately prior to purification, the conditioned media was thawed at 37°C and centrifuged at 15,000 revolutions per minute (rpm) for 15min to remove cellular debris and then concentrated at 4°C in a stirred cell concentrator under nitrogen gas pressure with 10kDa cut-off YM-10 regenerated cellulose ultrafiltration membrane, and then further concentrated with

10kDa cut-off Amicon centrifugal filter device according to the manufacturer's protocol (Millipore),

Post translational γ-glutamyl carboxylation of the FX Gla domain is essential for functional FX as it is responsible for calcium-dependent anionic phospholipid (aPL) binding. When calcium interacts with the Gla domain at N-terminal residues 1-11, an Ω loop is formed and three hydrophobic residues Phe4, Leu5, & Met8 protrude, enabling an interaction with aPL (93). Incomplete carboxylation of the Gla domain is a common problem with recombinant FX (94). Wild-type FX and recombinant FX with carboxylated Gla domain was purified from media using functional affinity purification by binding to aPL. rFX (250 nM) in media was incubated with $CaCl_2$ (2.5 mM) and LMV (1 mM) for 5mins at room temperature, then centrifuged at 13,000 rpm for 5 mins to pellet bound rFX to LMV. The Supernatant was kept and stored at -20°C for subsequent Western blot analysis. The pellet was washed by centrifugation three-times with $CaCl_2$ (2.5mM) in HBS and these washes were also kept and stored for Western blot analysis. The final pellet was resuspended with EDTA (5 mM) in HBS to dissociate the bound rFX from aPL and the sample was centrifuged at 13,000 rpm to pellet the LMV. Purified rFX in EDTA (5 mM) was stored at -80°C. All samples were quantified and evaluated by Western blot and if necessary due to low yield (<20 ng/µl), concentrated using a 10kDa cut-off Microcon centrifugal filter device according to manufacturer's protocols (Millipore). Purified commercial normal plasma derived FX (nFX) was also subjected to the same conditions and stored in EDTA (5 mM).

2.5 rFX Procoagulant function

2.5.1 RVV-X activation of rFX

The purified recombinant wild-type FX (WTFX), recombinant β-mutants (rFX), and plasma-derived nFX (200 nM) were incubated with RVV-X (125 nM), $CaCl_2$ (10 mM), and LMV (1 mM) for up to 30mins. Samples were taken at a range of time points and pipetted into Laemmli sample buffer to stop the reaction and then heated at 95 °C for 5 mins. The samples were run on 10% acrylamide SDS-PAGE and then transferred to PVDF for Western blot analysis using the same protocol as in section 2.4.3.1.

2.6 rFX fibrinolytic function

2.6.1 Plasmin-mediated cleavage and Lys-plasminogen binding

To establish the roles of the four basic residues at the β-peptide cleavage site in FXaα to FXaβ conversion and generation of Xa33/13, plasmin-mediated FXa fragmentation of each of the rFX mutants were followed by plasmin incubation time course and Western blot analysis. rFX (200 nM) was incubated with RVV-X (125 nM), $CaCl_2$ (10 mM), and LMV (1 mM) at room temperature for a period of time optimized for each mutant to minimize FXaα to FXaβ conversion during activation, while still allowing for maximal activation of rFX. The rFXa was then incubated with plasmin (100nM) up to 30mins and samples were taken at a range of time points and immediately added to Laemmli sample buffer to stop the reaction. The samples were then run at 100ng total FX protein per lane on 10% acrylamide gels by SDS-PAGE, transferred to PVDF membranes and Western blot analysis was conducted as in 2.4.3.1 to visualize the FXa

fragments. A second set of activated rFX was incubated with plasmin (100 nM) in the presence of EDTA (20 mM) to observe the differing FXa fragmentation without $CaCl_2$ (Figure 5).

To determine which of the basic residues in the β-peptide region was responsible for binding Pg, and which of the FXa fragments could bind Pg, ^{125}I-Pg was used for ligand blots on the same PVDF membranes used for Western blots. In order to have enough protein available to bind ^{125}I-Pg, 300ng of total FX protein per lane was loaded. Membranes were blocked overnight with bovine serum albumin (BSA; 10 mg/mL) in Tris-buffered saline (TBS; Tris-HCl (50 mM), NaCl (150 mM), at pH 7.4). The next day the PVDF membranes were probed with ^{125}I-Pg (50nM) at room temperature for 1 hour. The blots were then washed thrice with TBS and then placed between transparencies. Sheets of XAR film (Kodak) were exposed to the membranes for up to 2-3 weeks in metal film cassettes with Quanta III intensifying screens (Dupont). The film was developed and then analysed using a ChemiGenius imaging system. Band intensity was standardized to a constant amount of nFXa for all Western and ligand blots.

2.6.2 Tissue-type plasminogen activator-mediated plasmin generation

To compare the enhancing ability of the nFX, WTFX and different rFX β-mutants in tPA-mediated plasmin generation, the increase of plasmin activity over time was measured using a chromogenic assay. In a 96-well flat bottom plate, nFX, WTFX and rFX (100 nM) in HBS/0.1% PEG-8000 were incubated with RVV-X (125 nM), $CaCl_2$ (10 nM), and SUV (0.1 mM) to activate at room temperature for 10mins to minimize FXaα to FXaβ conversion during activation. Lys-plasminogen (0.5 μM) was added and then the reaction was initiated with the addition of tPA (10 nM) and incubated up to 40 mins. At a range of time points, 10 μL samples were taken and added to 190 μL chromogenic substrate for plasmin S-2251 (200uM substrate in HBS/PEG-8000 and EDTA (20 mM)) and measured kinetically for 1 min at 405nm using SpectraMax microplate

reader (Molecular Devices). At the same time, another set of 10 µL samples were taken and added to Laemmli sample buffer for Western blot analysis. Assays were done in duplicate over three experiments and standard error of the mean and post-hoc t-tests comparing each group to one another were used to assess statistical significance.

3. Hyperfibrinolysis in Hemophilia Patients

3.1 Overview and specific goals

Enhanced clot-dissolving (i.e. fibrinolysis) capabilities may provide an explanation for the relative protection from CVD in the hemophilia population. This has led us to analyze the initiating enzyme of fibrinolysis and regulators of the fibrinolysis pathway. tPA initiates fibrin clot degradation in the presence of a C-terminal Lys-containing cofactor by activating Pg to plasmin (Pn). PAI-1 is an important tPA inhibitor, which forms an irreversible complex with the active site of tPA to prevent Pn generation. Activated TAFI (TAFIa) removes the C-terminal lysine residues necessary for Pg- and tPA-binding to cofactors and thus also inhibits clot-dissolution. Hemophilia patients generate lower levels of thrombin due to low FVIII & FIX levels. Since TAFI is activated by thrombin/thrombomodulin, it is anticipated that the hemophilia patients will have lower levels of TAFIa compared to controls.

Hypothesis: To explain their lower propensity for cardiovascular disease, hemophilia patients have enhanced fibrinolysis, which reduces thrombosis.

Specific objectives are:

1. To determine whether there is a difference in specific fibrinolysis protein and activity levels between a cohort of hemophilia subjects and controls: i) tPA, ii) PAI-1, and iii) TAFI.
2. To determine whether patients with hemophilia demonstrate evidence of enhanced fibrinolysis activity in the clot lysis assay, in comparison to age, gender and CV risk factor matched controls.

3.2 Results

The clinical characteristics of the 25 hemophilia subjects are presented in Table 2. The sample included a broad range of ages with approximately one-third having hemophilia B (expected prevalence ~15-20%) and 17/25 had mild-moderate hemophilia.

Age (years)	Median: 44 (IQR 37,55), Range: 24-71
Type of Hemophilia	18 Hemophilia A, and 7 Hemophilia B
*Severity of Hemophilia	12 Mild, 5 Moderate, 8 Severe
Factor VIII or IX Usage (IU/kg/year)	Median: 129.5 (IQR 0,1110.5), Range: 0-5235.5
Cardiovascular Risk Factors (Number of Patients, %)	
Diabetes	1, 4%
Hypertension	4. 16%
Elevated cholesterol	3, 12%
Current smokers	3, 12% cigarette smokers, 1, 4% marijuana smoker
Family History of CVD	7, 28%

Table 2: Clinical characteristics of the hemophilia patients enrolled in study

Cardiovascular risk factors were used to match three control individuals for each patient. *Severity is classified by baseline FVIII or FIX level; Mild = FVIII or FIX: 0.06-0.40 IU/mL, Moderate = FVIII or FIX: 0.01-0.05 IU/mL and Severe = FVIII or FIX: < 0.01 IU/mL.

3.2.1 tPA, PAI-1 and TAFI antigen

Antigen levels of tPA, PAI-1 and TAFI were measured in the 25 hemophilia patient plasmas compared to 75 controls. The patient group includes both the hemophilia A and B patients and all severities. The mild and moderate severities were combined for simplicity since there were no differences between the two groups. The control group consists of three cardiovascular risk matched non-hemophilia individuals per patient. IMUBIND® tPA, IMUBIND® Plasma PAI-1, and IMUCLONE® TAFI ELISA kits were purchased from American Diagnostica (Stanford, USA) and conducted according to the manufacturer's protocols. There was no significant difference found between hemophilia patients (A and B combined) and controls for all three antigens: tPA ($p = 0.428$), PAI-1 ($p = 0.195$), and TAFI ($p = 0.230$). Severe hemophilia patients were found to have a

significantly lower level of tPA Ag than the mild-moderate patients (Mean: 2.9 ± 2.2 ng/ml, 6.0 ± 2.6 ng/ml respectively; $p < 0.01$). PAI-1 Ag was also found to be significantly lower in severe hemophilia patients compared to mild-moderate patients (Mean: 26.6 ± 6.8 ng/ml, 48. 3 ± 20.1ng/ml respectively; $p < 0.01$). There was no significant difference in TAFI Ag between the mild-moderate and the severe hemophilia groups (Table 3 and Figure 11).

	Patient		Control	P Value (T Test)
tPA Ag (ng/ml)	5.1 ± 2.7		5.7 ± 2.6	0.428
	Mild-Mod	Severe		
	6.0 ± 2.6	2.9 ± 2.2		Mild-Mod vs Severe: $p < 0.01$
PAI-1 Ag (ng/ml)	40.7 ± 17.9		46.4 ± 21.4	0.195
	Mild-Mod	Severe		
	48.3 ± 20.1	26.6 ± 6.8		Mild-Mod vs Severe: $p < 0.01$
TAFI Ag (% Pooled Normal Plasma)	83.2 ± 14.8		78.0 ± 20.9	0.230
	Mild-Mod	Severe		
	86.6 ± 12.8	79.8 ± 18.9		Mild-Mod vs Severe: $p = 0.315$

Table 3: Summary of fibrinolysis antigen results

tPA, PAI-1 and TAFI Ag were measured by ELISA and no difference was found between the patient and control groups. Severe hemophilia patients were found to have significantly lower tPA and PAI-1 Ag than the mild-moderate patients.

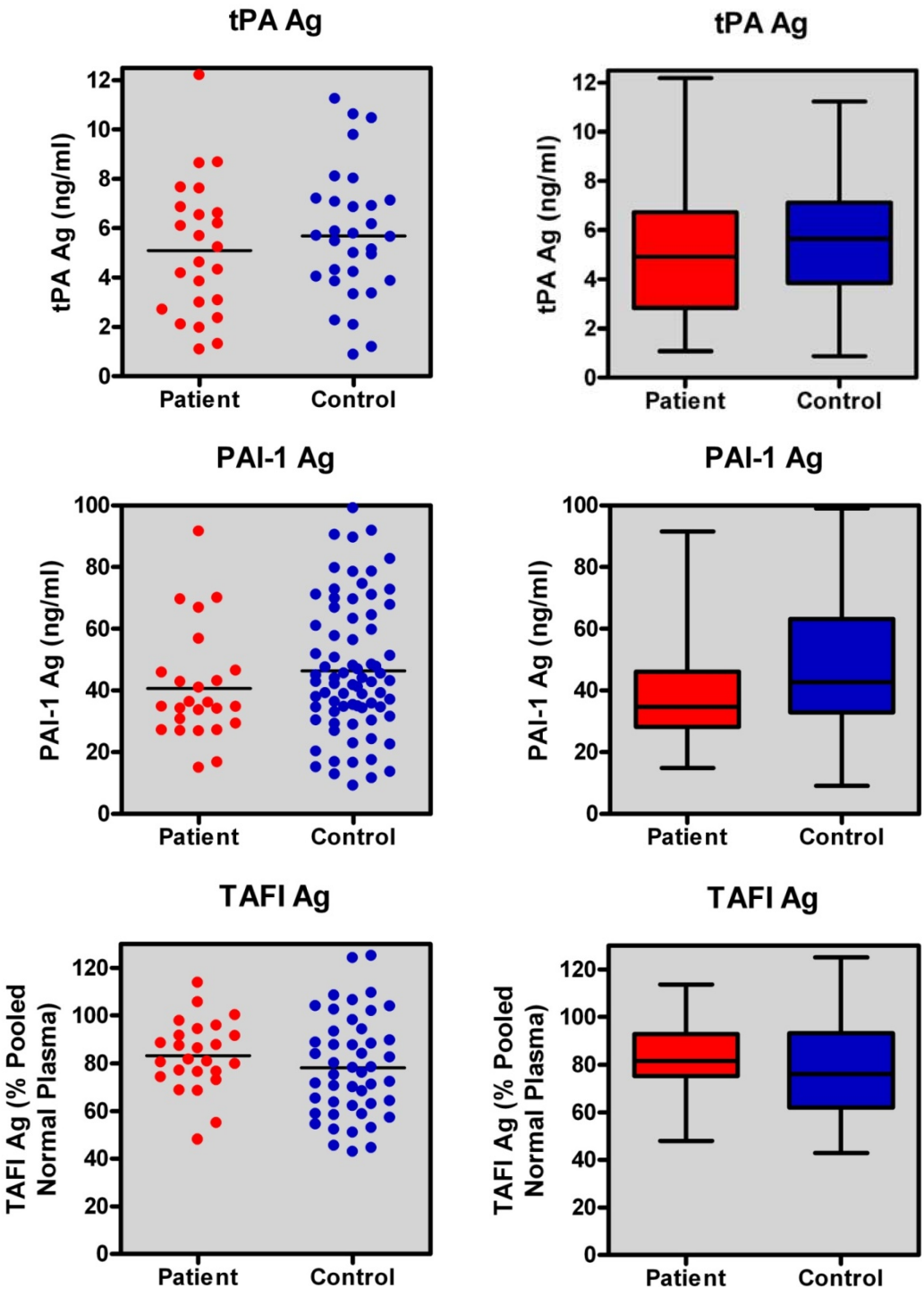


Figure 11: tPA, PAI-1, and TAFI Ag levels are normal in adult male hemophilia patients

tPA, PAI-1, and TAFI Ag were measured using sandwich ELISA. The wells of a 96 well plate were coated with a primary antibody which captures the Ag. A second primary antibody, this time conjugated to HRP binds the captured antigen.

3.2.2 PAI-1 activity

PAI-1 Activity was measured with the SPECTROLYSE® PAI Activity kit from American Diagnostica (Stanford, USA), a two-stage indirect chromogenic assay conducted according to protocol. In the first stage, a fixed amount of tPA is added to react with PAI-1 in the sample, and the sample is acidified to destroy α2AP. In the second stage, residual tPA activity is measured by adding Pg in excess and using a chromogenic substrate for Pn to measure how much is converted by tPA. α2AP was removed in the first stage because it would bind and inhibit the Pn being generated and measured. PAI-1 activity was significantly lower in the hemophilia patients compared to the controls assayed (mean: 9.5 ± 7.1 IU/ml, 25.0 ± 20.9 IU/ml respectively; $p < 0.01$) (Figure 12 and Table 4). When stratified according to severity of hemophilia, PAI-1 activity was found to be significantly lower in severe patients compared to patients with mild-moderate hemophilia (Mean: 3.8 ± 2.8 IU/ml, 12.5 ± 7.3 IU/ml, respectively; $p < 0.01$).

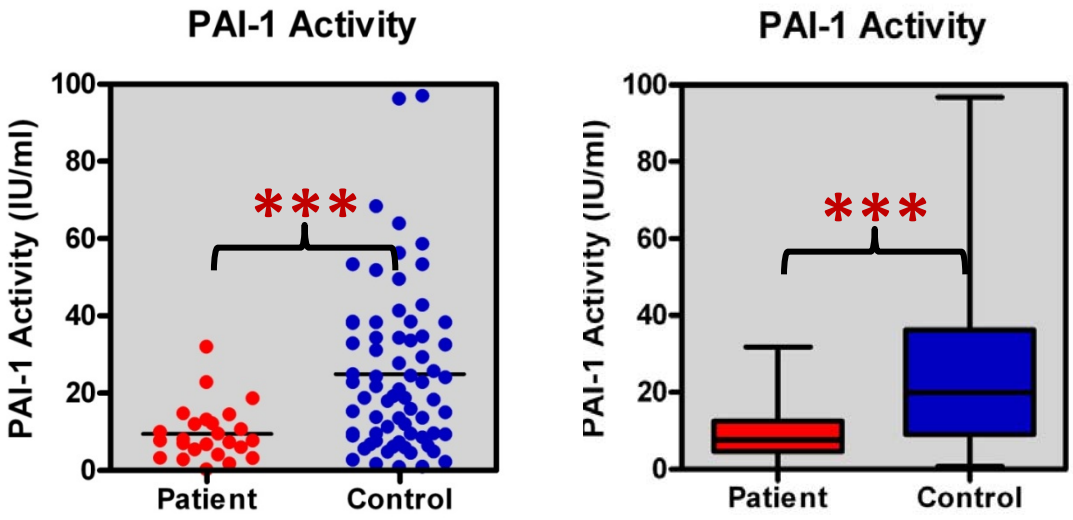


Figure 12: Adult male hemophilia patients have decreased PAI-1 activity

PAI-1 Activity was measured using a two-stage indirect chromogenic assay. In the first stage a fixed amount of tPA was added to react with PAI-1 in the sample, and the sample was acidified to destroy α2AP. In the second stage, residual tPA activity was measured by adding Pg in excess and using a chromogenic substrate for Pn to measure how much is converted by tPA. α2AP was

removed in the first stage because it would bind and inhibit the Pn being generated and measured.

3.2.3 Thrombin/thrombomodulin activatable pro-TAFI and intrinsic carboxypeptidase N-like activity

ACTICHROME® TAFI Activity Assay kit was purchased from American Diagnostica (Standford, USA) with the intent to measure TAFIa activity, but was found to not be properly designed for measuring only TAFIa activity, but rather for thrombin/thrombomodulin activatable zymogen TAFI (pro-TAFI). Two sets of samples are assayed simultaneously; one set is activated by adding a recombinant thrombin/thrombomodulin complex, and the other is left unactivated. Carboxypeptidase activity including activated TAFIa is measured using a specific TAFI substrate from American Diagnostica. Intrinsic Carboxypeptidase N-like activity was measured using the unactivated samples, and the difference between the activated and unactivated sets corresponds to the pro-TAFI that is activatable by the recombinant thrombin/thrombomodulin complex.

Thrombin/thrombomodulin activatable pro-TAFI in the hemophilia patients was significantly lower compared to the controls (mean: 39.6 ± 7.1 μg/ml, 44.2 ± 7.7 μg/ml respectively; $p = 0.018$). Conversely, intrinsic carboxypeptidase N-like activity measured in the same assay was significantly higher in the hemophilia patients compared to controls (mean: 11.9 ± 4.3 μg/ml, 7.6 ± 2.1 μg/ml respectively; $p < 0.01$) (Figure 13 and Table 4). When stratified according to severity of hemophilia, patients with severe hemophilia had significantly lower thrombin/thrombomodulin activatable pro-TAFI compared to mild-moderate hemophilia patients (32.4 ± 4.6 μg/ml, 42.3 ± 6.7 μg/ml respectively; $p < 0.01$). There was no significant difference between the severities of hemophilia in intrinsic carboxypeptidase N-like activity (Mild-Mod: 13.0 ± 3.1 μg/ml, Severe: 10.1 ± 7.1 μg/ml; $p = 0.299$)

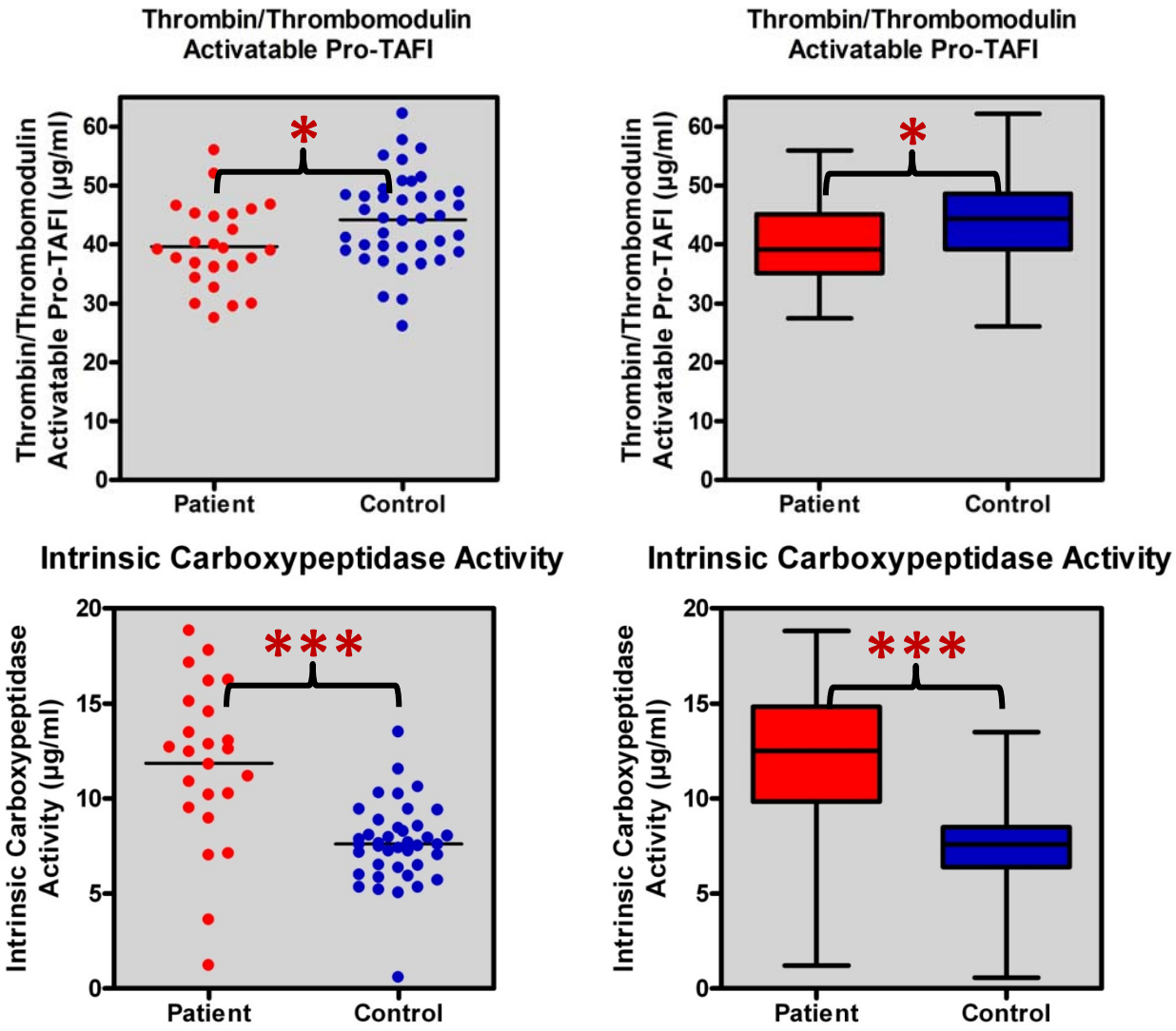


Figure 13: Adult male hemophiliacs have decreased thrombin/thrombomodulin activatable pro-TAFI and increased intrinsic carboxypeptidase N-like activity

Two sets of samples are assayed simultaneously; one set was activated by adding a formulated thrombin/thrombomodulin complex, and the other was left unactivated. Activated TAFI was measured using a specific TAFI substrate that measures carboxypeptidase activity. Intrinsic carboxypeptidase N-like activity was measured using the unactivated samples, and the difference between the activated and unactivated sets corresponds to the pro-TAFI that is activatable by the thrombin/thrombomodulin complex.

	Patient		Control	P Value (T Test)
PAI-1 Activity (IU/ml)	9.5 ± 7.1		25.0 ± 20.9	<0.01
	Mild-Mod	Severe		
	12.5 ± 7.3	3.8 ± 2.8		Mild-Mod vs Severe: $p < 0.01$
Thrombin/Thrombomodulin Activatable Pro-TAFI (µg/ml)	39.6 ± 7.1		44.2 ± 7.7	0.018
	Mild-Mod	Severe		
	42.3 ± 6.7	32.4 ± 4.6		Mild-Mod vs Severe: $p < 0.01$
Intrinsic Carboxypeptidase N-like Activity (µg/ml)	11.9 ± 4.3		7.6 ± 2.1	<0.01
	Mild-Mod	Severe		
	13.0 ± 3.1	10.1 ± 7.1		Mild-Mod vs Severe: $p = 0.299$

Table 4: Summary of fibrinolysis activity results

PAI-1 activity, activatable TAFI, and intrinsic carboxypeptidase activity were measured using American Diagnostica kits according to manufacturer protocols. PAI-1 Activity and thrombin/thrombomodulin activatable pro-TAFI were found to be significantly lower in the patients compared to controls, with the lowest levels found in severe patients compared to mild-moderate patients. Intrinsic carboxypeptidase N-like activity was found to be significantly increased in patients.

3.2.4 Overall fibrinolytic activity by plasma clot lysis assay

To observe overall fibrinolytic activity of hemophilia patients compared to controls, plasma lysis assays were conducted. TF-containing Innovin at two different dilutions was used to initiate clot formation to allow participation of both the intrinsic and extrinsic coagulation pathways. The 1 in 4,000 dilution of Innovin contains a high TF concentration allowing one to model the FVIII independent extrinsic pathway, while the 1 in 16,000 dilution represents the FVIII dependent intrinsic pathway. A physiologically relevant concentration of tPA (75 pm) was added and Raleigh light scattering measuring clot turbidity was used to follow clot dissolution. Patient and control samples were run in duplicate at 37°C in 96-well-plates, sealed with a transparent adhesive plastic to prevent evaporation. Clot turbidity was measured at 405 nm using a

SpectraMax microplate reader (Molecular Devices) over several days. Half-lysis times, the time it takes to reach 50% clot dissolution, were calculated using Graphpad Prism 4 software (Figure 14). Max OD represents the clot amount and structural density of the initial formed clot.

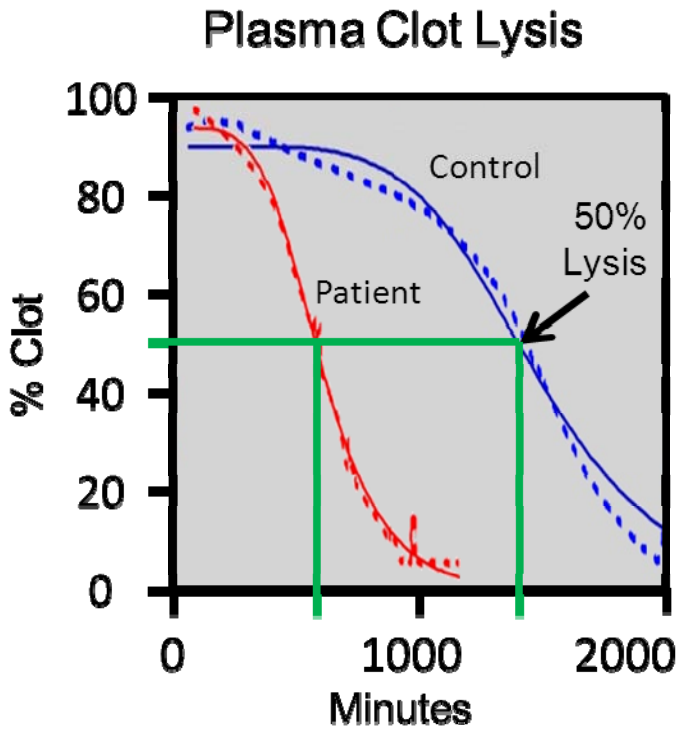


Figure 14: Plasma lysis example/Matched pair patient lyses faster than control

This is an example of a matched pair of patient and control results. Clot turbidity was measured at 405nm using a SpectraMax microplate reader (Molecular Devices) over several days. Half-lysis times, the time it takes to reach 50% clot dissolution, were calculated using Graphpad Prism 4 software.

There was no difference found in half lysis time nor max clot turbidity between plasmas clotted with 1:4000 dilution of Innovin compared to a 1:16000 dilution in both the hemophilia patients (1420 ± 501 mins, 1098 ± 366 mins respectively; p = 0.607) and controls (1926 ± 491 mins, 1996 ± 642 mins, respectively; p = 0.932). A relatively small sample size and wide variability within both the hemophilia and control groups made it difficult to evaluate significant differences, however there was a trend of hemophilia patient plasmas having enhanced fibrinolysis compared to controls (mean: 833 ± 1188 mins, 1377 ± 1483 mins respectively; P = 0.134). 50% of patient plasmas lysed at least 2-fold faster compared to controls, 39% had at least a 4-fold enhancement, and 22% had as much as a 10-fold enhancement. There was no significant difference, even upon detailed analysis, in the initial clot amount and structural density as represented by Max OD (mean: 0.5 ± 0.1, 0.5 ± 0.1 respectively; P = 0.209). There was also no difference between the different severities of hemophilia in half lysis time (Mild-Mod: 656 ± 923, Severe: 1387 ± 1751; p = 0.289) and Max OD (Mild-Mod: 0.5 ± 0.1, Severe: 0.5 ± 0.1; p = 0.416). All plasma lysis data results are summarized in Figures 15 and 16, and Table 5.

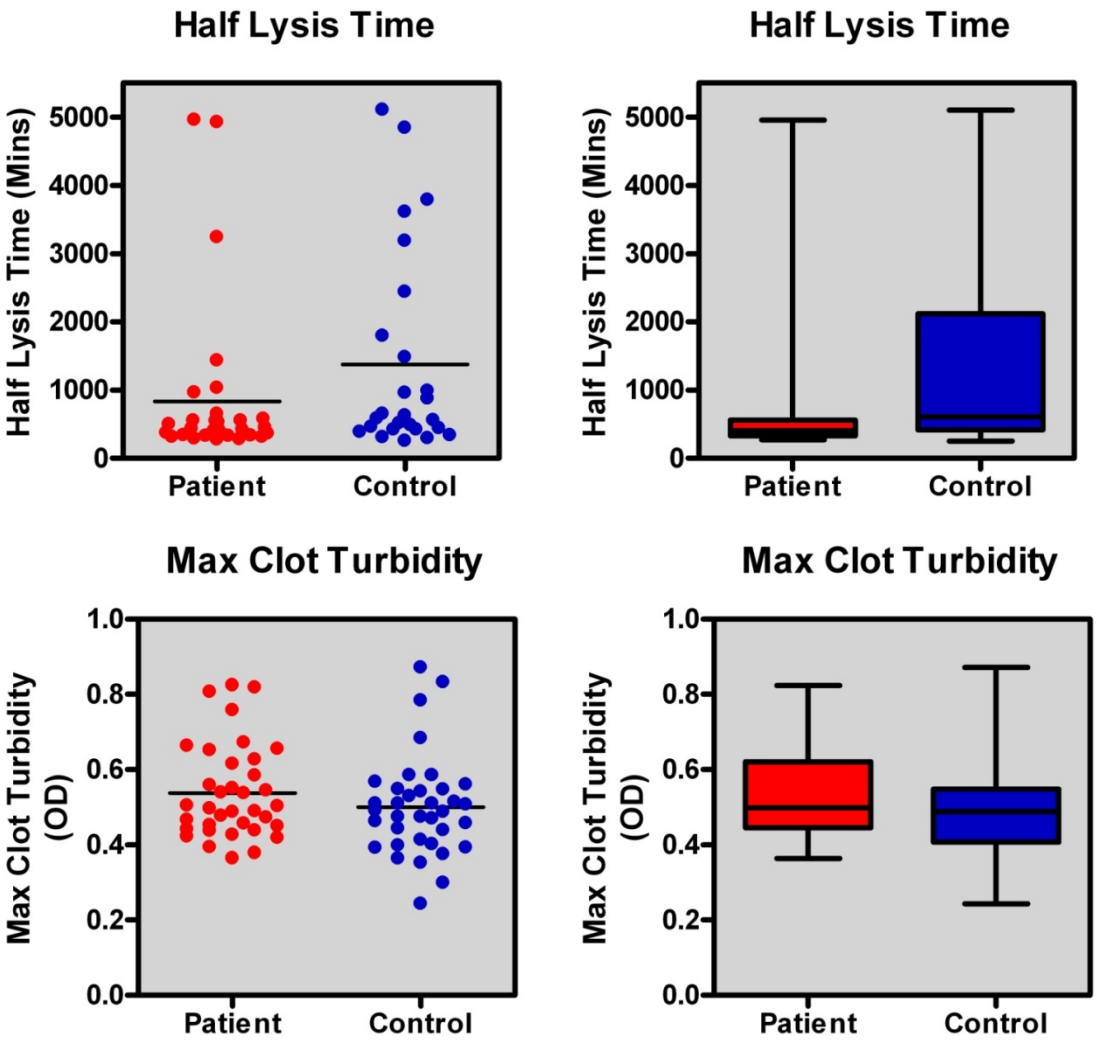


Figure 15: Half lysis time in patients are not significantly shorter than in controls

Half lysis times, the time it takes to reach 50% clot dissolution, were calculated using Graphpad Prism 4 software. Max clot turbidity representing initial clot amount and structural density was measured by Max OD.

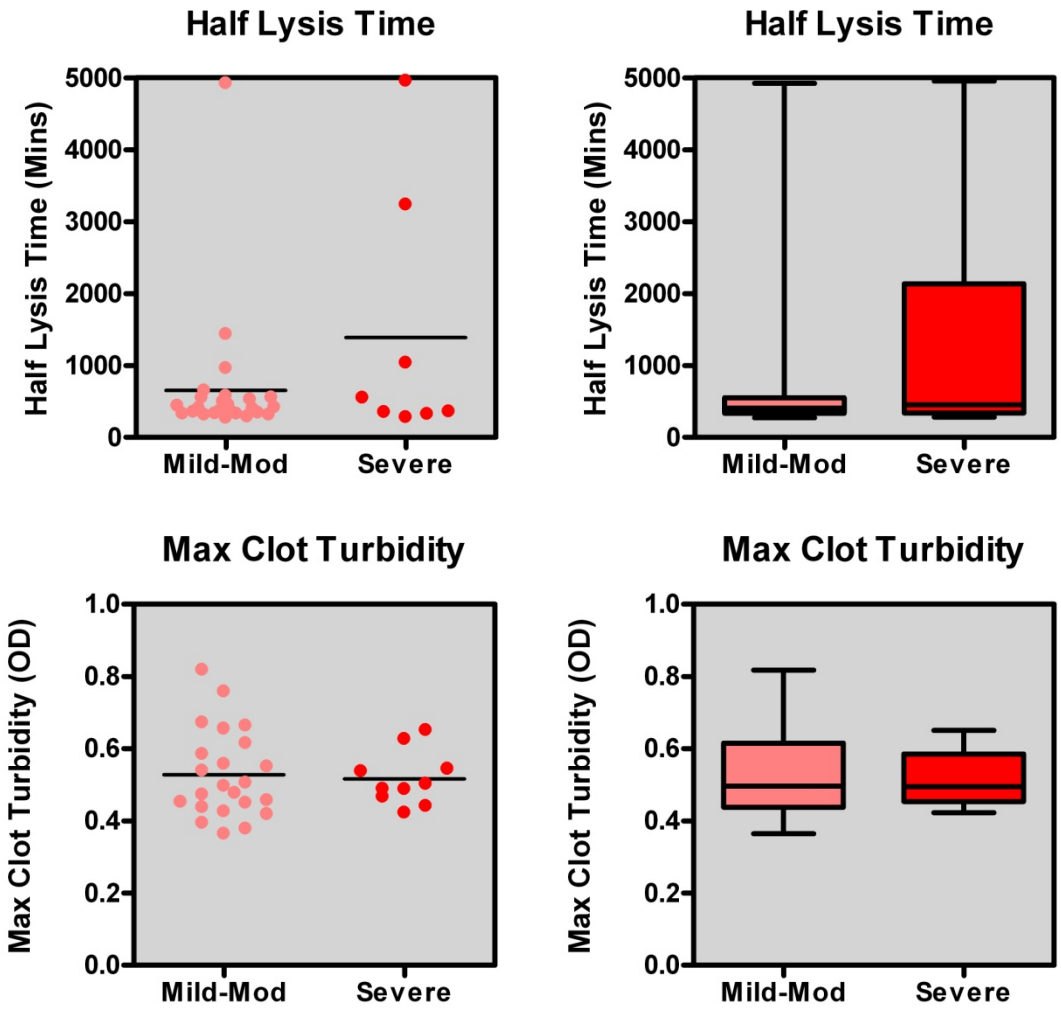


Figure 16: No significant differences found in half lysis time nor max clot turbidity between severities of hemophilia

Half lysis times and Max clot turbidity were calculated as in Figure 15 and stratified by severity of hemophilia.

	Patient		Control		P Value
Innovin Dilution	**1:4000**	**1:16000**	**1:4000**	**1:16000**	
Half Lysis Time (mins)	1420 ± 501	1098 ± 365	1926 ± 491	1996 ± 641	Patient: 0.607; Control: 0.932
	833 ± 1188		1377 ± 1483		0.134
	Mild-Mod	Severe			
	656 ± 923	1387 ± 1750			Mild-Mod Vs Severe: p = 0.289
Max OD	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	Patient: 0.115; Control: 0.390
	0.5 ± 0.1		0.5 ± 0.1		0.209
	Mild-Mod	Severe			
	0.5 ± 0.1	0.5 ± 0.1			Mild-Mod vs Severe: p = 0.416

Table 5: Summary of plasma clot lysis results

There was no significant differences found in half lysis times or max OD between patients and controls. There was also no difference between the two dilutions of Innovin which represented the involvement of the intrinsic and extrinsic pathways.

3.3 Discussion

During the tainted blood tragedy of the 1980s and 1990s, many people who received blood products were infected with HIV and hepatitis C. Hemophilia patients were among those who depended on these contaminated blood products, and this population unfortunately suffered great morbidity and/or loss of life. In present day, the rigorous screening of blood donors and samples, as well as the use of recombinant factor replacement therapeutics instead of plasma-derived factors, have minimized the threat of pathogen infection. We are now observing age-related disorders in hemophilia patients such as CVD, for which people with inherited hemophilia have a decreased risk of mortality. This project has looked at markers of fibrinolysis, tPA, PAI-1, and TAFI, as well as plasma clot lysis in hemophilia patients to help explain the relative protection from CVD.

3.3.1 Effect on tPA, PAI-1, and TAFI antigen levels

The tPA Ag levels of 5.1 ± 2.7 ng/ml and 5.7 ± 2.6ng/ml in patients and controls respectively were comparable to those previously in the literature (~5ng/ml) (1). It is unknown why there are lower levels of tPA in patients with severe hemophilia. This decrease in tPA could be a compensatory response to increased bleeding risk in patients with severe hemophilia, achieved by reducing fibrinolysis.

PAI-1 Ag levels of 40.65 ± 17.98 ng/ml and 46.39 ± 21.41 ng/ml in patients and controls respectively were also comparable to those of reference plasma levels published in literature (~26-47ng/ml) (55-57). It is unclear why there are lower levels of PAI-1 Ag in severe hemophilia patients compared to the other severities. This may contribute to an increased bleeding diathesis in severe patients.

Quantification of TAFI has been difficult historically because of the lability of TAFIa. The three forms of TAFI: Pro-TAFI, TAFIa, and TAFIai, are difficult to measure separately and as a result, total TAFI Ag is measured as a percentage of pooled normal plasma (%PNP). The conversion from % PNP (unit of measure given by American Diagnostica kit) to µg/ml is unknown. The TAFI Ag levels 83.2 ± 14.8% PNP and 78.0 ± 20.9% PNP, in patients and controls respectively, fell in the range of healthy individuals 41-259% PNP (95). This is consistent with previous findings of normal total TAFI Ag in hemophilia (39). The wide range of TAFI Ag levels found in the literature might be due to the ELISA kit from American Diagnostica measuring all forms of TAFI including those that have become inactive.

3.3.2 Effect on fibrinolysis inhibitors: PAI-1, TAFI, and carboxypeptidase activity

PAI-1 activity of 25.0 ± 20.9 IU/ml in the controls was comparable to reference levels in the literature (~24 IU/ml) (58). PAI-1 activity was significantly lower in the hemophilia patients at 9.5 ± 7.1 IU/ml across all severities compared to controls, with the lowest levels in the severe patients at 3.8 ± 2.8 IU/ml. Lower PAI-1 fibrinolysis inhibitor activity might contribute to enhanced fibrinolysis in hemophilia patients. Severe patients had significantly lower levels than both the mild and moderate patients and this could also be contributing to increased bleeding diatheses, although we did not have complete age of first bleed data, which has been found to correlate with bleeding heterogeneity (96). This decrease in PAI-1 activity in severe patients is opposite to what has been found in the past with regards to hemorrhagic phenotypes [more severe hemorrhagic phenotypes (16.9 AU/L) compared to mild-moderate hemorrhagic (8.9 AU/L) and non-hemophilia groups (6.5 AU/L)] (51).

Thrombin/Thrombomodulin activatable pro-TAFI in the hemophilia patients was significantly lower compared to the controls (mean: 39.6 ± 7.1 µg/ml, 44.2 ± 7.7 µg/ml respectively; p =

0.018), and again was lowest in severe hemophilia patients at 32.4 ± 4.6 μg/ml. This decrease in pro-TAFI in hemophilia is consistent with a previous study by Antovic et al (39). Lower levels of pro-TAFI suggests there will be less TAFI activated and lower activity of this fibrinolysis inhibitor as well. This along with a lower level of PAI-1 activity supports the hypothesis that hemophilia patients have hyperfibrinolysis due to less inhibition from fibrinolysis inhibitors. In addition to decreased levels of fibrinolysis inhibitors, some other factors that could contribute to hyperfibrinolysis in hemophilia patients include:

- Decreased initial clot amount - lower FVIII and FIX activity, especially in severe hemophilia patients would lead to lower generation of thrombin and thus decreased clot formation. Yet, in our experiments, max OD demonstrates that initial clot turbidity is comparable between patients and controls even though patient plasma was taken at therapeutic trough levels.
- Weak clot structure - Although clot turbidity is the same, perhaps if we visualize the patient clots under a scanning electron microscope (SEM) we may see that hemophilia patients have looser clots of a lower density. Other studies looked at clot structure by SEM in hemophilia A and B and have shown that the clots are porous with short, thick fibrin fibers and reduced polymerisation (97,98).
- Increased Pg or Pn – there has been no evidence of this in the literature as these have not been measured specifically with regards to hemophilia patients.
- Other cofactors for tPA and Pg in the vicinity of the clot, such as FXa fragments – discussed in the second project.

Grunewald et al found increased tPA, PAI-1 and TAFI in more severe hemorrhagic phenotypes within the severe hemophilia A and B population (51). They suggested that the

increased PAI-1 activity is a response to their observed increased tPA antigen. The high levels of pro-TAFI is suggested by Grunewald et al to be a response to a greater demand for fibrinolytic inhibition from incomplete clot formation and associated bleeding. However, with decreased levels of thrombin in severe hemophilia there is less activation of TAFI leading to the loss of fibrinolytic inhibition. TAFI is also known to play an anti-inflammatory role, inhibiting pro-inflammatory mediators bradykinin, and complement anaphylatoxins C3a and C5a (99,100). As mentioned earlier, the arbitrarily defined "intensely hemorrhage phenotype" which is based on the number of joint bleeds (>3 joints affected) might be representative of a group with increased inflammation. Inflammation is associated with increased tPA, PAI-1 (87) and TAFI (88), which is exactly what they observed in the "intensely hemorrhagic" group. Their study was limited to a small group of patients in one clinic with confounding factors such as hepatitis C and HIV (19/21 had hepatitis C and 2 had HIV) and has not been confirmed or re-assessed in another setting.

Intrinsic carboxypeptidase N-like activity, which includes both TAFIa and plasma carboxypeptidase N was found to be significantly higher in the hemophilia patients compared to controls (mean: 11.9 ± 4.3 μg/ml, 7.6 ± 2.1 μg/ml respectively; $p < 0.01$). Since zymogen TAFI was found to be lower in patients, this increase is likely due in most part to carboxypeptidase N (CPN) which is always active unlike TAFI which normally circulates as a zymogen (101) and spontaneously becomes inactive with a short half-life of 10 mins. It is unclear why CPN would be increased in hemophilia while TAFI, also known as carboxypeptidase U or B2, is decreased. This could be a compensatory effect in hemophilia patients where CPN generation is increased to take over the role of the lacking carboxypeptidase U. While both have been shown to significantly decrease plasminogen binding to cells at physiological plasma concentrations, only TAFIa is able to inhibit whole blood clot lysis initiated by tPA (101). Thus, since CPN does not

inhibit clot lysis, our result of increased intrinsic carboxypeptidase activity in hemophilia patients does not contradict the conclusion of hemophiliacs having enhanced fibrinolysis due to reduced inhibition.

3.3.3 Effect on plasma clot lysis

In the plasma clot lysis assays we looked at overall fibrinolysis activity in the hemophilia and control plasmas. Looking at the two different dilutions of Innovin allowed us to see the effect of FVIII on the rate of fibrinolysis. There was no difference between the two Innovin groups in half lysis times nor max clot turbidity in both the hemophilia patients ($p = 0.607$) and controls ($p = 0.932$). This suggests that the lower level of FVIII in the hemophilia patients is sufficient to generate the normal amount of initial clot, and that FVIII does not have an effect on fibrinolysis. Half lysis times in the hemophilia patients on average were 40% shorter than in the controls (mean: 833 ± 1188 mins, 1377 ± 1483 mins respectively; $P = 0.134$). 50% of patient plasmas lysed at least 2-fold faster compared to controls, 39% had at least a 4-fold enhancement, and 22% had as much as a 10-fold enhancement. This trend supports the idea that hemophilia patients have enhanced fibrinolysis, which could explain their relative protection from CVD. There was no significant difference in initial clot amount and density as shown by Max OD, however visualization with SEM might reveal any differences in density of the clot that can contribute to faster clot dissolution (97,98). There was also no statistical difference between the different severities of hemophilia in half lysis time and Max OD, however there was a trend of mild patients having shorter lysis times. The mild patients were less likely to receive coagulation factor replacement or prophylaxis, and this may be a reason for shorter lysis times. Low numbers of samples and wide variation due to some patients being on coagulation replacement

therapy may have contributed to the lack of significant differences, but there was a visible trend in shorter lysis times in hemophilia patients compared to controls.

3.4 Summary

Hemophilia patients have been shown to have a lower risk of mortality from cardiovascular disease independent of their baseline coagulation FVIII or FIX level. We hypothesized that decreased inhibition of fibrinolysis or hyperfibrinolysis could explain this relative protection from cardiovascular disease death. In this thesis, I have evaluated several key components of the fibrinolysis pathway, tPA, PAI-1, and TAFI, both at the antigen and activity levels, and also investigated overall fibrinolysis by measuring plasma clot lysis. There were no significant differences found in antigen levels for tPA, PAI-1, or TAFI between hemophilia patients and cardiovascular risk matched-controls. However, I have shown that there is a significant decrease in TAFI and PAI-1 activity in hemophilia patients. This decrease in fibrinolysis inhibitor activity supports the hypothesis that hemophiliacs have enhanced clot-dissolving, due to attenuated down-regulation of fibrinolysis. On the other hand, I also discovered that total intrinsic carboxypeptidase activity, which predominantly includes plasma CPN, was higher in hemophilia patients compared to controls. The total intrinsic carboxypeptidase activity, is likely comprised of mostly CPN, since I have already shown that there are decreased levels of zymogen TAFI in hemophilia patients. TAFI circulates as a zymogen and once activated to TAFIa has a short half-life of 10 mins, where it spontaneously becomes inactive. Conversely, CPN is always active; thus the intrinsic carboxypeptidase activity is likely due to the always active CPN rather than the short-lived TAFIa. While both have been shown to significantly decrease Pg binding to cells at physiological plasma concentrations, only TAFIa is able to inhibit whole blood clot lysis initiated by tPA (101). Since CPN does not inhibit clot lysis, our result of increased intrinsic carboxypeptidase activity in hemophilia patients does not contradict the conclusion of hemophiliacs having enhanced fibrinolysis due to reduced inhibition.

I have also demonstrated the trend of enhanced fibrinolysis in hemophilia patients through faster plasma clot lysis in hemophilia patients than in controls. 50% of hemophilia patent plasma lysed 2-fold faster than controls, and 22% had up to a 10-fold enhancement. However, the small sample size and wide variation in both sets of patients and controls might have contributed to the lack of statistical significance. The broad range of plasma clot lysis times within the hemophilia group alone could correlate to varying bleeding diathesis. We attempted to assess age of first clinical consequence as a measure of bleeding severity; however we were only able to gather data for five of the patients, which was insufficient to observe a correlation.

These findings suggest that lower levels of PAI-1 activity and activatable TAFI are associated with hyperfibrinolysis in hemophilia patients. This is supported by the trend toward a more rapid fibrinolysis in hemophilia patient plasma. Overall these results suggest that independent of the severity of FVIII or FIX deficiency, hemophilia patients have enhanced fibrinolysis, a concept that requires further exploration as a potential explanation for relative protection from CVD as well as for heterogeneity in bleeding tendency.

4. Role of Factor Xa β-peptide Excision in Fibrinolysis

4.1 Overview and specific goals

Previous work in our lab has shown that clotting factor Xa (FXa) fragments can serve as cofactors for tPA that contribute to initiating plasmin generation. In particular, Pn-mediated FXa fragments with newly exposed C-terminal lysines, FXaβ and Xa33/13, have both been shown to enhance fibrinolysis in purified fibrin lysis assays. My work investigates the importance of β-peptide excision in fibrinolysis, which may be cleaved at four different basic amino acid residues by Pn, one of which is an arginine.

Hypothesis: i)The β-peptide, when cleaved at C-terminal lysine (Lys), will be an effective auxiliary cofactor and enhance fibrinolysis; ii) The β-peptide, when cleaved at C-terminal arginine (Arg) will not be an effective auxiliary cofactor given that Arg only weakly binds to Pg compared to Lys; iii) If prevented from being excised, FXaα will not provide auxiliary cofactor activity unless cleaved by Pn at K330 to Xa33/13 (or elsewhere) exposing a C-terminal lysine.

Specific objectives are:

1. To determine the role of the four basic amino acid residues (K427, R429, K433, K435) in FXaα to FXaβ conversion by plasmin in the presence or absence of aPL and EDTA
2. To generate the "KR4βQ" mutant that will lack all four basic amino acid residues for β peptide cleavage to evaluate the requirement for β peptide excision in Xa33/13 generation; and
3. To generate "R429K" mutant which will have the Arg429 residue changed to Lys, leaving only Lys as a possible cleavage site for β peptide excision, and thus will only produce FXaβ with C-terminal Lys.

4. To quantify the function and compare FXaα (KR4βQ mutant), FXaβ with C-terminal Lys (R429K), and FXaβ with C-arginine (K3βQ) in:

   i. Xa40 or Xa33/13 generation by plasmin in the presence or absence of aPL & EDTA

   ii. Participation in fibrinolysis: Pg binding, and Pg activation/Pn generation

4.2 Results

4.2.1 Molecular biology of factor X

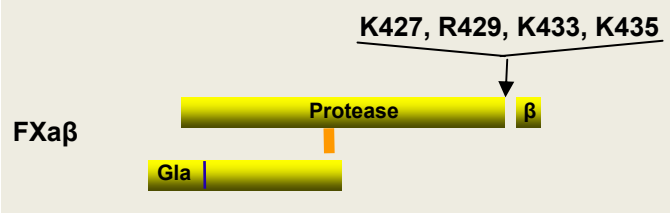
4.2.1.1 Site-directed mutagenesis and expression of FX mutants

The seven FX mutants studied in this project were made through the combined efforts of Dr. Mitra Panahi, a former postdoctoral fellow in our laboratory, Dr. Amanda Vanden Hoek, former PhD student from our laboratory, and me (Figure 17). Initial DNA work for the four single point mutants changing Lys or Arg, to Gln (K427Q, R429Q, K433Q, and K435Q), was done by Dr. Panahi, and expression by Dr. Vanden Hoek and myself. Gln was chosen as a substitute, instead of the common Ala, to neutralize the basic residue positive charge so that size of the side chain could be preserved. DNA work for the triple point mutant changing the three Lys to Gln (K3βQ) was done by Dr. Vanden Hoek and expression was done by both of us. I completed all DNA and expression work for the fifth single point mutant altering Arg429 to Lys (R429K) and the quadruple point mutant changing all four basic residues to Gln (KR4βQ), and also transfected a new set of WTFX clones to try to improve the activation yield. DNA from six of each of rFX transfected (R429K and KR4βQ) were sequenced and a query was conducted with a basic local alignment search tool (BLAST) for FX amino acid sequence using Protein BlastX. 5 of 6 were successfully changed in each set (Table 6).

Figure 17: Factor Xa Mutants

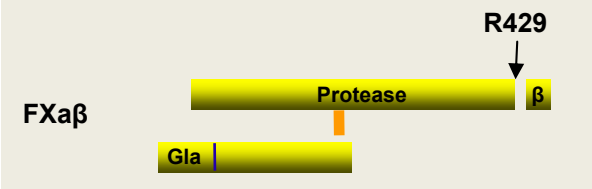
Mutants Available From Pryzdial Lab Members:

Single Point β Mutants - K427Q, R429Q K433Q, or K435Q



Each have one of the basic residues replaced by glutamine (Q) so that it cannot be cleaved by Pn at that point (i.e., only cleaves after the other three basic residues). Through this we can find out whether one particular basic residue specifically affects cleavage by Pn and the resulting tPA auxiliary cofactor activity.

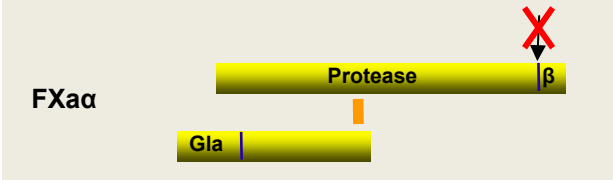
K3βQ Mutant



All three Lys are replaced by Q, so that the only FXaβ formed by Pn cleavage results in C-terminal arginine (Arg) at residue 429. This will determine whether C-terminal Arg also has tPA auxiliary cofactor activity, or if Lys is absolutely necessary for this function.

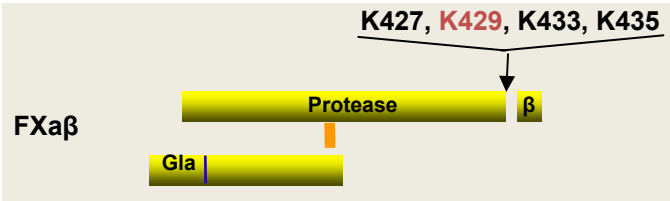
Mutants Generated For This Project:

KR4βQ Mutant – stabilized α



All four of the basic residues are replaced by glutamine, thus preventing β peptide excision. This mutant will show whether the FXaα form can confer auxiliary cofactor activity, and whether β peptide excision is necessary for Xa33/13 generation.

R429K Mutant



The Arg 429 is replaced by Lys, leaving only Lys as a possible cleavage site for β peptide excision, and thus any cleavage that produces FXaβ will give rise to a C-terminal Lys. This mutant may consequently have enhanced tPA cofactor activity compared to WTFXa.

Mutant	Wild-Type Amino Acid Sequence	rFX Amino Acid Sequence	Success?
Arg429Lys (R429K)	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTRGL PKAKSHAPEVITSSPLK	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTKGL PKAKSHAPEVITSSPLK	Yes
	VTRFKDTYFVTGIVSWGEGCA RKGKYGIYTKVTAFLKWIDRS MKTRGLPKAKSHAPEVIT	VTRFKDTYFVTGIVSWGEGCA RKGKYGIYTKVTAFLKWIDRS MKTKGLPKAKSHAPEVIT	Yes
		No F10 sequence found with BLAST query	No
	KDTYFVTGIVSWGEGCARKGK YGIYTKVTAFLKWIDRSMKTR GLPKAKSHAPEVITSSPL	KDTYFVTGIVSWGEGCARKGK YGIYTKVTAFLKWIDRSMKTK GLPKAKSHAPEVITSSPL	Yes
	QGDSGGPHVTRFKDTYFVTGI VSWGEGCARKGKYGIYTKVTA FLKWIDRSMKTRGLPK	RGTAGARTSPASRTKYFVTGI VSWGEGCARKGKYGIYTKVTA PLKWIDRSMKTKGLPQ	Yes
	DTYFVTGIVSWGEGCARKGKY GIYTKVTAFLKWIDRSMKTRG LPKAKSHAPEVITSSPLK	DTYFVTGIVSWGEGCARKGKY GIYTKVTAFLKWIDRSMKTKG LPKAKSHAPEVITSSPLK	Yes
Lys427Gln/Arg429Gln/ Lys433Gln/Lys435Gln (KR4βQ)	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTRGL PKAKSHAPEVITSSPLK	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMQTQGL PQAQSHAPEVITSSPLK	Yes
	GPHVTRFKDTYFVTGIVSWGE GCARKGKYGIYTKVTAFLKWI DRSMKTRGLPKAKSH	GPHVTRFKDTYFVTGIFSWGE GCAPKGKKTIFTSVSTFFLWI *RFM*NQFLFHANKH	No
	VTRFKDTYFVTGIVSWGEGCA RKGKYGIYTKVTAFLKWIDRS MKTRGLPKAKSHAPEVIT	VTRFKDTYFVTGIVSWGEGCA RKGKYGIYTKVTAFLKWIDRS MQTQGLPQAQSHADPGLA	Yes
	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTRGL PKAKSHAPEVITSSPLK	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMQTQGL PQAQSHAPEVITSSPLK	Yes
	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTRGL PKAKSHAPEVITSSPLK	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMQTQGL PQAQSHAPEVITSSPLK	Yes
	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMKTRGL PKAKSHAPEVITSSPLK	TYFVTGIVSWGEGCARKGKYG IYTKVTAFLKWIDRSMQTQGL PQAQSHAPEVITSSPLK	Yes

Table 6: Sequencing Results of FX mutant DNA

rFX mutant DNA was extracted with QIAprep spin miniprep kit (Qiagen - Ontario, Canada) and sequenced. The DNA sequences were aligned for amino acid sequence using Protein Blast X on the PubMed website.

4.2.1.2 Clone selection

Several clone colonies of each rFX were isolated and grown to ~80% confluence and then serum deprived in expression media for protein production. Conditioned media was collected and analyzed by Western blot to quantify the FX antigen expressed for each clone. A standard curve of titrated purified FX was used to quantify antigen based on densitometry (Figure 18). Factor Xa clotting activity for each clone was estimated using two standard curves of normal plasma and normal FX with known specific activity (HTI), and PT measured with the ST4 coagulation analyzer (Diagnostica Stago). Specific activity (units/mg) was used to represent the amount of functional protein present for each clone. This specific activity was calculated with the activity in units/ml from PT divided by FX antigen in mg/ml quantified by Western blot. For each mutant, two to three clones were selected that displayed a combination of high antigen production and high specific activity for large scale expression and purification for future experiments (Figure 19).

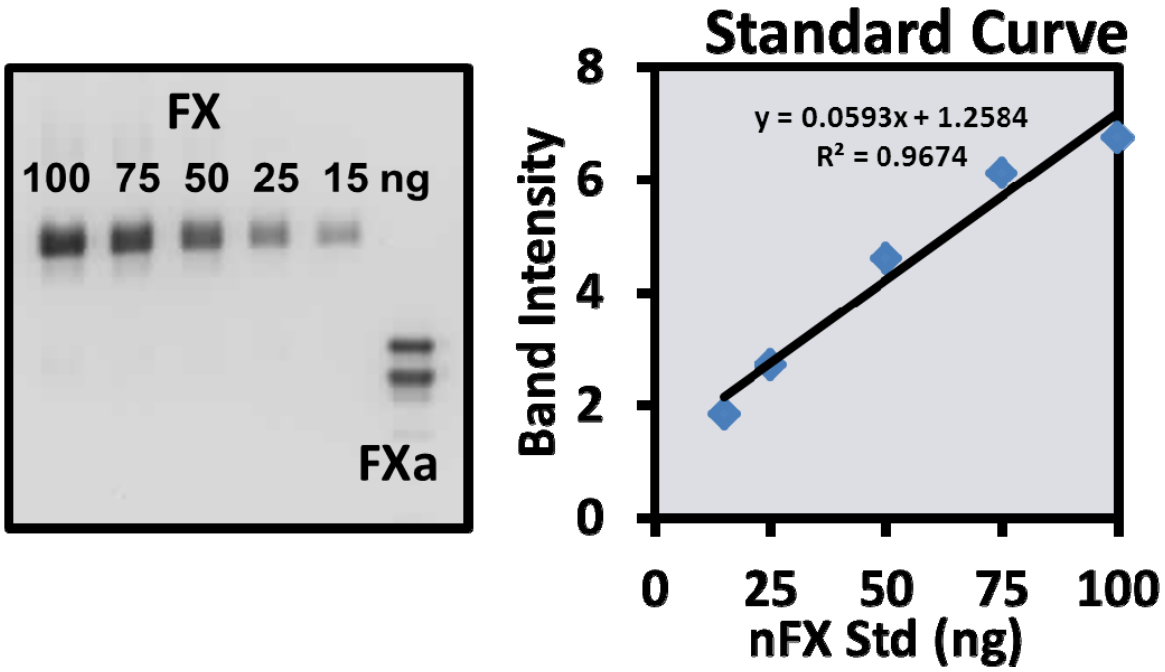


Figure 18: FX antigen in media was quantified by densitometry

rFX mutants were expressed in transfected HEK 293 cells. rFX protein was collected in media and measured by quantitative Western blot analyses. A representative standard curve is shown after densitometry of titrated purified FX.

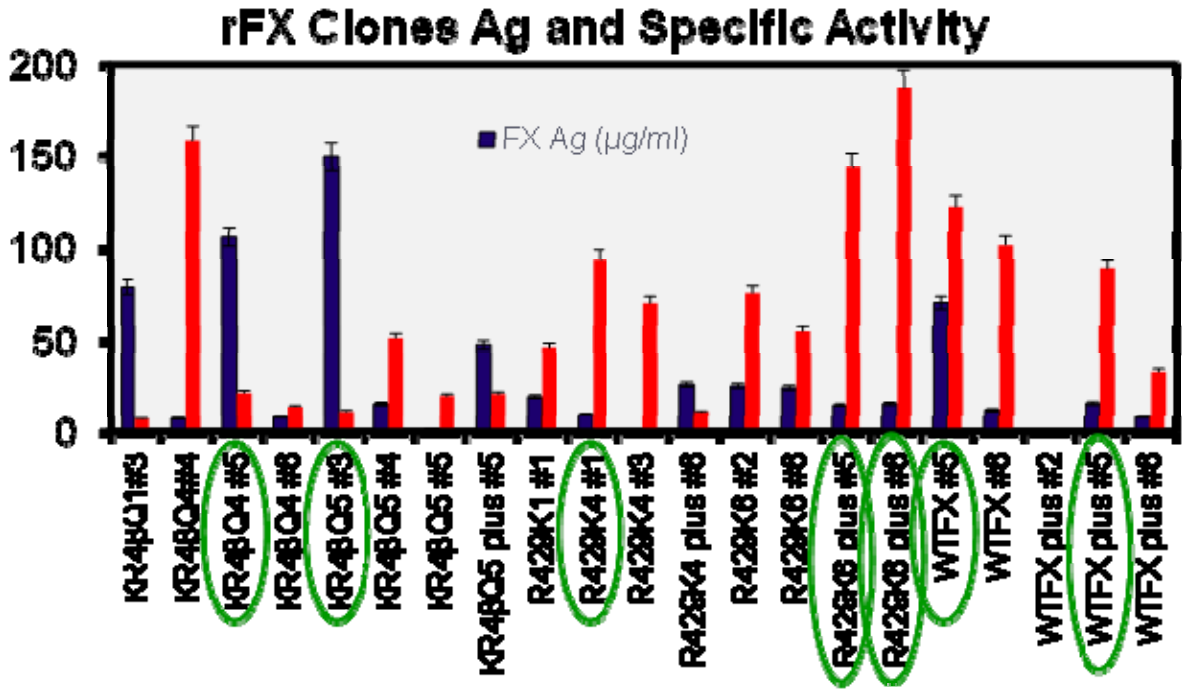


Figure 19: rFX clones were selected for high specific activity and antigen production

rFX protein in media from each clone was quantified by Western blot analysis and densitometry as in Figure 18. FXa activity was measured by prothrombin time assay. Specific activity was then calculated from the antigen and activity for each clone. Two to three clones for each mutant that demonstrated high antigen production and high specific activity, with emphasis on the latter, were selected for large scale expression and purification for experiments (circled in green)

4.2.1.3 Large-scale expression and purification of FX mutants by binding to anionic phospholipid vesicles

Once clones were selected, production of each was scaled up. To purify functional WTFX and rFX β-mutants from the conditioned media, we took advantage of the selective interaction of aPL with only a fully functional γ-glutamyl carboxylated Gla domain of FX. This allowed the incompletely carboxylated form to be separated from the properly modified protein (Figure 20). Although variable for each clone, as much as 50% was not activatable by RVV-X prior to removal by this aPL purification methodology.

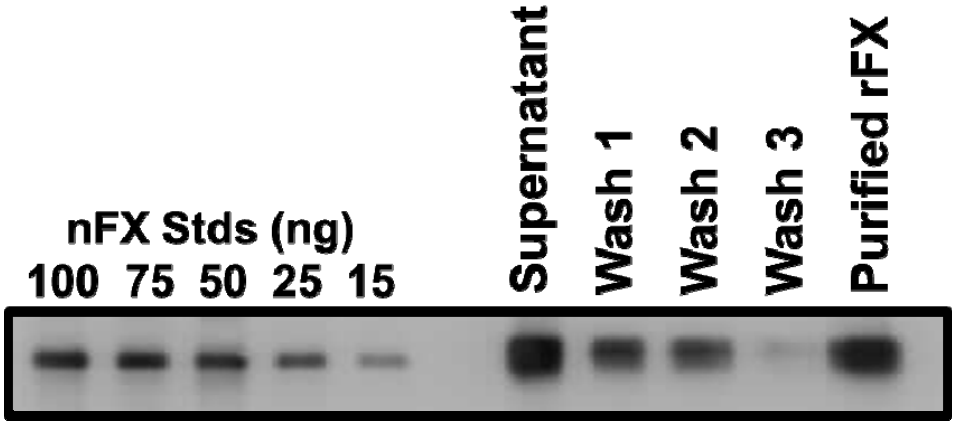


Figure 20: rFX Purification by functional affinity binding to aPL

rFX protein in media was purified by functional binding to aPL. The Western blot shows a standard of purified nFX and an example of a rFX purification at each step protocol after equilibration with aPL in the presence of $CaCl_2$. The centrifugal supernatant washes were in the presence of $CaCl_2$ and the final purified rFX was released into the supernatant with EDTA.

4.2.2 rFX Procoagulant function

4.2.2.1 RVV-X activation of rFX

The purified WTFX and rFX mutants, and nFX were treated with RVV-X in the presence of $CaCl_2$ and aPL, and samples were taken at specific time points for Western blot analysis to follow the kinetics of activation. Interestingly, mutation of the β-peptide cleavage site was found to alter the efficiency of activation peptide excision by RVV-X (Figure 21). The K427Q and R429Q mutants showed WT-like activation by RVV-X. Compared to the other mutants, K433Q, K435Q, and quadruple point mutant KR4βQ, resulted in delayed excision of the activation peptide by RVV-X. There is no plasmin present in this experiment, thus any FXaα to FXaβ conversion is due to autoproteolysis alone. Comparison of the rate of FXaα to FXaβ conversion is complicated by the differing rates of RVV-X activation among the rFX mutants. Negative control KR4βQ mutant with all four possible β-peptide excision sites changed to Gln remained in α-form throughout the experiment. K3βQ mutant, with only R429 available for β-peptide cleavage also remained in the α form suggesting that there is no autoproteolytic cleavage at R429. Thus, it was concluded that there is no autoproteolysis occurring at any site other than the three Lys K427, K433, K435. These data not only demonstrated an effect of the β-peptide on cleavage of the activation fragment, but also established experimental parameters to treat the rFX with RVV-X to optimize conversion to FXaα with minimal FXaβ to subsequently study the effects on plasmin-mediated cleavage.

4.2.2.2 rFX specific activity

The specific activity was derived using PT assays and antigen quantification for each of nine rFX produced. The WTFX clone that was selected, had comparable specific activity to nFX. Similar to excision of the activation fragment by RVV-X, mutation of the β-fragment cleavage

site affected FX activity in the PT assay. R429Q, K427Q, and R429K were found to have somewhat higher specific activities than nFX and WTFX by as much as 1.5-fold for R429Q. Conversely, K435Q and KR4βQ appeared to be significantly inhibited with only 10% PT activity compared to nFX (Figure 22).

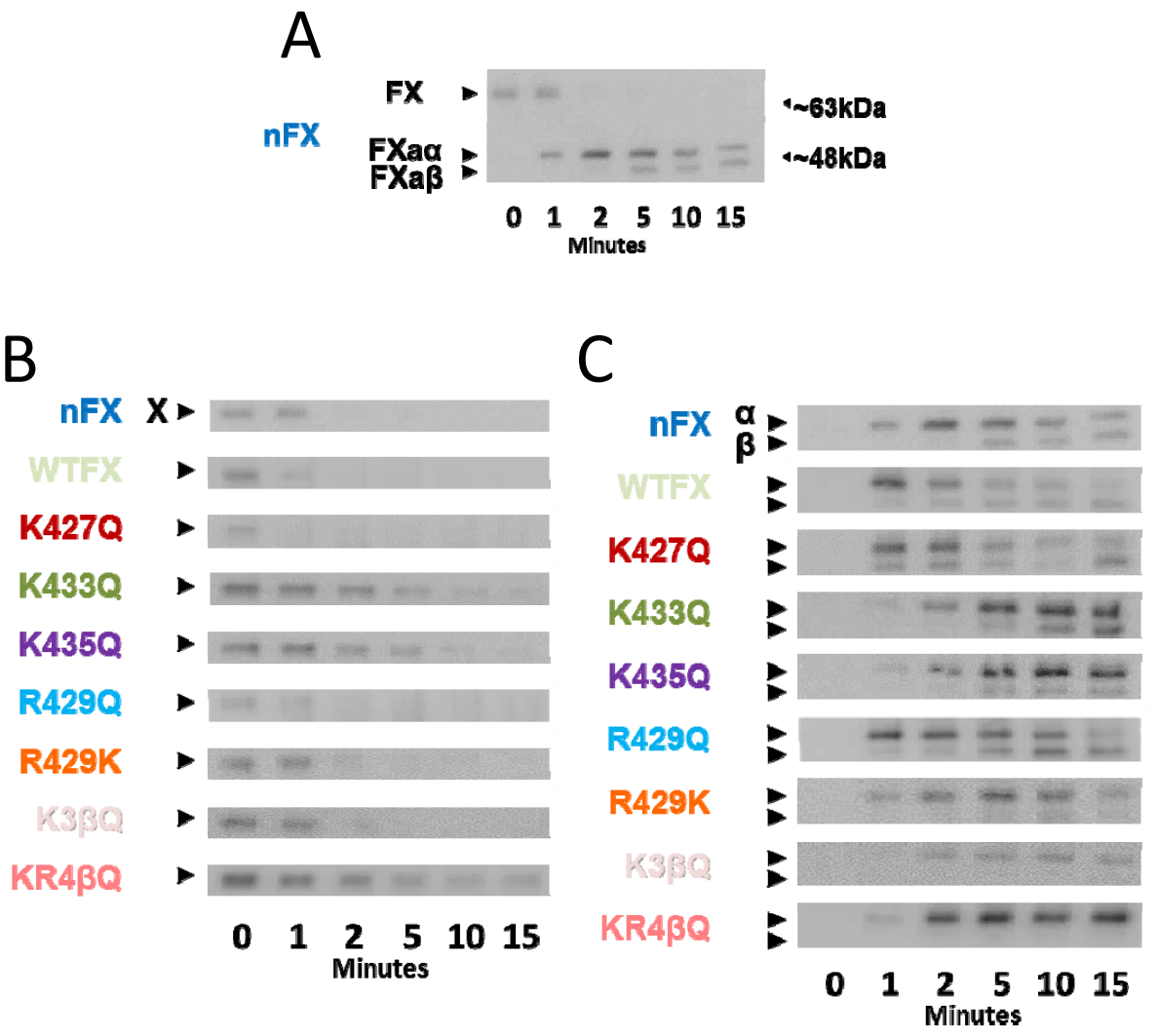


Figure 21: Mutation of the β-peptide cleavage site affects excision of the activation peptide by RVV-X

FX was treated with RVV-X in the presence of $CaCl_2$ and aPL. Samples were analysed by Western blot using a FX antibody. A. A sample Western blot of nFX demonstrating the cleavage of FX to FXaα and FXaβ. B. Western blot slices showing FX of all rFX mutants and plasma derived nFX. C. Western blot slices showing FXaα and FXaβ bands of all rFX mutants and nFX.

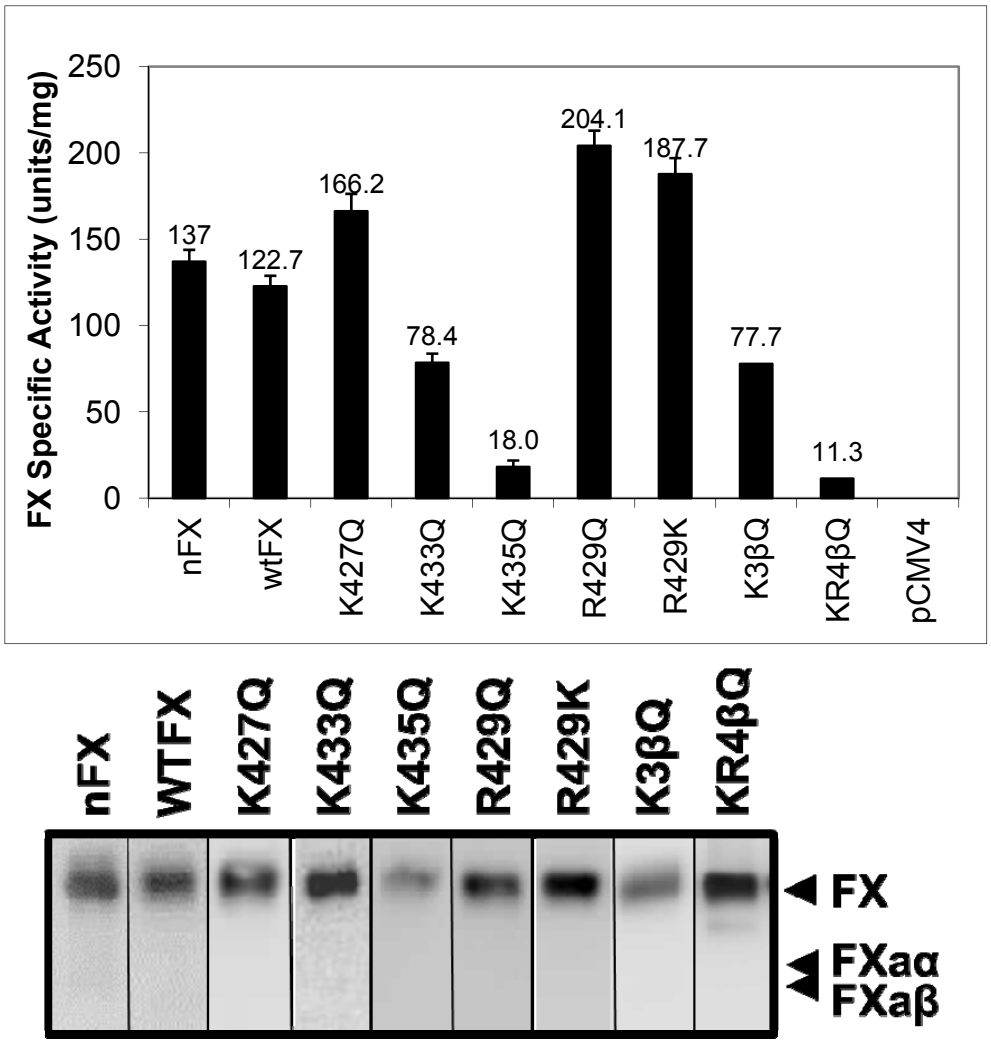


Figure 22: Specific activity of final selected clones

One clone of each mutant was selected and activity was measured by PT assays as in Figure 19. The lower panel shows Western blot of the rFX from collected media used for quantification and demonstrating lack of FXa and other fragments. rFX antigen was measured using an average of four lanes (only one of four shown) with the same volume of media loaded for each mutant.

4.2.3 rFX fibrinolytic function

4.2.3.1 Plasmin-mediated cleavage and Lys-plasminogen binding

Each of the activated rFX mutants (200 nM) were incubated with plasmin (100 nM) under two conditions to observe the diverse FXa cleavage products that may arise: one in the presence of $CaCl_2$ (10 mM) and aPL (1 mM), and another in the presence of EDTA (20 mM) and aPL (1 mM) (Figure 5). Plasmin digestion time courses on rFX after RVV-X pre-treatment for the optimal times determined above in media were subjected to Western blot analysis with 100ng total FX per lane (Figure 23). FXaα to FXaβ conversion results almost exclusively in these experiments from Pn-mediated β-peptide excision rather than autoproteolysis (102). KR4βQ which was designed to have no basic residues available for cleavage in the β-peptide region remained as intact FXaα after 30mins. These blots also showed that the triple point mutant, K3βQ, which only has R429 available for cleavage, was also resistant to FXaβ production, with only 8-9% conversion of the FXaα after 30mins, indicating that the R429 residue is a highly unfavorable plasmin cleavage site. Of the single point mutants, K435Q FXaα persisted the longest during plasmin treatment, suggesting that K435 is the preferred cleavage site in the presence of $CaCl_2$ and aPL. Unexpectedly, the R429K mutant was the most susceptible to autoproteolytic cleavage of FXaα with ~40% already as FXaβ at 0 mins, meaning it converted during the initial treatment with RVV-X. Neither of the K3βQ and KR4βQ mutants generated the Xa33 fragment after 30mins, and all of the other rFX mutants generated less Xa33 than nFX, indicating a prerequisite for β-peptide removal prior to Xa33/13 production.

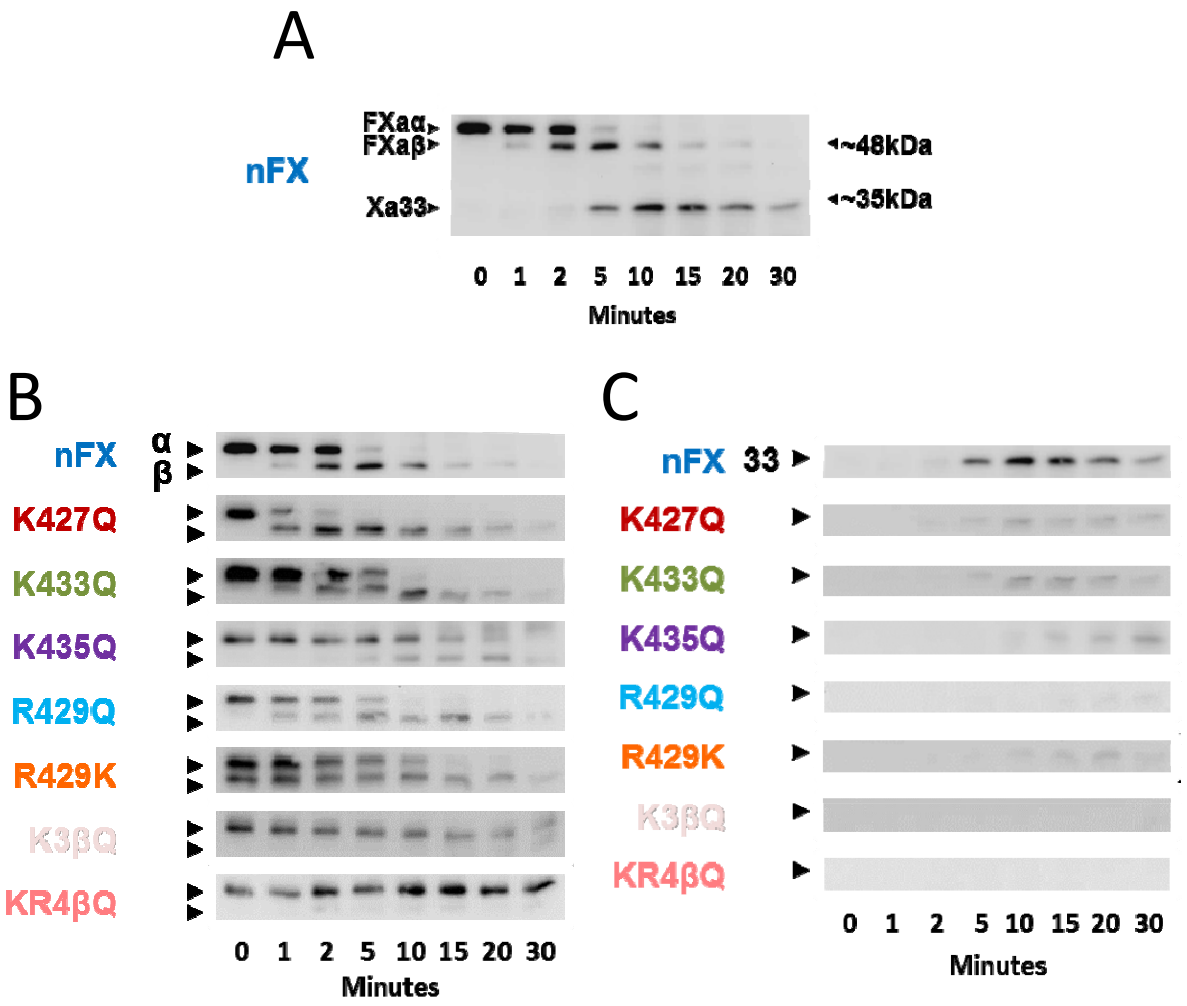
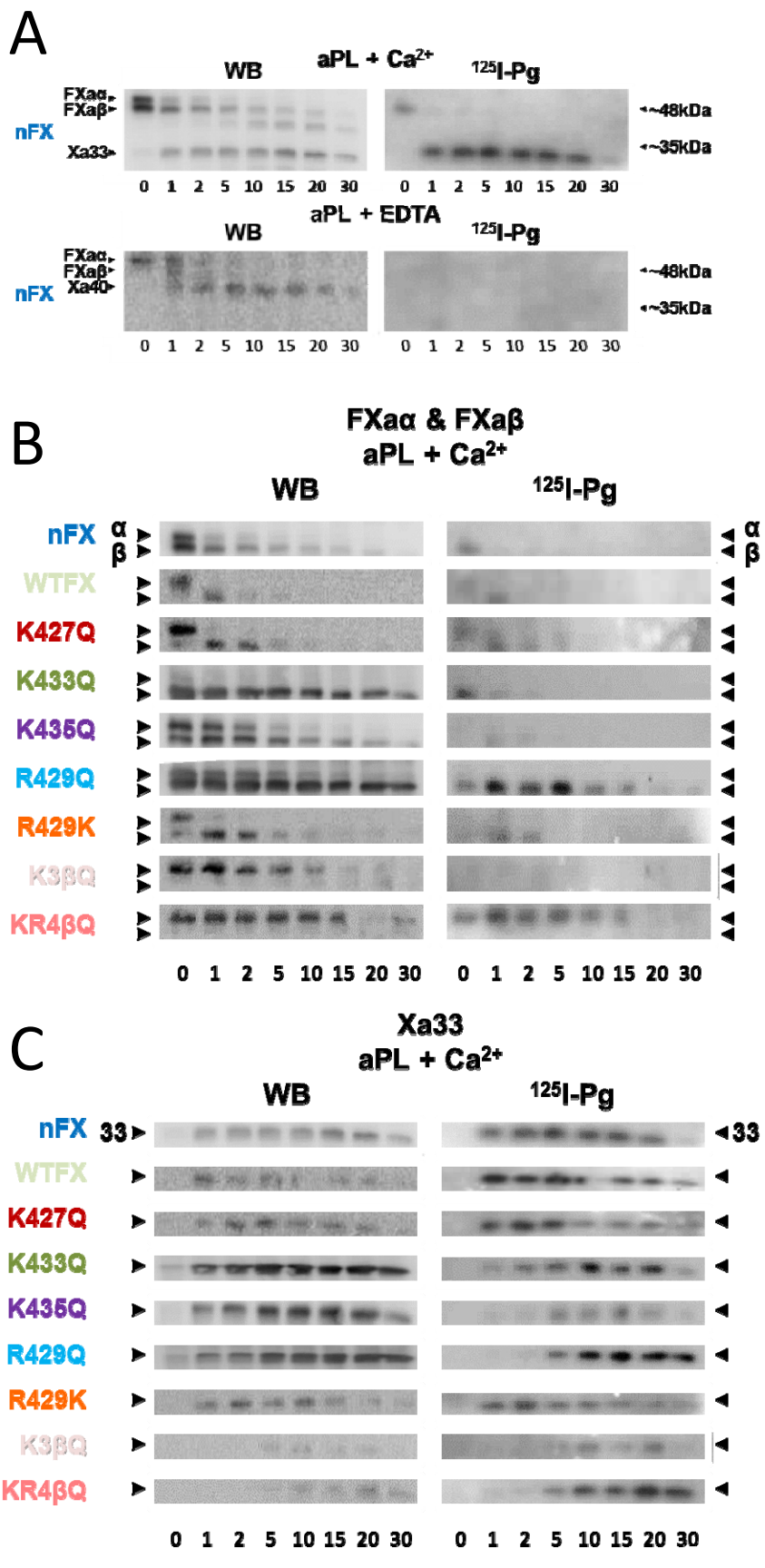


Figure 23: In the presence of $CaCl_2$ and aPL, K435 is the prevalent Pn cleavage site

Activated rFX mutants (200 nM) in media were incubated with Pn (100 nM) in the presence of $CaCl_2$ (10 mM) and aPL (1 mM). Samples were taken at specific time points and Western blot analyses were conducted using antibody to FX and scanned by densitometry. 100 ng of total FX is loaded into each lane. A. A sample blot of nFX demonstrating the cleavage of FXaα to FXaβ and Xa33. B. Western blot slices showing FXaα and FXaβ of all rFX mutants and plasma derived nFX. C. Western blot slices showing Xa33 of all rFX mutants and nFX.

To correlate Pn-mediated rFXa fragments to function in the fibrinolysis pathway, ^{125}I-Pg (50nM) binding was followed by ligand blotting with 300ng total FX per lane (Figure 24). Three-times the previous amount of FX (as in Figure 23) was used in order to visualize the ^{125}I-Pg binding. FXaβ fragments in all the mutants bound ^{125}I-Pg. In the presence of $CaCl_2$ and aPL, the K435Q mutant showed a delay in the disappearance of FXaα and subsequent Xa33 production and also revealed the least intense signal of ^{125}I-Pg binding of all the single point mutations. The R429Q mutant showed delayed Xa33 production and appeared to have the greatest amount of ^{125}I-Pg bound to FXaβ. Xa33 also bound ^{125}I-Pg for all the mutants, with K435Q and K3βQ revealing the least intense signal, although a proportionately high amount of Xa33 antigen was present for the former. Curiously the KR4βQ mutant, which has all four basic residues changed to Gln to prevent FXaα to FXaβ conversion, had a ^{125}I-Pg-binding fragment that appeared to be the same size as FXaα on blot analysis, as well as a ^{125}I-Pg-binding Xa33 fragment that did not appear in the unpurified plasmin digests. FXaα did not bind Pg in nFX, WTFX or any of the other mutants.

Under plasmin incubation conditions in the presence of EDTA, none of the FXa fragments bound appreciably to ^{125}I-Pg. The K3βQ mutant with only R429 available for cleavage, showed FXaβ production. R429K demonstrated FXaβ production and trace amounts of ^{125}I-Pg binding. There were also trace amounts of ^{125}I-Pg binding to a fragment of similar size to FXaα in KR4βQ. The R429Q mutant, which lacked the R429 cleavage site, was also able to generate FXaβ in the presence of EDTA, however there was still undetectable ^{125}I-Pg binding


A
WB
aPL + Ca2+
125I-Pg
FXaα
FXaβ
nFX
Xa33
~48kDa
~35kDa
0 1 2 5 10 15 20 30
aPL + EDTA
Xa40
B
FXaα & FXaβ
aPL + Ca2+
WB
125I-Pg
nFX
WTFX
K427Q
K433Q
K435Q
R429Q
R429K
K3βQ
KR4βQ
α
β
C
Xa33
aPL + Ca2+
33

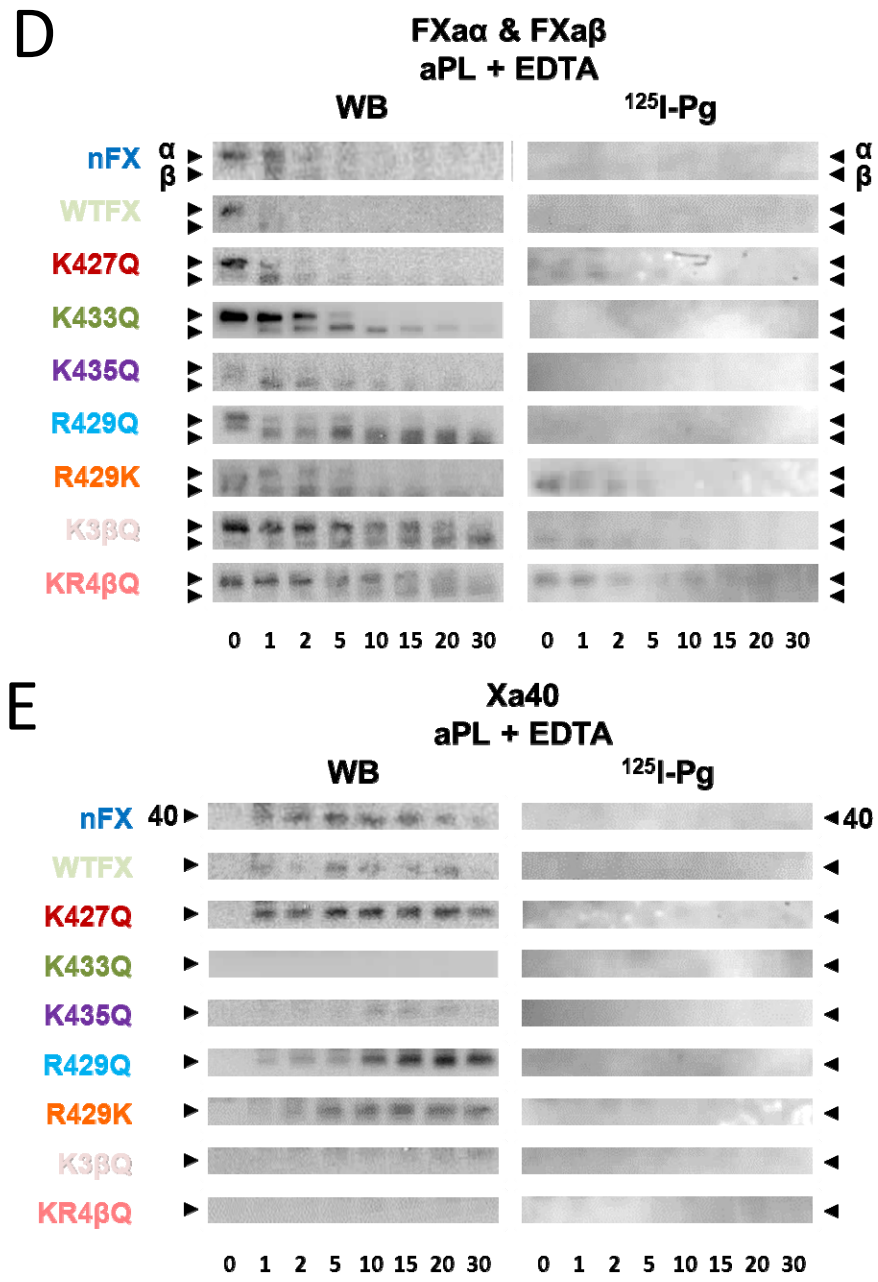


Figure 24: FXaβ and Xa33 fragments bind ^{125}I-Pg

RVV-X-treated rFX mutants (200 nM) were incubated with plasmin (100 nM) in the presence of $CaCl_2$ (10 mM) and aPL (1 mM), or in the presence of EDTA (20 mM) and aPL (1 mM). Samples were taken at specific time points and ^{125}I-Pg was used for ligand blot. Western blot analyses were conducted simultaneously using a FX antibody. 300 ng of FX was loaded into each lane. A. A sample set of Western blots (WB) and ^{125}I-Pg ligand blots demonstrating the cleavage of FXaα to FXaβ and Xa33 or Xa40 in the presence of $CaCl_2$ or EDTA. B. WB and ^{125}I-Pg ligand blot slices showing FXaα and FXaβ of all rFX mutants and plasma derived nFX demonstrating Pg binding in

the presence of $CaCl_2$ and aPL. C. WB and ^{125}I-Pg ligand blot slices showing Xa33 of all rFX mutants and nFX demonstrating Pg binding in the presence of $CaCl_2$ and aPL. D. WB and ^{125}I-Pg ligand blot slices showing FXaα and FXaβ of all rFX mutants and nFX demonstrating Pg binding in the presence of EDTA and aPL. E. WB slices showing FXaα and FXaβ of all rFX mutants and plasma derived nFX and ^{125}I-Pg ligand blot demonstrating Pg binding in the presence of EDTA and aPL.

4.2.3.2 Effect of β-peptide cleavage site mutation on tissue-type plasminogen activator-mediated plasmin generation

To compare the auxiliary cofactor activity of the nFXa, WTFXa and rFXa β-peptide cleavage site mutants in tPA-mediated plasmin generation, FX (100 nM) was pre-activated with RVV-X (125 nM) as above, and then Lys-Pg (0.5 μM) was added. The reaction was initiated with tPA (10 nM). Samples were evaluated in a chromogenic assay for Pn and by Western blot for FX antigen at the indicated times (Figure 25). The greatest difference in Pn generation between WTFXa and several rFXa mutants was seen at the 30 min time point. The R429K mutant was found to significantly enhance plasmin generation by ~30% compared to WTFX after 30 ($p = 0.01$). KR4βQ was also found to further enhance plasmin generation compared to WTFX by ~45% at 30mins ($p = 0.04$). K435Q mutant showed decreased Pn generation compared to WTFX, showing ~20% at 30 mins ($p = 0.01$). Western blot analysis on these plasmin generation time courses showed K435Q was the slowest at converting FXaα to FXaβ with only 73% of total FX still remaining in FXaα form by 30 mins (Figure 26).

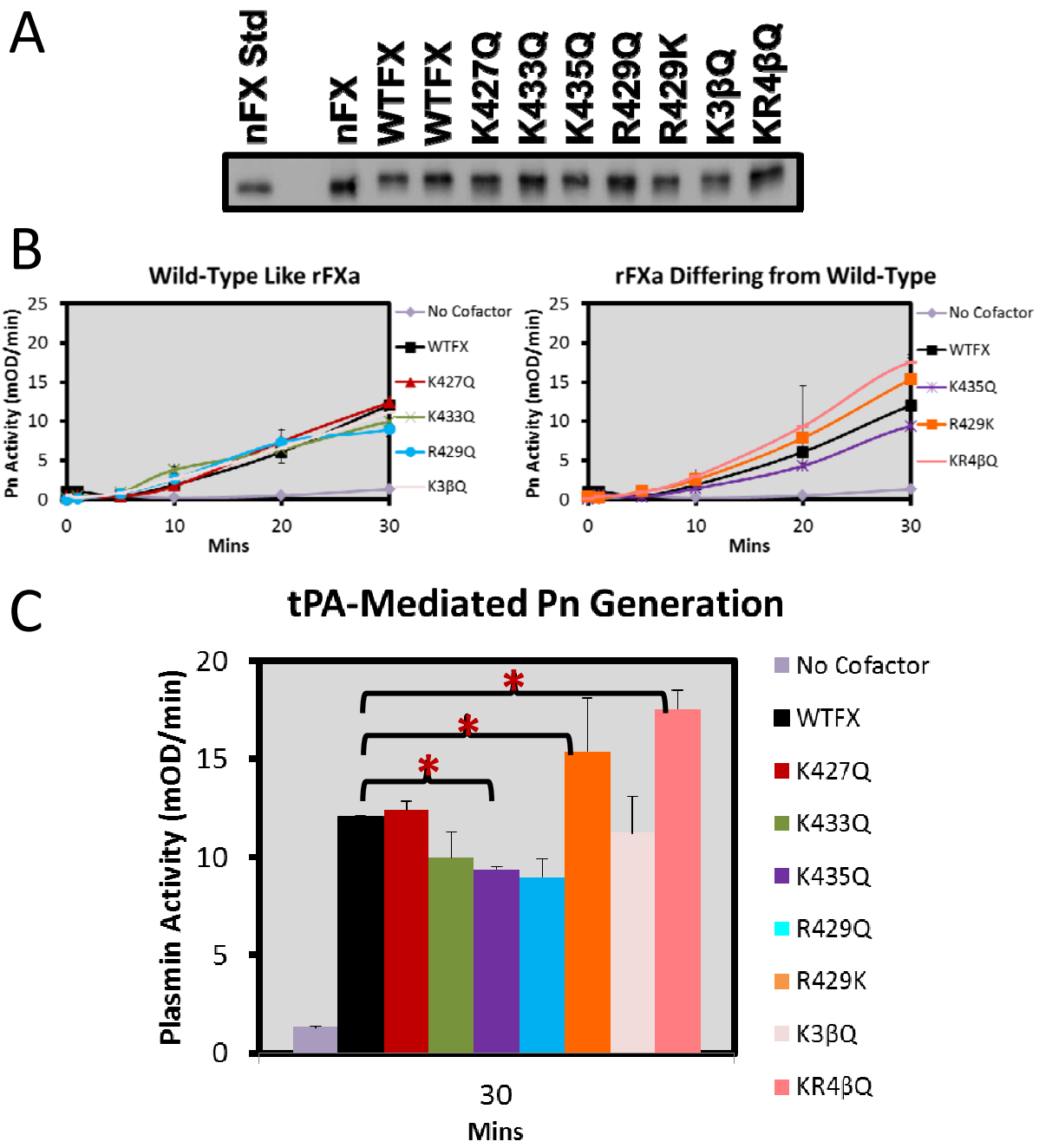


Figure 25: R429K & KR4βQ have increased plasmin generation

A. Western blot showing equal amounts of initial nFX, WTFX, and rFX Ag. B. FX (100 nM) was pre-treated with RVV-X (125 nM) in the presence of $CaCl_2$ (10 nM), and SUV (0.1 mM) at room temperature for 10 mins. Lys-Pg (0.5 µM) was added and Pn generation initiated with tPA (10 nM). At indicated times plasmin activity was measured using a chromogenic assay. The left panel shows rFXa mutants that demonstrated wild-type like enhancement of Pn generation compared to no cofactor. The right panel shows rFXa mutants that showed different levels of enhancement compared to wild-type. C. Pn generated at the 30 min time point which demonstrates the greatest variation among the rFX compared to WTFX. The asterisk (*) indicates significance of $p < 0.05$.

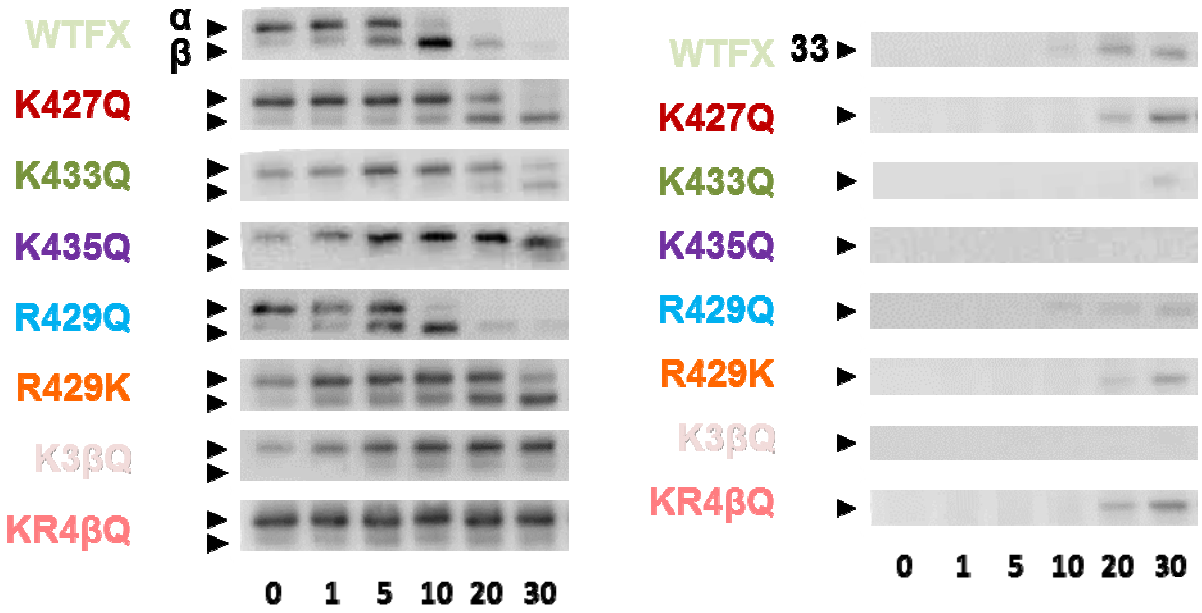


Figure 26: Western blots from plasmin generation

Samples from the plasmin activity assay in Figure 25 were evaluated by Western blot analysis and quantified by densitometry.

4.3 Discussion

Pn-mediated cleavage of FXa produces fragments FXaβ and Xa33/13 which have newly exposed C-terminal Lys that accelerate tPA in purified fibrin clot lysis assays. However, in plasma clot lysis, Xa33/13 is rapidly degraded and loses this fibrinolytic function. Thus, it appears that FXaβ is the fragment that is important for tPA auxiliary cofactor activity in vivo. The exact site of β-peptide excision is currently unknown, and this project has looked at the role of the four basic amino acid residues potentially cleaved to generate FXaβ; K427, R429, K433 or K435.

4.3.1 Effect on activation peptide cleavage

RVV-X activation time courses of the nFX, WTFX, and rFX revealed that activation peptide excision is affected by mutation of the β-peptide cleavage site. RVV-X binds at Ala417 to Leu431 on FX, which includes the β-peptide residues K427 and R429 (103). However, mutation of these two residues in the K427Q and R429Q mutants had no effect on RVV-X activation compared to wild-type. The mutants that did have an effect were the K433Q, K435Q, and quadruple point mutant KR4βQ, which caused delayed activation peptide excision by RVV-X. This suggests that the K433 and K435 sites may play a role in RVV-X binding site and thus on FX activation. There is no plasmin present in this experiment, thus any α to β conversion is due to autoproteolysis alone. Comparison of the rate of FXaα to FXaβ conversion is complicated by the differing rates of RVV-X activation among the rFX mutants. As predicted, the KR4βQ mutant with all four possible β-peptide excision sites changed to Q remained in the α form throughout the experiment. The K3βQ mutant, with only R429 available for β-peptide cleavage also remained in the α form suggesting that there is no autoproteolytic cleavage at R429. Thus, it was concluded that autoproteolysis may occur at Lys K427, K433, K435, but not at R429 in the presence of $CaCl_2$ and aPL.

4.3.2 Effect on specific activity

PT assays and antigen quantification were used to derive specific activity in the nFX, WTFX, and rFX. The WTFX clone selected had similar specific activity to nFX. Mutation of the β-peptide cleavage site was found to affect the specific activity of FX when clotting was initiated by TF. R429Q, K427Q, and R429K mutants were found to have higher specific activities than nFX and WTFX with the greatest at 1.5 fold in R429Q. Conversely, K435Q and KR4βQ was significantly inhibited with only 10% PT activity compared to nFX. This suggests that K435 plays an important role in FX procoagulant activity. Since these proteins were purified based on affinity for aPL, it is presumed that the extent of γ-carboxylation is comparable for each. Interestingly, it should be noted that this novel method excluded a large proportion of the rFX that could not be activated by RVV-X (not shown) and solved a significant technical challenge for this project.

4.3.3 Effect on β-peptide excision, Xa33 generation and ^{125}I-Pg binding

The conversion of FXaα to FXaβ in the Pn-mediated cleavage time courses results from both Pn-mediated β-peptide excision and autoproteolysis by FXaα and FXaβ (102). However the efficacy of Pn is 3 orders of magnitude higher than autoproteolysis and the relatively high concentration of Pn used (2:1 FX:Pn ratio) ensured that β-peptide cleavage was due to Pn. The KR4βQ mutant, designed to have no basic residues available for cleavage in the β-peptide region, remained in the intact FXaα form after 30mins in presence of aPL and $CaCl_2$. The triple point mutant, K3βQ, which only has R429 available for Pn cleavage, was also resistant to FXaβ production, with only 8-9% conversion of the FXaα after 30mins, indicating that the R429 residue is a highly unfavorable plasmin cleavage site. R429Q showed delayed Xa33 production and had the greatest amount of ^{125}I-Pg bound to FXaβ. These observations together suggest that R429 β cleavage site is maintained in a configuration that is protected from proteolysis in the

presence of aPL and $CaCl_2$. Furthermore, changing the unfavourable R429 to a Lys in R429K produced a hypercleavable mutant that appeared to be the most susceptible to autoproteolytic cleavage with approximately 40% of FXa already in FXaβ at the initial time-course sampling point, having converted during the initial RVV-X treatment in media when no Pn was present (Figure 23).

Of all the single point mutants, K435Q had the highest proportion of FXaα remaining by 30 mins in the presence of aPL and $CaCl_2$. This was consistent with less overall ^{125}I-Pg binding compared to the other mutants. This persistence of FXaα suggests that K435 is the preferred site of cleavage when FXa is bound to aPL. In agreement with this conclusion, a previous study published only as an abstract over 2 decades ago suggested that K435 is the probable major autolytic cleavage site in the production of FXaβ (104).

FXa fragments, FXaβ and Xa33/13, have been previously shown to bind Pg and C-terminal Arg does not bind Pg. (23,25,26). The R429Q mutant is unavailable for plasmin cleavage at R429. Interestingly, although similar to R429Q there was FXaβ evident during the experiment for K433Q and K435Q, the correlation of ^{125}I-Pg binding differed for R429Q, which was observed to persist for 30 min. This may suggest that while the initial FXaβ for the other mutants had a Pg-binding C-terminal Lys, such as K435 or K433, a subsequent cleavage exposed R429 to disable this function as shown by the loss of ^{125}I-Pg to FXaβ in K433Q, K435Q, and even nFX. At present we do not have a way to test this hypothesis without a double point mutation. Nevertheless, the K3βQ data suggest that R429 is a poor Pn cleavage site for β-peptide excision in the presence of aPL and $CaCl_2$. The cleavage of K435 excises a large O-linked carbohydrate and consequently exposes R429 for subsequent β-peptide cleavage (105-107). The exact site of O-linkage is unknown, but the possible sites: serine 436, Ser 444, Ser445, or Thr443; are all C-terminus to

K435. The hypothesis that O-linked carbohydrate causes steric hindrance to R429 cleavage can be tested in the future using O-glycosidase to remove the carbohydrate on FXaα. This may change Pn cleavage preference. Commercial concanavalin A Jack-bean lectin linked to biotin (108), which binds the specific carbohydrates on the β-peptide would be used to monitor the deglycosidation. As a positive control, we would expect that only FXaα binds to the lectin as FXaβ would have lost the O-linked carbohydrate upon β-peptide excision.

In addition to effects on FXaβ production, the mutations we studied affected subsequent cleavage to generate Xa33/13. In the plasmin digest experiments using rFX in media, all rFX generated less Xa33/13 than nFX and K3βQ and KR4βQ did not produce Xa33/13 fragments (Figure 23). From this the general conclusion is that β-peptide cleavage facilitates subsequent K330 cleavage producing Xa33. However, in the purified experiments in which we loaded more protein in the gel for WB, the Xa33 band was visible (Figure 24). There is nevertheless a clear delay in ^{125}I-Pg binding to Xa33 from 1 min to 5 min in the K3βQ and KR4βQ suggesting that our initial conclusion was correct. K3βQ and KR4βQ had delayed Xa33 production consistent with inhibited FXaβ production. Thus, β-cleavage is required for K330 cleavage. The K435Q and R429Q mutants also had delayed Xa33-binding to ^{125}I-Pg.

The KR4βQ was designed to replace all four available β-peptides with non-cleavable sites, thus stabilizing the FXaα-form. FXaα does not bind to Pg as shown for nFX and in previous work by our lab (23,25,26).However, the FXaα that is derived from KR4βQ unexpectedly was found to bind to ^{125}I-Pg. There are two basic residues that might be responsible for a new fragment close to the β-peptide region: K420 and R424. However a cleavage at one of these sites would result in a smaller fragment predicted to run lower than FXaβ on SDS-PAGE, not comparable to FXaα. The more feasible explanation is predicted from the cDNA sequence of the F10 gene, which

suggests that FXaα is anticipated to have a C-terminal Lys (K448) (109,110). This has previously been suggested to be excised by carboxypeptidases upon secretion from cells into plasma to account for the lack of Pg- binding to nFXaα and nFX (21,23,24). The constructs used here to produce rFX indeed encode for this C-terminal Lys. The mutations in KR4βQ may prevent its excision in cell culture by endogenous enzymes and be responsible for the observed lack of interaction with ^{125}I-Pg.

In the presence of EDTA, none of the single point Gln mutations, WTFX or nFX plasmin-mediated FXaβ that were produced bound significantly to ^{125}I-Pg, until R429 was mutated to Lys. Since all mutants produced still enabled production of FXaβ, this observation suggested that R429 is the preferred Pn cleavage site in the absence of aPL-binding (Figure 4). In this context, aPL could be localizing the fibrinolytic role of FXa and acting as an allosteric transmolecular switch altering the cleavage site at the β-locus from R429 to K435 providing fibrinolytic function only when aPL is exposed and providing a procoagulant surface (Figure 28). K3βQ with only R429 available produced FXaβ with trace amounts of ^{125}I-Pg binding. R429K is likely being cleaved at the K429 site which is responsible for the ^{125}I-Pg to FXaβ here. Curiously the R429Q mutant which has no R429 available produced a FXaβ, but had no ^{125}I-Pg binding. This suggests that an unpredicted Arg was cleaved rather than one of the three Lys, to account for the lack of ^{125}I-Pg binding. One possibility is that an additional site at nearby R424 may be involved when multiple mutations are introduced. This FXaβ could be from the suggested alternative Arg cleavage site, or is actually one of the expected Lys FXaβ in an orientation on the PVDF membrane with the Lys blocked from Pg binding. More rigorous solution phase studies are required with a homogeneous orientation of FXaβ with C-termini exposed for binding such as with surface plasmon resonance experiments as done in the Pryzdial lab in the past (26,27). For the KR4βQ

mutant, the FXaα C-terminal Lys K448 may account for ^{125}I-Pg-binding, as also suggested to explain the experiment conducted in the presence of $CaCl_2$.

4.3.4 Effect on plasmin generation

The tPA-mediated plasmin generation experiments showed the tPA cofactor activity of the rFX mutants. The greatest effect of β-peptide mutation on Pn generation was seen at the 30 min time point. R429K was designed to better understand the fibrinolytic enhancing function of FXa by changing the non-Pg-binding Arg into a Lys that could potentially bind Pg or tPA. The R429K mutant was found to significantly enhance plasmin generation by approximately 30% compared to WTFX ($p = 0.01$). Interestingly, the KR4βQ, which was not originally expected to have any Pg-binding properties, was also found to further enhance plasmin generation compared to WTFX by 45% at 30mins ($p = 0.04$). K435Q showed reduced Pn generation compared to WTFX by 20% at 30 mins ($p = 0.01$), but still enhanced Pn generation compared to no cofactor. This was consistent with the Western blot analysis on these plasmin generation time courses that showed K435Q also was the slowest at FXaα to FXaβ conversion with 73% of total FX remaining as FXaα by 30 mins (Figure 26). nFX, WTFX and all of the rFX mutants enhanced Pn generation compared to the no cofactor control. This suggests that Xa33 generation at least partially rescues C-terminal Lys accessibility that was lost due to K435Q mutation, which was slower in FXaβ generation, to also enhance Pn generation.

The C-terminal Lys on FXaβ enables Pg receptor and tPA cofactor function in fibrinolysis. There are several Pg receptors, such as annexin A2 tetramer and Plg-R_{KT} (111-114), that have been reported to activate Pg to Pn and protect Pn from inhibitors (115,116). However, these have been predominantly cell associated and likely affect Pn generation in tissue remodeling rather than its fibrinolytic role. The Pg/Pn pathway is not just involved in fibrinolysis, but in cell

invasion and extracellular matrix turnover as well (117-119). Common to all the Pg receptors, including FXa fragments, is the localization of the Pg and activator tPA to focus the Pn proteolytic activity only to the immediate environment.

4.4 Summary

The auxiliary cofactor model of fibrinolysis (Figure 3) proposed by our lab suggests that FX proteolytic fragments, FXaβ and Xa33/13, enhance fibrin clot dissolution by providing C-terminal Lys binding sites for sites Pg and tPA localized to the clot. These participate in fibrinolysis to initially increase the generation of Pn by tPA. Both FXaβ and Xa33/13 have been shown to enhance fibrinolysis in purified fibrin experiments, however, in plasma there is no effect from Xa33/13 (unpublished). This suggests that there is a mechanism in plasma that rapidly inactivates Xa33/13 and FXaβ is the predominant tPA accelerator. Prior to the work conducted comprising the current thesis, the site(s) of cleavage leading to β-peptide excision was unknown, with four possibilities: K427, R429, K433, and K435. I investigated the role of these four basic residues in FXaα to FXaβ conversion and subsequent participation in fibrinolysis through mutagenesis and followed, Pn-mediated cleavage, radiolabelled Pg binding, and tPA-mediated Pn generation. Our prior work demonstrated that Pn and autoproteolysis may either produce FXaβ, although the former is many orders of magnitude on a molar basis more effective.

I demonstrated that mutating individual residues to Gln to prevent cleavage did not prevent Pn-mediated FXaβ production, suggesting that all four can play a role in β-peptide excision to varying degrees. K435Q, mutation resulted in persistence of FXaα when treated with Pn. The loss of rapid C-terminal Lys accessibility in the K435Q mutant was reflected by decreased binding to ^{125}I-Pg. These results suggest that K435 is the preferred site for Pn-mediated FXaβ production under conditions that favour aPL-binding On the other hand, R429Q demonstrated the greatest amount of ^{125}I-Pg binding to FXaβ and the R429K mutant was able to convert FXaα to FXaβ more quickly by autoproteolysis than the other rFX mutants and both FXaα and FXaβ bound ^{125}I-Pg. The K3βQ mutant, which only had the R429 site available for plasmin cleavage in

this region was the slowest to produce FXaβ and did not bind ^{125}I-Pg. These findings together suggest that the R429 is an unfavourable plasmin cleavage site in the presence of aPL and $CaCl_2$ and confirms that C-terminal Arg is likely the basis for lack of binding previously observed when FXaβ is produced in the absence of $CaCl_2$ (23,25,26). When rFX, K3βQ and KR4βQ were converted to FXa and treated with Pn, they did not produce Xa33/13 fragments. This suggests that excision of the β-peptide facilitates subsequent K330 cleavage producing Xa33/13.

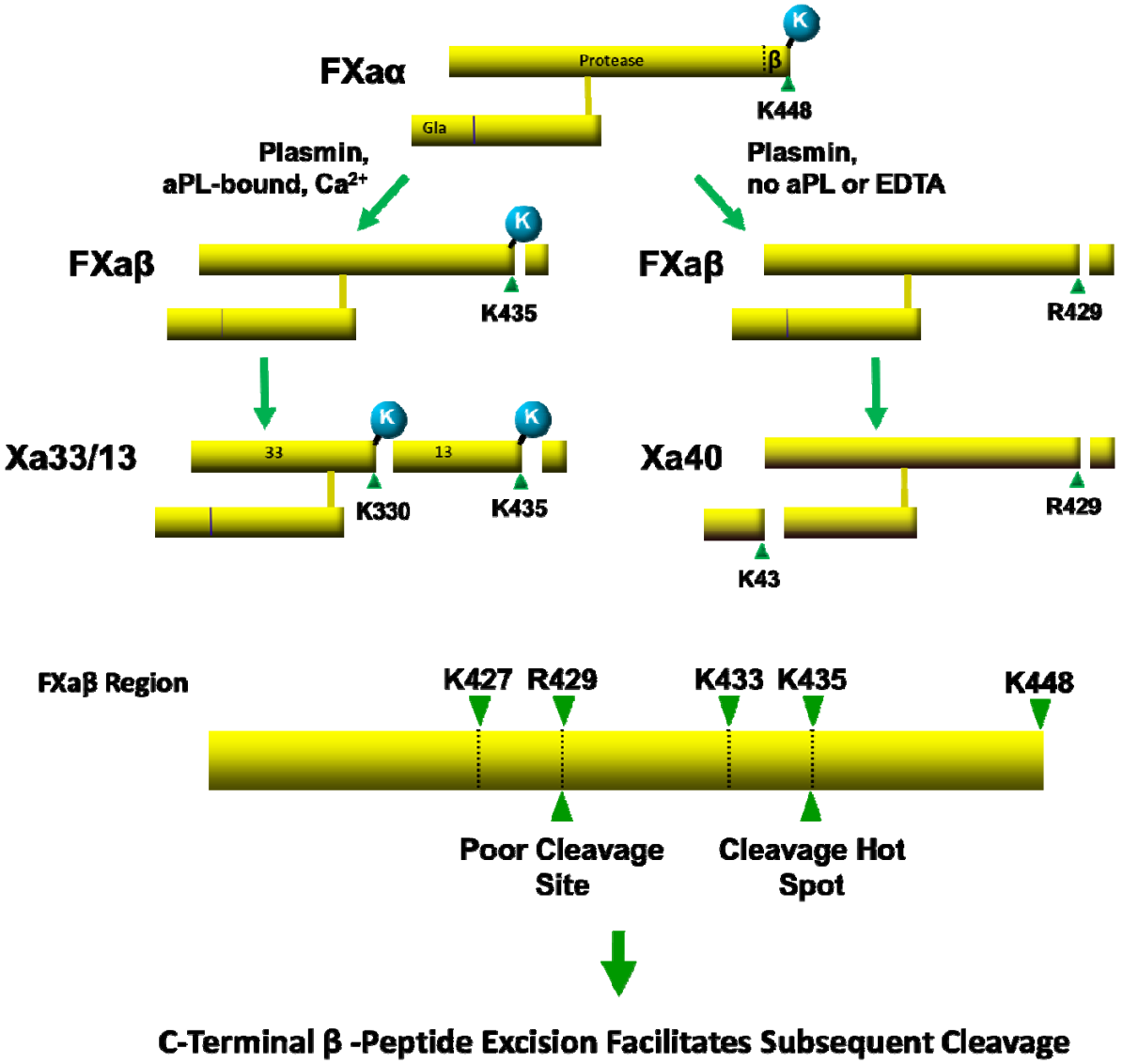
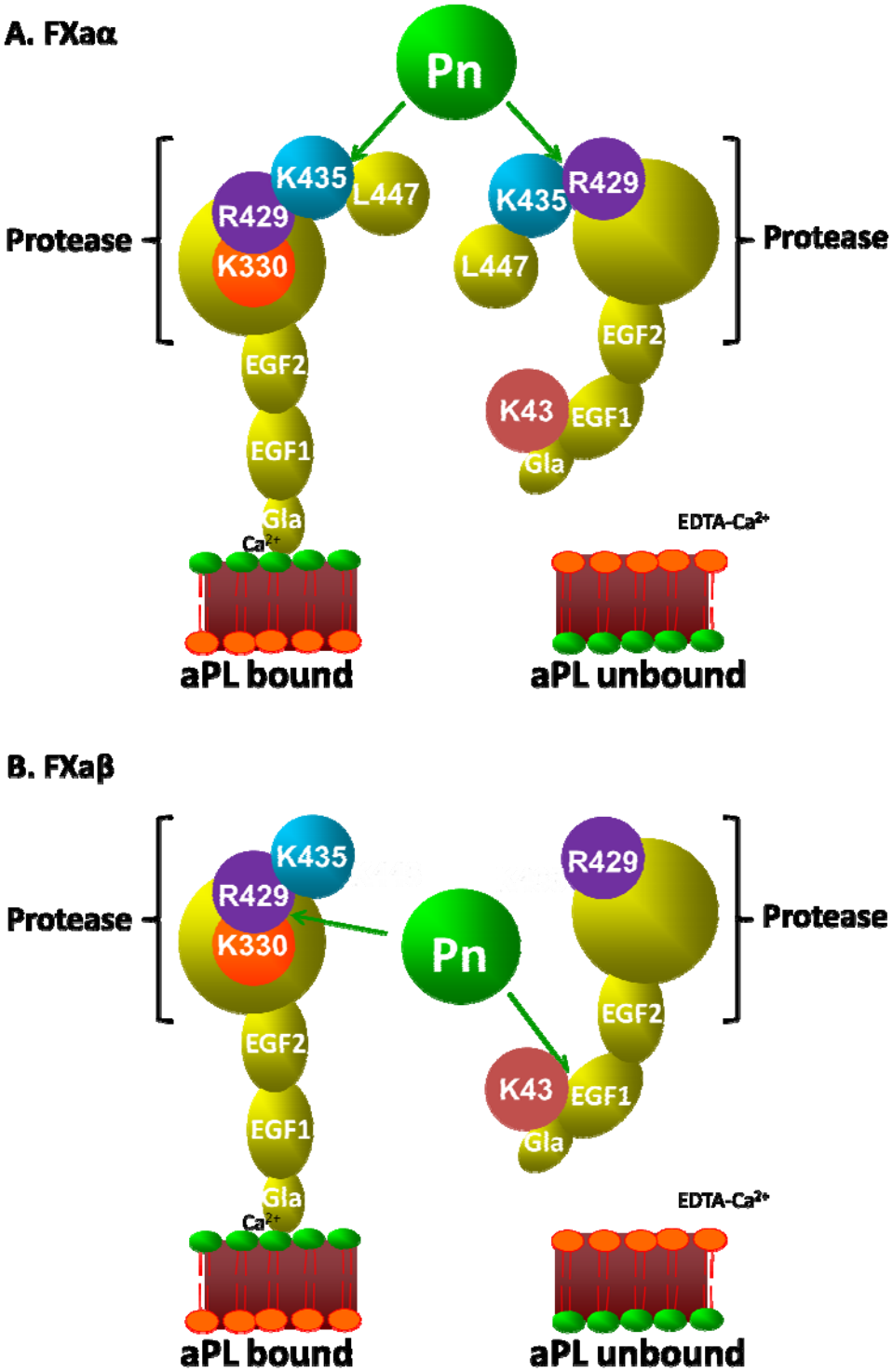


Figure 27: Summary of FXa β-peptide results

Through mutagenesis, plasmin-mediated cleavage, and radiolabelled Lys-Pg binding experiments, several conclusions can be made regarding the role of the basic residues in the β-peptide region of FXa. First, K435 is the preferred β-peptide cleavage site when FX is bound to aPL. R429 is a poor β-peptide cleavage site when bound to aPL. C-Terminal β-peptide excision facilitates Xa33 generation. A hypercleavable FX mutant, R429K, with an obligate C-terminal Lys was found to enhance tPA-mediated Pn generation. And lastly, we have also discovered that there is a C-terminal Lys on stabilized FXaα mutant which was also able to bind Pg and enhance Pn generation. K448 is predicted by cDNA, but not expected to exist in wild-type FXaα because of the quick action of cellular carboxypeptidases. The quadruple mutation stabilizing FXaα likely caused a conformational change in the folding of C-terminal region preventing the cleavage of K448.


A. FXaα
Pn
Protease
K435
L447
R429
K330
EGF2
EGF1
Gla
Ca2+
K435
R429
L447
EGF2
K43
EGF1
Gla
Protease
EDTA-Ca2+
aPL bound
aPL unbound
B. FXaβ
Protease
K435
R429
K330
EGF2
EGF1
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EDTA-Ca2+
aPL bound
aPL unbound

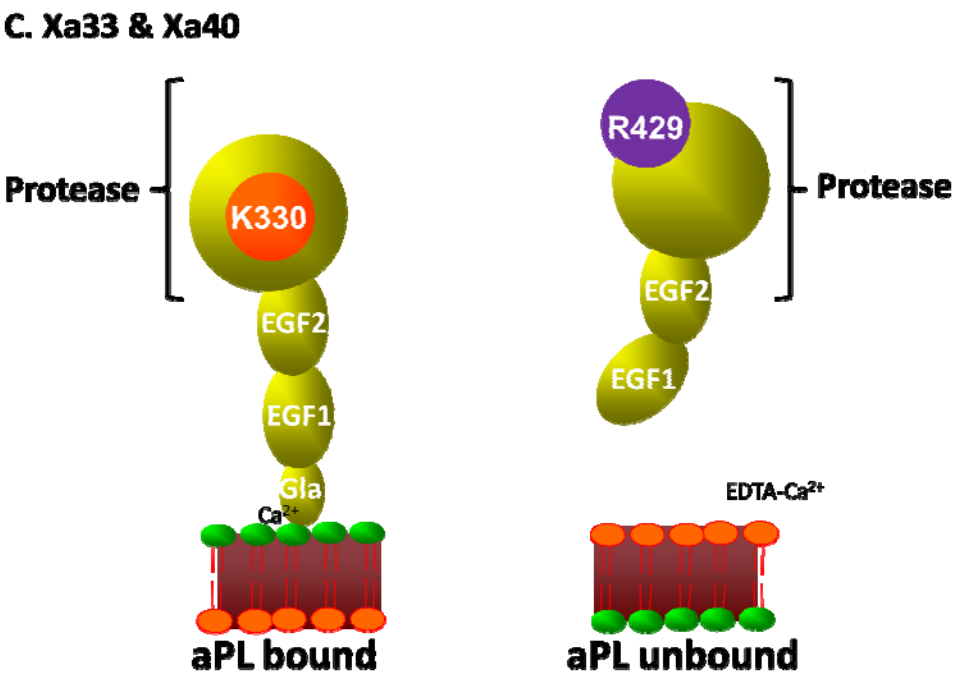

Figure 28: Anionic phospholipid acts as an allosteric switch altering the β cleavage site and affecting fibrinolytic function

A. When intact FXaα is bound to aPL in the presence of $CaCl_2$, a signal is relayed causing a conformational change in the protease domain exposing K435 as the preferred plasmin cleavage site for β-peptide excision. In the absence of aPL binding, R429 is the preferred plasmin cleavage site in the β locus. Probable C-terminal amino acids corresponding to cleavage sites are indicated.

B. aPL bound FXaβ has a C-terminal K435 that can bind tPA and Pg to enhance plasmin generation. In aPL unbound conditions, C-terminal R429 FXaβ is unable to bind tPA and Pg and does not confer fibrinolytic function.

C. FXaβ is further cleaved by Pn, producing FXa fragments in Xa33 and Xa40 in aPL bound and unbound conditions respectively. Xa33 with C-terminal K330 can bind tPA and Pg, while Xa40 cannot.

In the presence of EDTA, none of the plasmin-mediated FXa fragments bound significantly to ^{125}I-Pg, suggesting that R429 is the preferred Pn cleavage site in the absence of aPL binding (Figure 4). The trace amounts of ^{125}I-Pg binding to FXaβ binding in R429K mutant, and to the FXaα in KR4βQ are due to a C-terminal Lys. The R429Q and R429K mutants that lack the R429 site were still able to generate FXaβ in the presence of EDTA. This suggests that although R429 is the preferred β-peptide cleavage site under these conditions, one of the three Lys can still be cleaved to excise the β-peptide, and this is likely responsible for the trace amounts of ^{125}I-Pg binding we see here. For the KR4βQ mutant, the FXaα C-terminal Lys K448 could be binding the ^{125}I-Pg.

Finally, I investigated the role of the β-peptide residues in the participation of FXa in fibrinolysis as a tPA auxiliary cofactor through tPA-mediated Pn generation experiments. R429K was designed in an attempt to improve FXa as a fibrinolytic enhancer by changing the non-Pg binding R429 into Lys that could potentially bind and help activate Pg. We were successful in this venture with R429K enhancing plasmin generation by 30% compared to WTFX. Interestingly, the KR4βQ which has all four basic residues in the β-peptide region turned off and is stabilized in the FXaα form, was also found to further enhance plasmin generation compared to WTFX by 45% at 30mins. This further supports the idea that FXaα has a C-terminal Lys in this mutant, and it is also able to provide a site for Pg binding and activation. The K435Q mutant showed decreased Pn generation compared to WTFX supporting the conclusion that K435 is the preferred cleavage site in the β-peptide cleavage region and is predominantly responsible for the participation of FXaβ in fibrinolysis. These mutants warrant further study in clot lysis assays to see their fibrinolytic potential.

5. Future Studies

5.1 Bleeding diatheses and dissecting the intrinsic carboxypeptidase activity

I have shown here that there is enhanced fibrinolysis in hemophilia patients compared to age and cardiovascular risk-matched controls due to decreased inhibition of the fibrinolysis pathway from TAFI and PAI-1. Also, there was a trend toward shorter overall plasma clot lysis times in the hemophilia patients compared to controls. However, the small sample size of 25 patients in the Calgary cohort was possibly a limiting factor in attaining statistical significance. Fortunately, Dr. Jackson has organized a study in Vancouver with a much larger cohort that will expand on what we have learned from this small pilot study. The wide variation in the plasma clot lysis within the hemophilia group alone also contributed to the lack of statistical significance, which may be complicated by therapeutic factors remaining in the patients' circulation at the time of blood donation. Future studies could include bleeding scores to account for the differences in bleeding diatheses in patients, and perhaps increased bleeding will correlate with shorter plasma clot lysis times. The way we attempted to assign bleeding scores in this study was by patient age at the time of first clinical bleeding event, where earlier bleeding indicated a more severe phenotype. As evidenced by our difficulty with retrospectively gathering these data, a better measurement of bleeding tendency should be implemented such as the relatively comprehensive bleeding questionnaire used to diagnose von Willebrand disease (120). One can also attempt to normalize the clot lysis times in hemophilia patients by adding back purified FVIII or FIX to the plasma during clot formation.

The elevated intrinsic carboxypeptidase activity in hemophilia patients was an interesting result that should be further explored in more detail. I postulated that the majority of intrinsic

carboxypeptidase activity identified was likely CPN rather than TAFIa. One can exclude TAFIa with GEMSA and/or potato tuber carboxypeptidase inhibitor (PTCI) to only measure CPN and determine whether CPN is truly elevated in hemophilia patients. If this is true, CPN is possibly elevated as a compensatory mechanism for the decreased TAFI in hemophilia.

5.2 Fibrinolytic potential of the hypercleavable β-mutant and stabilized FXaα; stabilization of Xa33/13; C-terminal sequencing of FXa fragments

Through mutagenesis of the β-peptide region of FXa I have shown that K435 plays a main role in β-peptide excision and that R429 may be a hindrance to it. Specially designed fibrinolytic mutant R429K and stabilized FXaα (KR4βQ) mutant further enhance tPA-mediated Pn generation compared to nFX and the other β-peptide region mutants. This finding warrants further investigation into the functional fibrinolytic activity of these mutants in purified fibrin degradation and plasma clot lysis assays. And to elaborate on Pryzdial lab alum Dr. Vanden Hoek's research on the role of Lys330, new mutants can be made from these β-cleavage site mutants by adding the Lys330Gln mutation to turn off Xa33/13 generation and to prevent proteolytic degradation in plasma. This could lead to the development of a fibrinolytic therapeutic.

C-terminal sequencing of the FXaβ fragments in each of the mutants would be useful to unambiguously confirm that K435 is the preferred cleavage site, and that when it is not available R429 is cleaved site. Also C-terminal sequencing of KR4βQ should be conducted to demonstrate that FXaα has a C-terminal Lys as predicted from cDNA to explain our ^{125}I-Pg-binding data. N-terminal sequencing of the β-peptide has been attempted in the past by the Pryzdial lab, but was unsuccessful. A large quantity of the β-peptide would be needed and purification of the β-

peptide by the O-linked carbohydrate was difficult due to homogeneity. Another hurdle with this sequencing idea is that there is currently no standard method to do C-terminal sequencing. There are various novel C-terminal sequencing methods including manual enzymatic carboxypeptidases, acid hydrolysis, and mass spectrometry (121-125). When this type of sequencing becomes more widely available it can help confirm the cleavage sites I have suggested here.

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