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**Regulation of the Activation and Function of
Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)**

by

Tanya Marar

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

2016

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**Regulation of the Activation and Function of
Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)**

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This dissertation incorporates the outcome of joint research undertaken in collaboration with Anastassia Filipieva, Kara Picco, and Mousa Gawanmeh under the supervision of Dr. Michael B. Boffa. Primary contributions, experimental designs, data analysis, and interpretation were performed by the author. Additionally, manuscripts were written initially by the author and revised and edited by Dr. Michael B. Boffa.

- Collaboration with Anastassia Filipieva is covered in Chapter 3 of the dissertation; the contribution of the author was primarily through the provision of Figures 4-3, 4-4, and 4-5.
- Collaboration with Kara Picco is covered in Chapter 5 of the dissertation; the contribution was through generating preliminary results.
- Collaboration with Mousa Gawanmeh is covered in Chapter 3 of the dissertation; the contribution was through assistance in completing the cloning.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my dissertation, and have obtained written permission from each of the co-author(s) to include the above material(s) in my dissertation.

I certify that, with the above qualification, this dissertation, and the research to which it refers, is the product of my own work.

This thesis includes 2 original papers that have been previously published or is in preparation for publication in peer reviewed journals, as follows:

Dissertation Chapter	Publication Title and Full Citation	Publication Status
Chapter 2	Marar TT and Boffa MB. Identification of a Thrombomodulin Interaction Site on Thrombin-Activatable Fibrinolysis Inhibitor that Mediates Accelerated Activation by Thrombin. <i>J Thromb Haemost.</i> 2016	Accepted/In Press
Chapter 4	Marar TT, Filipieva A, and Boffa MB. Identification of Heparin Interaction Sites on Thrombin-Activatable Fibrinolysis Inhibitor that Modulate Plasmin-mediated Activation, Thermal Stability and Antifibrinolytic Potential.	In Preparation

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ABSTRACT

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that provides a molecular link between the coagulation and fibrinolytic systems. TAFI is activated through proteolytic cleavage by thrombin and plasmin although they are relatively weak activators. However, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin in the presence of glycosaminoglycans (GAGs), such as heparin, are able to greatly accelerate the activation of TAFI to generate the enzyme activated TAFI (TAFIa). Additionally, the presence of GAGs has been shown to stabilize the enzyme, TAFIa, increasing its half-life. TAFIa possesses basic carboxypeptidase activity which down-regulates fibrin clot lysis by removing carboxyl-terminal lysine and arginine residues from partially degraded fibrin thereby, attenuating the positive feedback in the fibrinolytic cascade that is essential for plasminogen activation. The work in this dissertation is focused on understanding the regulation of TAFI activation and evaluating TAFIa activity. Through construction of mutant variants of TAFI in putative interaction regions with TM and GAGs, we have identified important regions on the TAFI structure that mediate the accelerated activation by the thrombin-TM complex and the acceleration of plasmin-mediated TAFI activation by GAGs and the stabilization of the TAFIa enzyme. We found that the region on TAFI that showed the strongest TM dependence was focused around the activation domain of TAFI, which would be near where the c-loop of EGF-3 of TM would contact TAFI. We also identified residues on TAFI that could be part of a heparin binding site that allows GAGs to mediate the acceleration of plasmin-mediated TAFI activation and stabilization of the TAFIa enzyme. As well, we have designed a novel fluorescence based assay for the measurement of the basic carboxypeptidase activity of TAFIa, which has significantly increased sensitivity compared to the currently available carboxypeptidase B (CPB) substrates. Taken together, the results of the studies described will aid in further propelling the determination of the mechanism of TAFI activation by the respective activators as well as provide a sensitive tool for monitoring the carboxypeptidase activity of TAFIa.

DEDICATION

*I dedicate this work to my parents, Munir and Suzan, and my brother Raymond for their
love and support*

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AAFK	anisylazofornyl-lysine
AAFR	anisylazofornyl-arginine
ϵ -ACA	ϵ -aminocaproic acid
ADP	adenosine diphosphate
APC	activated protein C
AT	antithrombin
BAP	barium adsorbed plasma
BHK	baby hamster kidney cells
cDNA	complement deoxyrinonucleic acid
CPB	carboxypeptidase B
CPR	carboxypeptidase R
CPU	carboxypeptidase U
DIC	disseminated intravascular coagulation
DMEM	dulbecco's modified eagle medium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EPCR	endothelial protein C receptor
FDPs	fibrin degradation products
FV	coagulation factor V
FVa	activated coagulation factor V
FVII	coagulation factor VII
FVIIa	activated coagulation factor VIIa
FVIII	coagulation factor VIII
FVIIIa	activated coagulation factor VIIIa
FIX	coagulation factor IX
FIXa	activated coagulation factor IXa
FX	coagulation factor X
FXa	activated coagulation factor Xa
FXI	coagulation factor XI
FXIa	activated coagulation factor XIa
FXII	coagulation factor XII

FXIIa	activated coagulation factor XIIa
FXIII	coagulation factor XIII
FXIIIa	coagulation factor XIIIa
GAGs	glycosaminoglycans
GEMSA	2-guanidinoethylmercaptosuccinic acid
Gla	γ -carboxyglutamate residues
GlcA	β -D-glucuronic acid
GlcN	α -D-glucosamine
GP	glycoprotein
GP Ia/IIa	glycoprotein Ia/IIa receptors
GP Ib–IX-V	glycoprotein Ib–IX-V complex
HBS	HEPES-buffered saline
HBST	HEPES-buffered saline/0.01% (v/v) Tween 80
HEK293	human embryonic kidney cells
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-ethansulfonic acid)
Hipp-Arg, HR	hippuryl-L-arginine
6 \times His tag	hexa histidine tag
HPLC	high-performance liquid chromatography
IdoA	α -L-iduronic acid
k_{cat}	maximum rate of enzyme catalysis per unit enzyme
k_{cat}/K_M	catalytic efficiency
K_M	Michaelis-Menten constant
K_d	dissociation constant
LCI	leech carboxypeptidase inhibitor
LETS	Leiden Thrombophilia Study
LMWH	low molecular weight heparin
β ME	2-mercaptoethanol
MERGEPTA	2-mercaptomethyl-3-guanidinoethylthiopropionic acid
NCS	newborn calf serum
NO	nitric oxide
Opti-MEM	reduced serum minimal essential medium
PAI-1	plasminogen activator inhibitor-1
PAR1, PAR4	protease-activated receptors

PC	protein C
PC/PS	phosphatidylcholine/phosphatidylserine
PGI ₂	prostacyclin
Plg	plasminogen
Plummer's inhibitor	DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid
PMSF	phenylmethanesulfonyl fluoride
PPAck	D-phenylalanylprolylarginyl chloromethylketone
pro-pCPB	procarboxypeptidase B
PSF	penicillin/streptomycin/fungizone
PTCI	potato tuber carboxypeptidase inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpins	serine protease inhibitors
TAFI	thrombin-activatable fibrinolysis inhibitor
TAFIa	activated TAFI
TAFIai	inactivated TAFIa
TAMRA	tetramethylrhodamine
TCI	tick carboxypeptidase inhibitor
TdP	TAFI-deficient plasma
TdBAP	TAFI-deficient barium adsorbed plasma
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
tPA	tissue-type plasminogen activator
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
UH	unfractionated heparin
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VFKck	D-Val-Phe-Lys chloromethylketone
vWF	von Willebrand factor

Chapter 1

General Introduction

1.1 Introduction

Haemostasis is a protective mechanism that prevents significant blood loss upon vascular injury while maintaining the fluidity of blood elsewhere in the circulation [1, 2]. It involves the rapid and highly localized formation of a stable haemostatic plug to efficiently seal the lesion in the vessel wall. Haemostasis involves a balance between the opposing pathways of the coagulation cascade involved in fibrin clot formation and the fibrinolytic cascade involved in clot dissolution. Each pathway comprises many different components which require coordinated activity in addition to interplay between the systems. The entire process is tightly regulated and ensures the clot is removed in a timely manner following repair to the damaged tissue. These pathways are constantly active and tightly controlled through a balance of anticoagulant and procoagulant activities. Different stimuli can disturb and shift the balance which could either lead to inappropriate clot formation or inability to form a stable clot causing thrombosis or excessive blood loss, respectively [1-4].

1.2 Overview of Haemostasis

Haemostasis encompasses many complex processes, all occurring simultaneously, in order to seal the rupture in the damaged blood vessel. This is accomplished by three general components: the vascular wall, platelets, and the coagulation pathway [2]. Endothelial cells, lining the inner surface of the blood vessels, play an important role in maintaining the haemostatic balance. The endothelium expresses various anticoagulant and procoagulant molecules and their distribution varies throughout the vasculature [5]. Under normal physiological states, the balance is in favour of anticoagulant activities by

maintaining the blood in a fluid state and preventing clot formation. The intact endothelium mediates its anticoagulant activities by releasing vasodilators (prostacyclin (PGI₂), nitric oxide (NO)), preventing platelet adhesion and activation (PGI₂, NO, ecto-adenosine diphosphatase), preventing coagulation (surface expression of thrombomodulin, heparin-like molecules, endothelial protein C receptor (EPCR), and secretion of tissue factor pathway inhibitor (TFPI)), and preventing fibrin deposition (secreting tissue-type plasminogen activator (tPA)). Injury to the endothelium causes a shift in the balance in favour of procoagulant activities to promote clot formation and prevent excessive blood loss. The procoagulant activities include promoting platelet adhesion and activation (producing von Willebrand factor (vWF)), enhancing coagulation (induced by bacterial endotoxin or cytokines to express tissue factor (TF) and coagulation factor V (FV)), and inhibiting clot dissolution (secreting plasminogen activator inhibitor-1 (PAI-1) [5-9].

The endothelial cells are located at the interface between the flowing blood and the sub-endothelial structures deep within the layers of the blood vessel wall. In normal circumstances, plasma proteins, platelets and cellular elements suspended in flowing blood never make sustained contact with the endothelial surface due to the anticoagulant properties of healthy endothelium [1, 7]. The endothelium provides a protective layer from the basement membrane and extracellular matrix located in the intimal layer immediately beneath the endothelium. The extracellular matrix is composed of adhesive proteins such as collagen, fibronectin, laminin, vitronectin, and vWF, allowing platelet adhesion in the event of denudation or injury of the endothelium. In addition, the vessel

wall contains TF which is a membrane protein located on smooth muscle cells, fibroblasts, and macrophages that initiates blood coagulation [1].

Following vascular injury, the first step is immediate constriction of the damaged blood vessels. Vasoconstriction temporarily decreases blood flow within the vessel to immediately limit bleeding [6]. The lesion in the vessel wall results in exposure of the subendothelial extracellular matrix allowing platelets to make contact with these structures thereby triggering adhesion. Platelets possess many different receptors on their surface, allowing them to bind to collagen or other adhesive proteins such as vWF. In high shear conditions found in arteries and the microcirculation (blood velocity between $300\text{-}1600\text{ s}^{-1}$), vWF is essential for platelet adhesion to the sub-endothelium; vWF acts as a bridge by binding to collagen and platelet membrane glycoprotein Ib–IX–V (GP Ib–IX–V) complex [7, 10]. In low shear conditions as in veins (blood velocity approximately $20\text{-}200\text{ s}^{-1}$) platelet adhesion is mediated through other platelet receptors, such as glycoprotein Ia/IIa (GP Ia/IIa) and $\alpha 2\beta 1$ integrin interaction with collagen [10]. Platelet adhesion results in activation of the platelets causing a change in shape, from discoid shape to formation of pseudopodia giving them a spikey appearance and allowing them to spread out on the surface of the injury [11].

Platelet activation results in intracellular signalling which stimulates release of a variety of substances from their granules. There are two types of platelet granules important in haemostasis. Platelet alpha granules contain fibrinogen, fibronectin, coagulation factors V and VIII (FVIII), platelet factor 4, and growth factors among other substances. Platelet dense granules contain platelet activators or agonists such as adenosine diphosphate (ADP), ionized calcium, and serotonin [12]. Release of these

platelet agonists, in particular ADP, serves to recruit and activate additional platelets at the injury site. This promotes platelet aggregation by allowing them to adhere to the layer of platelets already present, eventually forming a loose platelet plug, the first haemostatic structure. In addition, ADP mediated platelet activation induces a conformational change of the platelet glycoprotein IIb/IIIa allowing activated platelets to form a high affinity bond to fibrinogen as well as bind to vWF, fibronectin, and vitronectin. The platelets ability to bind to fibrinogen, forming a bridge between adjacent activated platelets, allows them to stick to one another and contributes to spreading the platelet aggregates over the exposed subendothelium. The platelet plug temporarily reduces blood loss, but is not enough to seal the lesion in the vessel wall. Since vasoconstriction and platelet plug formation occur very rapidly they are often grouped together and referred to as *primary haemostasis*. The next step of haemostasis known as the coagulation cascade or *secondary haemostasis* is what reinforces and strengthens this platelet plug [7, 12].

1.3 The Coagulation Cascade

The coagulation pathway is a very intricate process and comprises a series of zymogen to enzyme conversions. The coagulation pathway was initially represented as a “cascade” or “waterfall” model which was an important advance in understanding the complicated process [13, 14]. However, this model was found not to be representative of *in vivo* clinical observations thus was refined as a cell-based model which is a better predictor of the *in vivo* phenomena. The cell-based model emphasizes that different cell surfaces serve to control and regulate the coagulation process. The cell-based coagulation

model is viewed as occurring in three overlapping phases: initiation, amplification, and propagation (Figure 1-1) [15].

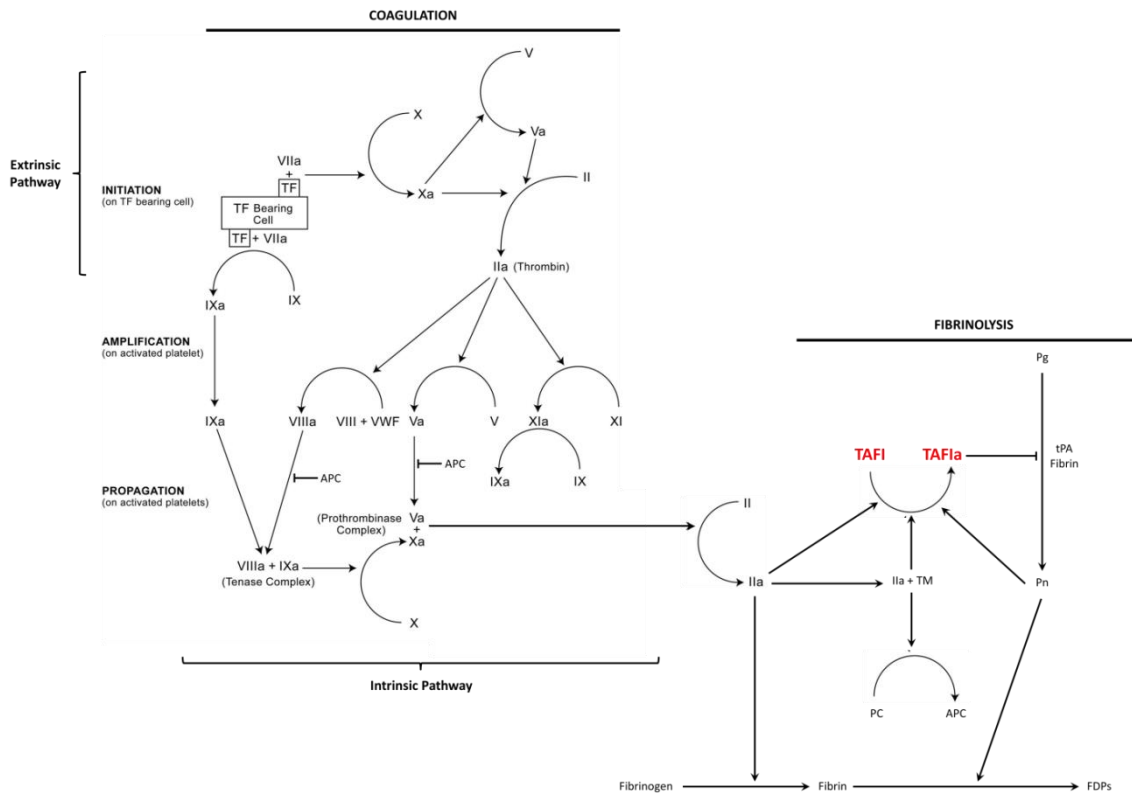


Figure 1-1. The coagulation and fibrinolysis systems. The cell based model of haemostasis consists of the initiation, amplification and the propagation phase. The initiation pathway of coagulation, traditionally known as the *extrinsic pathway*, generates a small amount of thrombin (IIa) that is able to convert the soluble plasma protein fibrinogen into insoluble fibrin fibres to promote fibrin clot formation. The large burst of thrombin produced by the intrinsic pathway consolidates the fibrin clot, partly through the activation of thrombin-activatable fibrinolysis inhibitor (TAFI). When thrombin binds to thrombomodulin (TM) forming a complex, it loses its procoagulant abilities and instead functions as an anticoagulant or antifibrinolytic enzyme through its ability to activate protein C (PC) or TAFI, respectively. Fibrin degradation is accomplished by plasmin (Pn) which is formed through activation of plasminogen (Plg) by tissue plasminogen activator (tPA) with the fibrin surface acting as an important cofactor. Partial degradation of fibrin by plasmin results in a form of fibrin that exposes carboxyl-terminal lysine and arginine residues. Exposure of the basic residues enhances the cofactor function of fibrin promoting the positive feedback mechanism of plasminogen activation. TAFIa attenuates this positive feedback by removing the carboxyl-terminal basic residues. Therefore, the TAFI pathway inhibits plasminogen activation and down-regulates fibrinolysis. The figure was adapted and modified from reference [3].

The coagulation process begins when there is a breach in the vascular endothelium and the flowing blood is exposed to the extravascular tissue, more specifically to cells expressing TF. TF is an integral membrane protein expressed by fibroblasts, macrophages, and smooth muscle cells and during inflammatory states is expressed by activated endothelial cells and monocytes [16-18]. Activated factor VII (FVIIa) circulating in the plasma, representing about 1% of the total and the remaining 99% as FVII zymogen, rapidly binds to TF [19]. The TF-FVIIa complex activates the plasma zymogens, factor IX (FIX) and factor X (FX), to their activate serine protease forms, FIXa and FXa, on the cell surface. At high concentrations of TF, FX is activated by the TF-FVIIa complex whereas at low concentrations of TF, FIX is mainly activated [20, 21]. The zymogen FVII is also able to bind to TF where then FIXa and FXa, in addition to FVIIa, can feedback amplify the system by activating FVII bound to TF [22]. Factor Xa is then able to interact with its cofactor FV on the cell surface and activate it. The interaction of FXa-FVa complex with prothrombin forms the prothrombinase complex, activating prothrombin to generate a small amount of thrombin. This pathway of thrombin generation is called the initiation pathway and is traditionally known as the *extrinsic pathway* of coagulation since it involves extravascular elements [15, 23].

Formation of the thrombin enzyme is key in coagulation as it has many important functions in haemostasis including activation of platelets, conversion of the plasma protein fibrinogen to fibrin which is involved in forming a fibrin net around the platelet plug, and feedback amplification of the coagulation pathway [22]. The TF-FVIIa complex functions on TF bearing cells and in close proximity to the partially activated platelet surface at the site of injury. This localization is an important step in amplification of the

procoagulant stimulus. The FX activated on TF bearing cells is quickly inhibited by plasma protease inhibitors TFPI and antithrombin (AT) if it diffuses away from the cell surface and into the blood. This is a vital and reoccurring theme in coagulation to localize reactions to the surface of cells to protect them from inhibition. TFPI and AT can interact with FXa directly inhibiting it; in addition, the TFPI-Xa complex can inhibit the TF-FVIIa complex [24, 25]. Nevertheless, the remaining FXa on the TF bearing cell surface is able to produce enough thrombin to fully activate the neighbouring platelets through their protease-activated receptors (PAR1 and PAR4), increasing their adhesion, as well as activate factors V, VIII, and XI (FXI) on the platelet surface [26-28]. Moreover, the platelet receptor GP Ib-IX can not only bind vWF but also thrombin since both proteins have distinct binding sites on the receptor therefore both can bind simultaneously [29]. This allows thrombin to activate coagulation factors directly on the platelet surface. For example, FVIII exists in plasma bound to vWF as a noncovalent complex since FVIII is highly unstable. When vWF binds to GP Ib-IX receptor, it brings FVIII in close proximity to thrombin allowing it to activate FVIII resulting in dissociation of FVIIIa from vWF [30, 31].

Activation of platelets allows a release of various substances from their granules including ionized calcium, partially activated FV, and FVIII as previously mentioned. Factor Xa and thrombin can fully activate FV released from platelets. Platelet activation also results in exposure of negatively charged phospholipids on their membrane which provide a surface for coagulation factors to assemble and localize their activity [15, 32]. Coagulation factors VII, IX, X and prothrombin interact with the negatively charged phospholipid through their amino-terminal γ -carboxyglutamate (Gla) residues. These

coagulation factors are referred to as vitamin K-dependent factors since they require vitamin K for the proper synthesis of their Gla domain and calcium is essential for the proper folding of the domain. Calcium also mediates the binding of the proteins to the phospholipid surface through the interaction of one calcium ion with two γ -carboxyl groups from the Gla domain [33]. In addition, activation of platelets exposes protein receptors or binding sites on their surface for coagulation factors to interact with high affinity [15, 34-37]. Another reoccurring mechanism in coagulation is the assembly of complexes consisting of enzyme, substrate and cofactor on the cell surface to allow the proper positioning of the enzyme-cofactor-substrate to enhance catalytic activity and facilitate efficient activation. This localized assembly sets the stage for large amount of thrombin generation on the platelet surface referred to as propagation [15].

The small amount of thrombin formed from the extrinsic pathway is sufficient to form a fibrin net around the platelet plug, entrapping various blood cells to produce a thrombus. However, this amount of thrombin is not enough to stabilize the fibrin clot. The generation of large amounts of thrombin is necessary to protect the clot from degradation and only takes place after the formation of the fibrin clot through feedback amplification of the coagulation pathway [15, 22, 23]. This pathway of thrombin generation is called the propagation phase or the *intrinsic pathway* since coagulation is initiated by components within the vasculature.

The intrinsic pathway can initiate coagulation *in vitro* through the activation of coagulation factor XII (FXII) upon contact with polyanionic surfaces such as glass or kaolin in the so-called the contact pathway [38, 39]. The binding of FXII to negatively charged surfaces leads to auto activation to form activated factor XIIa (FXIIa). Factor

XIIa converts prekallikrein to kallikrein which in turn proteolytically cleaves high-molecular weight kininogen to release the vasodilator bradykinin. Additional FXII activation occurs through a positive feedback loop following formation of kallikrein [38]. Factor FXII can also be activated through contact with pathophysiological activators such as platelet polyphosphates, DNA, and RNA [40-42]. Generation of FXIIa as well as kallikrein leads to activation of FXI to FXIa [38]. As previously mentioned, FXI can also be activated directly by thrombin independent of the contact pathway components. In fact, individuals with a deficiency of FXII or other proteins of the contact pathway (prekallikrein or high-molecular weight kininogen) do not result in bleeding diathesis which indicates the contact pathway is not vital for physiological haemostasis [43]. However, this pathway may be significant in the setting of iatrogenic thrombosis such as occurs from indwelling catheters.

During the propagation phase, factor IXa that was activated on TF bearing cells is able to diffuse to the activated platelet surface since it is not rapidly inhibited by AT or other plasma protease inhibitors. Factor IX can also be directly activated on the platelet surface by thrombin. Then, factor IXa can interact with its cofactor FVIIIa to form the tenase complex, which activates FX to FXa [15, 27]. Formation of FXa by the tenase complex is approximately 50-fold more efficient than that formed by the extrinsic pathway [27, 44]. Factor Xa formed directly on the surface of the platelet allows it to quickly form a complex with its cofactor FVa to assemble the prothrombinase complex allowing a burst of thrombin formation close to the injury to allow stabilization of the thrombus. Amplification of thrombin generation occurs through factor XI. Factor XIa that was activated by thrombin on the platelet surface is able to directly activate FIX

providing additional FIXa to enhance the formation of the tenase complex. Thus, the coordinated assembly of the tenase and prothrombinase complex on the surface of activated platelets sets the stage for large scale thrombin production within the fibrin clot [15, 23].

Thrombin catalyzes the conversion of the soluble plasma protein, fibrinogen to fibrin, an insoluble protein. Fibrinogen is important in promoting platelet aggregation during primary haemostasis and fibrin is involved in forming a mesh around the platelet plug, entrapping various blood cells to stabilize the haemostatic plug during secondary haemostasis [1]. Fibrinogen is synthesized in the liver and is released into the bloodstream at high concentrations (1.5-3 mg/mL). Its structure is elongated and consists of three pairs of polypeptides called $A\alpha$, $B\beta$ and γ that are linked together at their amino-termini through disulfide bonds. The fibrinogen structure consists of three connected nodules: the central E nodule containing the amino-terminal residues of the polypeptides, and two distal D nodules. Thrombin cleaves fibrinogen releasing fibrinopeptide A and fibrinopeptide B from the amino-terminal central E nodule forming a fibrin monomer. Cleavage unmasks polymerization sites in the E nodule that allows fibrin monomers to interact laterally where the D nodule of one monomer can non-covalently interact with the exposed polymerization site on the E nodule of another monomer. The fibrin dimers then laterally associate to form protofibrils and polymerization continues to grow forming fibrin fibres varying in length and forming branches creating a fibrin mesh around the platelet plug [45]. The high concentration of thrombin generated inside the clot, activates factor XIII (FXIII), which is a transglutaminase enzyme found in plasma and released from the alpha granules of platelets. Factor XIIIa catalyzes isopeptide bonds between

adjacent D nodules in fibrin, cross-linking the fibrin fibers providing structural stability to the clot and increases the resistance of the clot to degradation [46, 47]. Activated FXIII (FXIIIa) also cross-links many other plasma proteins to fibrin incorporating them into the fibrin clot including fibronectin, α_2 -antiplasmin inhibitor [48], vWF [49], factor V [50], thrombospondin [51], and thrombin-activatable fibrinolysis inhibitor (TAFI) [52].

The rate of thrombin formation greatly influences the architecture of the fibrin clot and thus its stability. Studies have shown that fibrin clots formed during high rates of thrombin generation produce thinner fibrin fibers that are tightly packed and less porous and as a result were more resistant to degradation. On the other hand, low rates of thrombin generation tend to produce thicker fibrin fibers that are loosely packed and more porous and therefore susceptible to increased degradation [53].

1.3.1 The Anticoagulant pathway

The coagulation pathway is regulated at several stages during the progression of thrombin formation by different protease inhibitors or interference with the cofactor ability of some enzyme-substrate complexes. The anticoagulant pathways that regulate coagulation include: TFPI, AT, and protein C (PC).

TFPI is a protein that is synthesized and secreted into the circulation primarily by endothelial cells. TFPI is mostly found bound to heparan sulfate found on endothelial cells and bound to lipoproteins in the plasma; a small concentration is also present in the alpha granules of platelets. TFPI can inhibit FXa by directly binding to it and inactivating it. The TFPI-FXa complex can then inhibit TF-FVIIa complex thereby preventing the

activation of FX. TFPI can only inhibit the TF–FVIIa complex when in a complex with FXa [54].

AT is a serpin-type protease inhibitor that is recognized by activated coagulation plasma proteins FIXa, FXa, FXIa, and thrombin. AT can be found bound to glycosaminoglycans (GAGs) present on the surface of endothelial cells. It is a suicide substrate inhibitor that forms a 1:1 stable covalent complex with the activated coagulation factor and in the presence of GAGs the inhibition is greatly accelerated [3, 55]. AT inhibits free enzymes in plasma and is not able to inactivate enzymes in the tenase or prothrombinase complex or thrombin bound to fibrin since their active sites are less accessible even in the presence of GAGs [22].

Thrombomodulin (TM) is an integral membrane protein expressed on the cell surface of all endothelial cells in the vasculature [4, 5]. Any generated thrombin that diffuses from the fibrin clot is able to bind to TM located on intact endothelium. When thrombin binds to TM forming a complex, thrombin loses its procoagulant activities such as activation of platelets, activation of coagulation factors V, VIII, IX, XI, XIII, and cleavage of fibrinogen. Instead, thrombin bound to TM functions as an anticoagulant enzyme by activating the vitamin K-dependent PC to activated protein C (APC). The activation of PC is localized to the endothelial surface through binding to an EPCR in proximity to the thrombin-TM complex. APC with its cofactor, protein S, can cleave cofactors FVa and FVIIIa inactivating them thereby down regulating thrombin generation. APC can inactivate FVa and FVIIIa bound to the activated platelet surface as well as when they are assembled in prothrombinase or tenase complex, respectively [22,

56]. These anticoagulant pathways all aim to control and localize the coagulation pathway to the site of vascular injury.

1.4 The Fibrinolytic Cascade

The conversion of the plasma protein fibrinogen to fibrin results in the formation of a fibrin mesh around the platelet plug that provides the clot shape, strength, flexibility and stability. The fibrin mesh surrounding the clot acts as a scaffold to support endothelial cell growth and movement during wound healing [45]. The function of the fibrinolytic cascade is to promote fibrin clot dissolution and recanalization of the vessel wall. Fibrinolysis is analogous to the coagulation pathway in that it involves a series of zymogen to enzyme conversions, feedback amplification, and inhibition at different stages [1].

In normal circumstances, tPA is constitutively secreted into the circulation and is immediately inhibited by PAI-1 with a very low concentration of free tPA remaining [8]. The fibrinolytic system becomes activated when FXa and thrombin are continually released into the bloodstream stimulating the increased secretion of tPA from endothelial cells [57-59]. Therefore, initiation of fibrinolysis is dependent on the balance of tPA secretion and PAI-1. Another type of plasminogen activator is urokinase-type plasminogen activator (uPA) but it is only produced by perturbed endothelial cells. In addition, uPA is primarily involved in pericellular proteolysis during tissue remodelling and cell migration through binding to its receptor (uPAR) on the cell surface. On the other hand, tPA is mainly involved in dissolution of the fibrin clot through its ability to bind to fibrin [60].

Fibrinolysis begins when tPA and plasminogen bind to the fibrin surface. The binding of plasminogen to fibrin is mediated by its kringle domains also called lysine binding sites. tPA binds to fibrin via its finger domain and kringle domain 2. Fibrin functions as a cofactor to enhance plasminogen activation by providing a surface for tPA and plasminogen to bind forming a ternary complex consisting of tPA, plasminogen, and fibrin. The fibrin surface increases the local concentration of tPA and plasminogen and brings the active site of tPA in close proximity to the activation site of plasminogen. This allows tPA to catalyze the conversion of the plasma zymogen plasminogen to the serine protease plasmin. In the absence of fibrin, tPA mediated plasminogen activation is highly inefficient [61, 62]. Plasmin is the terminal enzyme in fibrinolysis which cleaves the fibrin mesh at basic residues within the coiled-coil regions linking the E and D domains, thereby producing varying sizes of soluble fibrin degradation products (FDPs). As plasmin digests the fibrin it exposes several carboxyl-terminal lysine and arginine residues which function as high affinity binding sites for tPA [63, 64] and plasminogen, accelerating fibrinolysis and stimulating positive feedback in the fibrinolytic cascade [65-68].

Native plasminogen inherently possesses a closed conformation and binding to fibrin, especially partially degraded fibrin with exposed carboxyl-terminal lysine residues, causes a conformational change in plasminogen allowing tPA better access to its cleavage site to yield plasmin [69]. Native plasminogen has a glutamic acid residue at its amino-terminus and is referred to as Glu-plasminogen. Plasmin can modify Glu-plasminogen by removal of the amino-terminal “tail” domain resulting in a plasminogen molecule, called Lys-plasminogen, with Lys77 at its amino-terminus. This conversion of Glu-plasminogen

to Lys-plasminogen enhances the positive feedback by altering the structure of plasminogen from a closed conformation to a more open conformation, exposing the tPA cleavage site and making it a better substrate for plasminogen activators [69, 70]. In addition, Lys-plasminogen's open conformation allows it to bind to fibrin with higher affinity than Glu-plasminogen [71-73]. tPA cleaves both Glu- and Lys-plasminogen in the latent trypsin-like protease domain to generate Glu- and Lys-plasmin, respectively [60].

1.4.1 Antifibrinolytic pathways

The fibrinolysis pathway is regulated at many levels by different inhibitors. Plasminogen activation is inhibited by PAI-1 by targeting the plasminogen activators tPA and uPA. Plasma- and platelet-derived PAI-1 inhibits the plasminogen activators in the circulation that diffuse away from the fibrin clot and is also capable of binding to the fibrin surface and inhibit tPA mediated plasminogen activation thereby preventing premature clot dissolution [74, 75]. Plasmin is primarily inhibited by α_2 -antiplasmin and to a lesser extent by α_2 -macroglobulin [76, 77]. Plasma- and platelet-derived α_2 -antiplasmin interact with plasmin by binding to its kringle domains therefore, plasmin bound fibrin is protected from inhibition allowing the plasmin generated on the fibrin surface to continue degradation [78-80]. However, factor XIIIa is able to cross-link α_2 -antiplasmin to fibrin which prevents plasmin from binding and prevents fibrin degradation [81]. Both PAI-1 and α_2 -antiplasmin are serpins that form a covalent complex with their target protease [82]. Overall, the relative concentrations and localization of plasminogen, plasminogen activators and inhibitors on the fibrin clot or in the vicinity greatly influences the extent and of clot dissolution [1].

1.5 The TAFI Pathway as a Link between Coagulation and Fibrinolysis

The coagulation and fibrinolytic systems may appear to be functioning independently of one another, but in fact many different components within the two systems interact and coordinate each other's activity. The purpose of the coagulation pathway is the formation of a stabilized fibrin clot through generation of the enzyme thrombin. Formation of the fibrin clot is subsequently followed by dissolution of the clot through fibrinolysis by the actions of the enzyme plasmin. Therefore, activation of the coagulation pathway and formation of thrombin is coordinated with the activation of the fibrinolytic pathway and formation of plasmin. For example, thrombin generated inside the clot can activate FXIII, which forms covalent bonds between the fibrin fibers and cross-links α_2 -antiplasmin to fibrin to provide structural stability to the clot and increase the resistance to fibrinolysis [46-48]. Coagulation proteins such as thrombin and FXa have been found to stimulate the increased secretion of tPA from endothelial cells thereby initiating fibrinolysis following formation of the fibrin clot [57-59]. On the other hand, plasmin formation can down regulate the coagulation pathway – and therefore thrombin formation – by proteolytically inactivating FXa, converting it into a fibrinolysis cofactor that is able to bind to plasminogen and accelerate plasmin generation by tPA [83-86]. In addition, plasmin modified FVa is able to accelerate tPA mediated plasminogen activation [87].

Another factor involved in maintaining haemostasis is TAFI. TAFI can be cleaved by thrombin [88] or thrombin in complex with TM [89] to activate TAFI. Plasmin is also able to cleave and activate TAFI [90]. Activated TAFI (TAFIa) attenuates fibrinolysis by interfering with the positive feedback mechanism of plasmin generation in the fibrinolytic

cascade. Therefore, TAFI is a molecular link between the coagulation and fibrinolysis pathways since activation by thrombin down regulates fibrinolysis [91].

1.6 TAFI

TAFI was discovered by four separate laboratories independently and therefore has been designated four different names. In 1989, Hendriks and coworkers first described the presence of a basic carboxypeptidase present in serum but not plasma and appeared following initiation of coagulation. Basic carboxypeptidases are hydrolytic enzymes that remove carboxyl-terminal lysine and arginine residues from protein and peptide substrates. They noticed the disappearance of the carboxypeptidase activity at 37°C and thus named it unstable carboxypeptidase (carboxypeptidase U, CPU) [92-94]. At the same time, Campbell and Okada identified an unstable carboxypeptidase in serum and found its activity was specific to arginine containing substrates and was named arginine carboxypeptidase (carboxypeptidase R, CPR) [95]. In 1991, Eaton and coworkers isolated a plasminogen-binding carboxypeptidase protein from plasma using plasminogen-Sepharose affinity chromatography. They cloned the cDNA encoding the protein from a human liver cDNA library and through sequence analysis found that it was homologous to pancreatic procarboxypeptidase B and therefore named the protein plasma procarboxypeptidase B (pro-pCPB) [96]. Bajzar and coworkers were initially trying to understand the mechanism of the profibrinolytic effect of the anticoagulant APC. They found that APC is not directly profibrinolytic but rather it prevents the formation of thrombin which prevented activation of a substance that inhibits fibrinolysis [97, 98]. On the basis of these observations they reported the isolation of the protein in 1995 and

named it thrombin-activatable fibrinolysis inhibitor (TAFI) based on its function. Amino acid sequence analysis indicated that TAFI was the same protein identified by Eaton and coworkers as plasma pro-pCPB [88]. Also, amino acid sequence analysis of CPU and CPR revealed that they are identical to pro-pCPB and TAFI [99, 100].

1.6.1 Properties and Expression of the TAFI Protein

TAFI circulates as a single-chain plasma zymogen in the bloodstream. TAFI is encoded by the *CPB2* gene which is located on chromosome 13q14.11 [100]. The *CPB2* gene encodes a 423-amino acid primary translation product that is referred to as a pre-proenzyme. The first 22-amino acids of the amino-terminus form a signal peptide which is cleaved off prior to secretion and the remaining 401-amino acid zymogen circulates in the blood plasma [96]. The mature TAFI protein consists of two structural domains: a 92-amino acid activation domain and a 309-amino acid catalytic domain. TAFI possesses a predicted peptide molecular mass of 45,999 Da however, the activation domain is *N*-glycosylated at four sites (Asn22, Asn51, Asn63, Asn86) causing it to migrate on SDS-PAGE as a ~60,000 Da protein. The glycosylation accounts for ~20% of the molecular weight of plasma TAFI [101, 102].

Amino acid sequence analysis revealed that TAFI is homologous to the family of zinc-containing metallo-carboxypeptidases including pancreatic carboxypeptidase A1, A2, B and mast cell carboxypeptidase A. The residues of zinc-dependent carboxypeptidases involved in coordinating the zinc ion, substrate binding as well as catalysis are all conserved in the TAFIa catalytic domain. The differentiating feature of these carboxypeptidases is their specificity for the carboxyl-terminal amino acid of the

substrate. Pancreatic carboxypeptidase B and TAFIa are specific for basic carboxyl-terminal amino acids whereas carboxypeptidases A are specific for aliphatic or aromatic amino acids [96, 103].

The bound Zn^{2+} in the active site of TAFI is coordinated to His159, Glu162 and His288. The residues involved in substrate binding are Arg235, Tyr341 and Asp348. Residue Asp348, found at the bottom of the channel (S1' subsite), is involved in forming a salt bridge with the basic side chain in the substrate (P1' residue), while residues Arg235 and Tyr341 assist in fixing the carboxyl-terminal group of the substrate. The key residue involved in substrate catalysis is Glu363. Residue Glu363 along with Zn^{2+} activate a water molecule to generate a hydroxyl nucleophile which attacks the carbonyl carbon on the substrate. This is followed by proton transfer and formation of a tetrahedral intermediate that subsequently collapses and breaks the peptide bond, resulting in the release of the carboxyl-terminal basic residue and formation of a new carboxyl-terminus [104].

The main source for the plasma pool of TAFI arises from expression of *CPB2* in the liver [103, 105]. The concentration of TAFI in plasma varies within the human population ranging between 73 and 275 nM [106, 107]. TAFI protein has also been identified within the alpha granules of platelets and is released upon platelet activation [108]. While the platelet pool of TAFI accounts for less than 0.1% of TAFI present in the vasculature, release of platelet-derived TAFI at the thrombosis site could have a significant impact on fibrinolysis [108, 109].

CPB2 mRNA and TAFI protein expression has recently been shown in megakaryocytes and macrophages, although it is significantly less than in hepatic cells

[110]. The presence of TAFI in megakaryocytes suggests that platelet-derived TAFI is synthesized by megakaryocytes. The role of the extra-hepatic pool of TAFI within macrophages is not known at this time but suggests that the TAFI pathway may be implicated in other processes besides fibrinolysis [110].

1.6.2 Activation of TAFI

The TAFI zymogen becomes activated through proteolytic cleavage of the activation domain at Arg92 to form the active 35,000 Da TAFIa carboxypeptidase B-like enzyme (Figure 1-2). This proteolytic cleavage results in the release of the activation domain and allows exposure of the active site cleft of TAFIa enabling interactions between its substrates to catalyze the removal of carboxyl-terminal arginine and lysine residues [88]. Thrombin and plasmin alone are capable of cleaving the Arg92-Ala93 scissile bond. However they are both weak activators of TAFI, as they require high concentrations of the enzymes and long incubations to form a sufficient amount of TAFIa [88, 96]. Thrombin in complex with the endothelial cell surface cofactor TM is capable of significantly enhancing the activation of TAFI. The thrombin-TM complex increases the catalytic efficiency (k_{cat}/K_M) of TAFI activation by over 1000-fold compared to thrombin alone [89]. On the other hand, activation of TAFI by plasmin can be accelerated in the presence of GAGs such as heparin. However, the catalytic efficiency of TAFI activation by plasmin in the presence of heparin is still approximately 10-fold lower than by thrombin-TM [90].

Based on this evidence it was assumed that the thrombin-TM complex is the physiological activator of TAFI. However, an *in vitro* clot lysis assay revealed that the

half-maximal effect of TAFIa on prolonging clot lysis time is attained at a concentration of 1 nM. Activation of 1 nM of TAFI corresponds to less than 2% of the the plasma pool of TAFI. These results suggest that even a small amount of TAFI activation is enough to significantly inhibit the fibrinolytic cascade [89]. For example, the high concentration of thrombin generated by amplification of the coagulation pathway through factor XI is sufficient to activate TAFI in the absence of TM. In fact, a TAFIa mediated inhibition of fibrinolysis has been demonstrated *in vitro* through clot lysis assays performed in the absence of TM but with the existence of an intact factor XI-dependent pathway [88, 106]. Likewise, experiments in a rabbit thrombolysis model indicated that inhibition of FXIa promotes thrombolysis in manner dependent on prevention of TAFI activation [111]. In addition, thrombin-TM mediated activation of TAFI would only occur on the surface of intact endothelium whereas sites of vascular injury that expose GAGs from the subendothelial matrix may be expected to promote plasmin-mediated activation [90, 112, 113]. Therefore, TAFI activation *in vivo* can be achieved by thrombin, thrombin-TM, and plasmin yet, the contribution of each of the activators to the TAFI pathway *in vivo* is unknown. Activation of the TAFI pathway *in vivo* is likely dependent on the on the site of thrombosis, the extent of activation of the coagulation and fibrinolytic cascades in addition to the phenotype of the endothelium [5, 8].

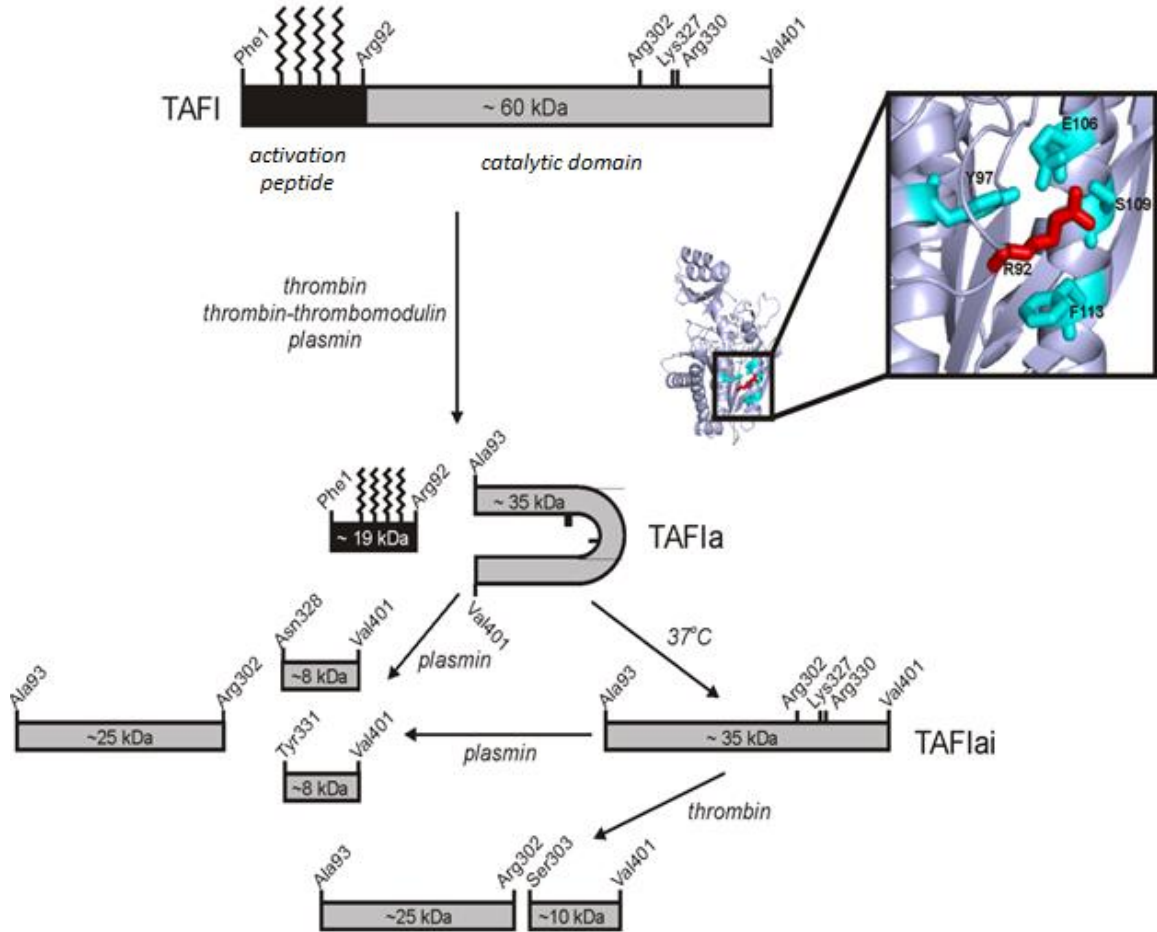


Figure 1-2. Activation of TAFI and inactivation of TAFIa. TAFI circulates as an inactive zymogen in the bloodstream, and becomes activated during blood clotting or fibrinolysis. The mature TAFI protein consists of a 92-amino acid activation domain (black) which is *N*-glycosylated at four sites, and a 309-amino acid catalytic domain (grey). TAFI becomes activated through cleavage at Arg92 by thrombin, thrombin-TM, or plasmin forming activated TAFI (TAFIa). TAFIa is unstable at body temperature possessing a half-life of approximately 8-15 minutes, and undergoes a conformational change (to form TAFIai) that is associated with the disappearance of its enzymatic activity. This conformational change also exposes residue Arg302 for cleavage by thrombin and accelerates cleavage by plasmin at residues Arg302, Lys327 and Arg330. The inset displays the location of Arg92 (red) and the side chains of residues in its vicinity (cyan). The figure was adapted and modified from reference [104]. Crystallographic data are from reference [114]. (PDB accession #3D66)

1.6.2.1 Thrombomodulin and TAFI activation

TM is a transmembrane protein expressed on the luminal endothelial surface lining the blood vessels within the vasculature. TM is found in all vascular beds including arteries, veins, and capillaries [4, 5, 115]. It is especially highly expressed in the microvasculature which makes up more than 99% of the endothelial surface area [116]. TM is a 559 amino acid transmembrane glycoprotein and is divided into 10 structural domains: an amino-terminal lectin-like domain (residues 1-222), six tandem epidermal growth factor (EGF)-like domains connected by small interdomain peptides (residues 227-462), a serine/threonine-rich domain (residues 463-497), a transmembrane domain (residues 498-521) and a short cytoplasmic tail (residues 522-559) (Figure 1-3) [115-117]. In addition, soluble forms of TM are found circulating in the blood due to proteolysis of the intact protein or damage to endothelial cells. Increased TM levels are found in disorders linked to vascular damage including infections, sepsis, and inflammation and have been recognized as a marker of disease [7, 9, 118-121].

Any thrombin generated at the injury site that diffuses into the blood either encounters AT to rapidly inhibit the protease preventing it from cleaving fibrinogen in the circulation or it binds to TM on the intact endothelium [15]. TM interacts with thrombin forming a high affinity 1:1 complex which possesses anticoagulant and antifibrinolytic properties. The thrombin-TM complex prevents the procoagulant functions of thrombin by accelerating PC activation whereas the antifibrinolytic properties are promoted through activation of TAFI and thereby down-regulating fibrinolysis [115, 116, 122, 123].

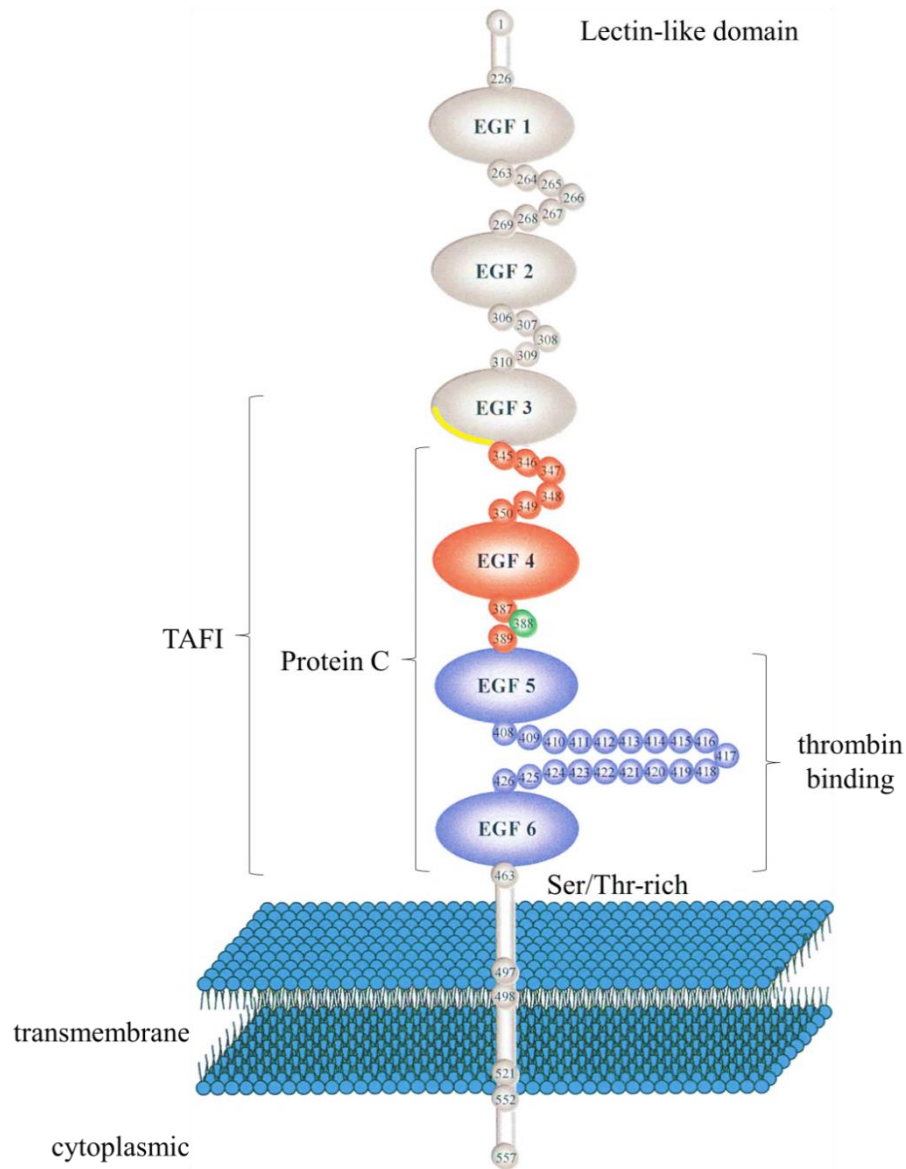


Figure 1-3. Structure of thrombomodulin (TM). The structure of TM can be divided into several domains. The extracellular amino-terminal domain of TM, which is directed towards the circulating blood, consists of a lectin-like domain, followed by six tandemly repeating epidermal growth factor (EGF)-like domains joined by small connecting peptides, a serine/threonine-rich domain, a transmembrane domain and a short cytoplasmic tail. The EGF domains 5 and 6 are primarily responsible for the interaction with thrombin (indicated in blue). The smallest primary structure of TM capable of efficiently activating Protein C requires the residues of EGF-4 through EGF-6 plus the six residues connecting EGF-4 to EGF-3 (indicated in red) whereas TAFI requires residues of the c-loop of EGF-3 through EGF-6 (indicated in yellow). The figure was adapted and modified from reference [124].

The structure of the thrombin-TM complex has been determined through X-ray crystallography of human α -thrombin bound to the EGF-like domains 4, 5 and 6 on TM [125]. Thrombin binds to TM EGF-5 and part of TM EGF-6 which is through interactions with the lysine and arginine residues on the anion binding exosite I on thrombin [125-127]. Additional structural elements of TM are required to allow efficient activation of PC and TAFI by thrombin and, interestingly, these elements differ. The smallest primary structure of TM capable of efficiently activating PC requires the residues of EGF-4 through EGF-6 plus the six residues connecting EGF-4 to EGF-3 whereas TAFI requires residues of the c-loop of EGF-3 through EGF-6. A report of alanine scanning experiments of residues within the c-loop of TM EGF-3 showed a 90% or greater reduction in TAFI activation but no effect on PC activation [128]. Modeling of the PC structure with the thrombin-TM complex showed that EGF-4 makes direct contact with the activation peptide of PC, most notably involving three consecutive lysine residues in the activation peptide [125]. Whether similar or analogous interactions occur between TM and TAFI is unknown.

1.6.2.2 Plasmin as an activator of TAFI

Plasmin is the ultimate enzyme of the fibrinolytic pathway. It is formed by conversion of the plasma zymogen plasminogen to the active serine protease, plasmin. Plasminogen is a single chain glycoprotein that is primarily synthesized by the liver and circulates in plasma at a concentration of 1.5 – 2 μ M. Plasminogen activators cleave plasminogen at the Arg561-Val562 bond to form the a two-chain plasmin protease. The structure of plasmin consists of the heavy chain containing the five kringle domains

(amino-terminal part of plasminogen) linked by a disulfide bond to a light chain containing the serine protease catalytic domain (carboxyl-terminal part of plasminogen). Kringle domains, also referred to as lysine binding sites, are triple loop structures stabilized by three disulfide bonds. Plasmin contains two lysine binding kringle domains (kringle 1 and 4) that exhibit high affinity to lysine and lysine analogs as well as internal and carboxyl-terminal lysine residues in proteins. The kringle domains of plasminogen/plasmin mediate its interaction with fibrin, cell surface receptors, and other proteins including its inhibitor α_2 -antiplasmin [60, 129, 130].

In addition to its roles in fibrin dissolution and promotion of positive feedback in the fibrinolytic cascade, plasmin also functions as an activator of TAFI. Thus, plasmin is also a negative regulator that attenuates plasmin generation in the fibrinolytic cascade by interfering with the positive feedback mechanism through TAFIa. Plasmin is a very poor activator of TAFI [60, 88]. An ideal plasmin substrate contains the following amino acids surrounding the scissile bond for optimal catalysis: an arginine or lysine residue at the P1 position, an aromatic residue at the P2 position (immediately amino-terminal to the P1 residue), arginine, lysine, or serine at the P1' position (immediately carboxyl-terminal to the P1 residue), and an arginine, lysine, or glycine at the P2' position. Plasmin is less selective at the P3 position of the substrate [131]. The P2 position in TAFI is a proline (Pro91) which makes TAFI a suboptimal substrate for plasmin. Additionally, the P1' and P2' amino acid residues in TAFI are not solvent exposed and therefore are not able to interact with plasmin [132]. However, the residues surrounding the cleavage site are not the only determinants of substrate specificity [133]. Since the interaction of plasmin/plasminogen with other proteins is mediated through their lysine binding kringle

domains, this too may be the mode of interaction between plasmin and TAFI. Plasmin's kringle domains may interact with TAFI through its surface exposed lysine residues [134]. At this time the structural basis for the interaction of plasmin with TAFI is unknown.

1.6.2.3 Glycosaminoglycans and their effect on TAFI/TAFIa

GAGs are long, unbranched polysaccharide chains consisting of repeating disaccharide units. The chains vary in length, and can contain between 80 to as many as 200 disaccharide units that occupy highly extended conformations. The disaccharide unit consists of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) which is usually sulfated and the second sugar is usually a uronic acid (glucuronic acid or iduronic acid) or galactose. There are sulfate or carboxyl groups on most of their sugars, rendering them highly negatively charged. GAGs are considered the most anionic molecules produced by animal cells. There are four main groups of GAGs which are categorized by their sugars, the type of linkage between the sugars, and the quantity and location of sulfate groups: (1) hyaluronan, (2) chondroitin sulfate and dermatan sulfate, (3) heparan sulfate and heparin, and (4) keratan sulfate. GAGs are found covalently linked to a core protein and as a whole are referred to as proteoglycans [135, 136]. The structure of a proteoglycan is highly variable in terms of the number and types of GAG chains, lengths, and the proportion of sulfated and non-sulfated groups along the chains. Proteoglycans are found on the luminal surface of endothelial cells as part of the glycocalyx, or in the subendothelial extracellular matrix which can be exposed upon vascular injury [135, 137, 138].

Two GAGs, heparan sulfate and heparin, have similar polysaccharide backbones but differ in terms in their cellular localization and structural modifications. Nearly all animal cells synthesize proteoglycans that contain heparan sulfate GAGs that are present on the cell surface of the endothelium and the extracellular matrix. By contrast, heparin is synthesized in cytoplasmic secretory granules by connective-tissue type mast cells [135, 139]. Heparan sulfate is found covalently attached to a variety of core proteins (syndecans 1-4, betaglycan, glypicans 1-6) whereas heparin is mainly attached to the serglycin core protein [135]. They are both composed of alternating units of α -D-glucosamine (GlcN) and uronic acid, β -D-glucuronic acid (GlcA) or α -L-iduronic acid (IdoA), that are connected by (1 \rightarrow 4) glycosidic linkages [135, 139]. Heparan sulfate and heparin differ in terms of the extent of sulfation. In heparin, the GlcN sugar is predominantly *N*-sulfated whereas the GlcN sugar in heparan sulfate varies containing both *N*-sulfated and *N*-acetylated and smaller amounts of *N*-unsubstituted sugars. Heparin is highly sulfated with typically more than 80% sulfation containing 2.3-2.8 sulfates/disaccharide, while heparan sulfate has less than 60% containing 0.6-1.5 sulfates/disaccharide. In addition, the molecular weight of heparin varies between 7 to 20 kDa whereas heparan sulfate varies between 10 to 70 kDa [135, 139, 140].

Due to the structural diversity of heparin and heparan sulfate they are able to bind and interact with a variety of proteins to regulate their function. The interaction between GAGs and their target protein is mediated through ionic interactions between the negatively charged sulfate groups and the positively charged basic amino acid residues (lysine, arginine, histidine in certain cases), respectively [139, 141]. In addition, hydrogen bonding is possibly involved in the interaction between heparin binding to proteins. For

example, heparin binds to arginine residues more tightly than lysine residues due to the stronger hydrogen bond between arginine's guanidinylate side chain and the heparin sulfate group as compared to the primary amino side chain of lysine residues [142]. The basic residues on the protein structure can appear as linear arrangements or in spatial folded clusters. Amino acid sequence alignments between various proteins known to bind GAGs were analyzed in order to find a GAG binding consensus sequence which could be used to identify potential GAG-binding regions in other proteins. Two GAG consensus sequences were determined: $-XBBXBX-$ and $-XBBBXXBX-$, where B and X represent basic and hydrophobic residues, respectively [139, 141, 143]. The consensus sequences were found to appear as both α -helical and β -strand conformations. For instance, the $-XBBXBX-$ sequence folded in β -strand conformation and the $-XBBBXXBX-$ sequence folded in α -helical conformation results in exposure of their basic amino acid residues leaving the hydrophobic residues oriented toward the interior of the protein. In addition, a third GAG binding consensus sequence $-XBBBXXBBBXXBBX-$ has been identified in vWF. The GAG binding consensus sequences can be helpful in identifying heparin-binding regions in proteins however, many proteins do not possess these sequences since many factors determine the ability of GAGs to interact with proteins [141, 143]. The specific folding conformation of the protein structure can bring distant basic residues in the primary protein sequence closer together in clusters on the protein surface to enable GAG binding. A minimum spacing of 20 Å of basic amino acids on the protein structure was noted to bind GAGs in some proteins irrespective of tertiary structure. Apart from the structure of the protein, the distribution and conformation of uronic acid residues and

amount and position of sulfate groups on the GAGs can influence the interaction with proteins [139, 144].

GAG-protein interactions are important in a variety of cellular functions including the regulation of coagulation. AT is an important anticoagulant serine protease inhibitor that is found circulating in plasma, bound to cell surface endothelial heparan sulfate or exposed GAGs present in the extracellular matrix. AT inhibits the active coagulation plasma proteins thrombin, FIXa, FXa, FXIa at a slow rate by forming an AT-protease complex but, in the presence of GAGs, this inhibition is dramatically accelerated [3, 55]. Heparin is able to bind to AT inducing a conformational change that accelerates AT-protease complex formation and thus inhibition [145]. This function of heparin underlies its role as an anticoagulant drug and as the template for related synthetic anticoagulants. The minimum number of polysaccharide units required to bind to AT is a highly sulfated pentasaccharide sequence. This binding is sufficient to accelerate FXa inhibition by AT but a longer sequence of polysaccharides consisting of 16 or more residues is needed to accelerate inactivation of thrombin as well as FIXa and FXIa [7, 139, 144]. Therefore, the interaction of GAGs with proteins necessitates a minimum length requirement for binding as well as function, and is specific to the GAG interacting protein. This underlies the function of the anticoagulant drug Fondaparinux, which is a synthetic heparin compound composed of a pentasaccharide sequence that exclusively accelerates the inhibition of FXa [4]. Heparin is a widely used anticoagulant drug for deep vein thrombosis however there are limitations such as short half-life, requirement for parenteral administration, individual variation in responses to heparin treatment as well as risk of hemorrhagic complications [146].

GAGs have been found to play a role in regulation of fibrinolysis. The presence of GAGs, such as heparin, has been found to regulate TAFI activation by accelerating its activation by plasmin as well as stabilizing the TAFIa enzyme [90]. In the same study, other GAGs including chondroitin sulfate, dextran sulfate, and heparan sulfate and keratan sulfate at high concentrations have been found to stimulate plasmin-mediated TAFI activation. Unfractionated heparin (UH) and low molecular weight heparin (LMWH) were also examined and no difference was found between them [90].

1.6.3 Inactivation of TAFI

A characteristic that differentiates TAFIa from other carboxypeptidases is its intrinsic instability. The intrinsic instability of TAFIa is temperature-dependent and causes the activity of TAFIa to spontaneously decay. At body temperature TAFIa possesses a half-life of approximately 8-15 minutes. The half-life of TAFIa is increased to about 2 hours at 22°C and at 0°C TAFIa is stabilized [102]. In contrast to the proteases of the coagulation and fibrinolytic pathways, no endogenous inhibitors of TAFIa have been described to this point. However, proteinaceous inhibitors of TAFIa have been found in the secretions of the leech *Hirudo medicinalis* [147] and the tick *Rhipicephalus bursa* [148] which would be expected to promote continued fluidity of blood during feeding from a host. The inactivation of TAFIa is associated with a dramatic structural change that results in a significant decrease in the intrinsic fluorescence signal of the enzyme [102, 149]. The intrinsic thermal instability of TAFIa is thought to be the regulatory mechanism of down-regulation of TAFIa activity *in vivo* [102, 149].

The crystal structures of human and bovine TAFI have provided insight into the structural basis for the intrinsic instability of TAFIa [114, 150]. The crystal structure of TAFI revealed that there is a highly dynamic region (residues 296-350) encompassing a loop, a surface-exposed helix, and another surface-exposed loop in the catalytic domain which is called the “dynamic flap” [114, 151]. Changes in conformation of the dynamic flap can plausibly be linked to loss of TAFIa activity because the flap encompasses part of the wall of the active site as well as the catalytically crucial Tyr341 and Asp348 residues. In the TAFI zymogen structure, the dynamic flap region is stabilized by interacting with the activation domain through hydrophobic interactions between Val35 and Leu39 in the activation domain and Tyr341 in the dynamic flap. Proteolytic cleavage of the activation domain by thrombin, thrombin-TM or plasmin releases the activation domain and increases the mobility of the dynamic flap. Eventually, an irreversible conformational change occurs in the dynamic flap, disrupting the catalytic site and resulting in the loss of TAFIa activity [114, 150]. Mutagenesis studies have shown that some residues mutated within this dynamic flap region greatly decrease the intrinsic stability of TAFIa which in turn reduces the antifibrinolytic potential of the enzyme [149, 152].

The proteolytic cleavage of TAFIa was initially thought to be associated with the inactivation of the enzyme. However, it has been shown that proteolytic cleavage of TAFIa by thrombin can only occur following the thermal inactivation of TAFIa. The conformational change in TAFIa exposes amino acid residue Arg302 causing it to be cleaved by thrombin [149, 153]. On the other hand, plasmin has been shown to cleave TAFIa at Arg302, Lys327 and Arg330 prior to its conformational change and thermal

inactivation of TAFIa can accelerate this cleavage [149, 154]. In addition, high concentrations of plasmin have been shown to cleave TAFI at Lys327 and Arg330 forming a 45,000 Da TAFI species that upon further cleavage at Arg92 would not result in the active enzyme. The ability of plasmin to cleave the TAFI zymogen and TAFIa prior to its thermal inactivation may be a process to regulate the level of active TAFIa (Figure 1-2) [154].

Although TAFIa does not possess an endogenous inhibitor *in vivo*, many exogenous inhibitors of TAFIa have been described based on its homology to the pancreatic carboxypeptidases. Several reversible competitive inhibitors of TAFIa exist including the lysine analog ϵ -aminocaproic acid (ϵ -ACA) and arginine analogs 2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MERGEPTA) and 2-guanidinoethylmercaptosuccinic acid (GEMSA) [92, 102, 155]. Some naturally occurring inhibitors include potato tuber carboxypeptidase inhibitor (PTCI) [156, 157], as well as those from leech (LCI) [147] and tick (TCI) [148]. Since TAFIa is a metallo-carboxypeptidase (it contains a zinc ion in its active site), it is therefore sensitive to chelating agents such as ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline [92]. In addition, TAFI contains eight cysteine residues and, based on homology to the pancreatic carboxypeptidase, six of the residues are involved in disulfide bonds within the catalytic domain and therefore would be susceptible to reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol (β ME) [92, 96].

An important characteristic of the competitive inhibitors of TAFIa is that they are also able to stabilize the conformation of TAFIa at saturating concentrations of the inhibitor thereby preventing it from thermal decay [102, 158]. In addition, they are able to

decrease the rate of proteolytic cleavage at Arg302 by preventing the structural change of TAFIa that is associated with inactivation making it more susceptible proteolysis [153, 155]. Studies of the effect of competitive inhibitors on fibrinolysis in the context of lysis assays revealed that the inhibitor can either promote or inhibit fibrinolysis, depending on its concentration [158]. The inhibitors display a biphasic antifibrinolytic effect where high concentrations of the inhibitor would bind all the available TAFIa and therefore promote fibrinolysis. On the other hand, lower concentrations of the inhibitor would result in a small concentration of TAFIa bound to the inhibitor, being stabilized and also released in a time-resolved manner, thus causing prolongation of fibrinolysis [159].

1.7 The Function of TAFI in Fibrinolysis

TAFIa possesses basic carboxypeptidase activity, catalyzing the removal of carboxyl-terminal basic residues from protein and peptide substrates. The basic carboxypeptidase activity of TAFIa is responsible for its antifibrinolytic activity through its ability to remove carboxyl-terminal lysine and arginine residues from partially degraded fibrin which are important for the development of positive feedback in the fibrinolytic cascade (Figure 1-4) [160]. TAFIa suppresses the positive feedback mechanism of plasmin generation through various mechanisms.

First, TAFIa interferes with the stimulation of plasminogen activation and thus the mechanism of positive feedback. The fibrin surface functions as a cofactor by enhancing plasminogen activation by tPA over 500-fold, thereby localizing plasmin formation to the site of the fibrin clot [62, 161]. Plasmin cleavage of the fibrin mesh results in the exposure of carboxyl-terminal basic residues, providing additional binding sites for tPA

and plasminogen through their kringle domains. These residues enhance the cofactor function of fibrin by approximately 3-fold, stimulating Glu-plasminogen activation [160, 162]. Additionally, the partially degraded fibrin promotes the conversion of Glu-plasminogen to Lys-plasminogen, enhancing the positive feedback in the fibrinolytic pathway [62, 161]. Lys-plasminogen is a better substrate for tPA due to its open conformation, rendering the cleavage site more accessible for tPA, as well as its higher binding affinity to the partially degraded fibrin [71-73]. TAFIa cleavage of these exposed carboxyl-terminal basic residues on fibrin significantly all but eliminates the fibrin cofactor activity for Glu-plasminogen activation along with down regulating the rate of conversion of Glu-plasminogen to Lys-plasminogen [160, 163]. Overall, TAFIa diminishes the positive feedback in plasminogen activation and substantially halts the progression of fibrinolysis.

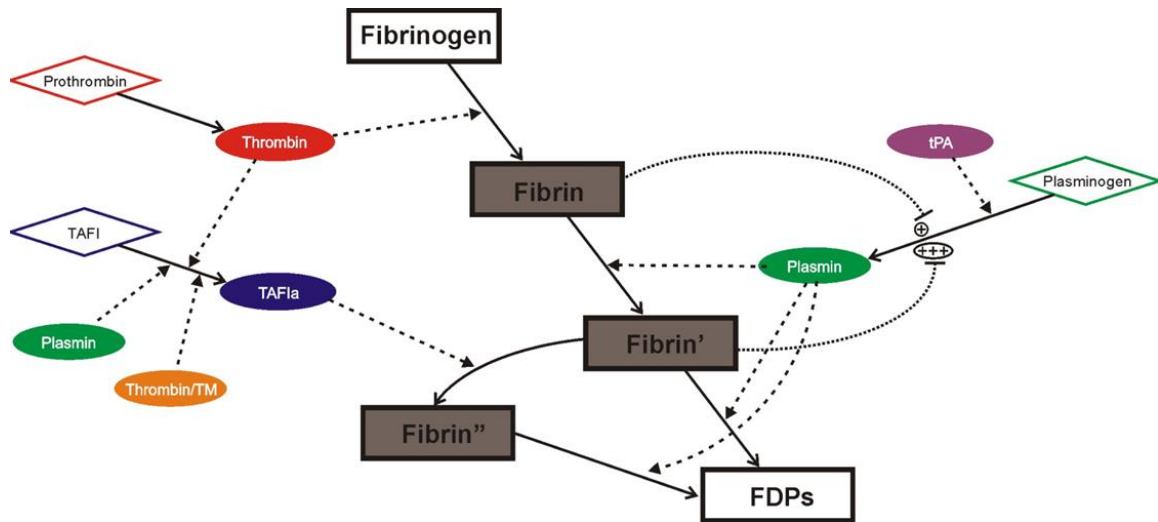


Figure 1-4. The role of the TAFI pathway in fibrinolysis. The coagulation and fibrinolytic pathways terminate in the formation of the enzymes thrombin and plasmin, respectively. Thrombin cleaves the soluble plasma protein fibrinogen to facilitate the formation of the insoluble fibrin clot. The fibrin surface functions as an essential cofactor for tissue-type plasminogen activator (tPA)-mediated activation of plasminogen to plasmin. Plasmin cleaves the fibrin fibers at specific lysine residues in the clot to form soluble fibrin degradation products (FDPs) thereby leading to clot dissolution. Proteolysis of fibrin by plasmin results in a form of fibrin (fibrin') with enhanced cofactor activity, relative to native fibrin, due to the newly-exposed carboxyl-terminal lysine residues. Activated TAFI (TAFIa), possessing basic carboxypeptidase activity, efficiently removes the carboxyl-terminal lysine residues from partially-degraded fibrin, thus yielding a form of fibrin (fibrin'') that possesses almost no cofactor activity. Thus, the TAFI pathway attenuates fibrinolysis by interfering with positive feedback in the fibrinolytic cascade that is necessary for the development of positive feedback in the fibrinolytic pathway. The figure was adapted and modified from reference [104].

Secondly, TAFIa suppresses fibrinolysis by eliminating the protective effect of partially degraded fibrin on plasmin [164]. When plasmin is formed, it remains associated with the fibrin surface through the exposed carboxyl-terminal basic residues by binding through its kringle domains. This restricts the activity of plasmin to the fibrin clot thus promoting fibrin clot dissolution [165]. Removal of the carboxyl-terminal basic residues on the fibrin surface by TAFIa decreases the ability of plasmin to bind to partially degraded fibrin allowing it to be more readily inhibited by circulating α_2 -antiplasmin. TAFIa decreases the local concentration of plasmin on the fibrin surface which prolongs the progression of fibrinolysis [166].

Finally, TAFIa down regulates fibrinolysis by decreasing the cooperative cleavage mechanism of plasmin-mediated fibrin degradation [167, 168]. It has been demonstrated that cleavage of all the fibrin chains is not required for complete fibrin clot dissolution. Instead, fibrin is solubilized when the α , β and γ chains of neighbouring protomers are cleaved resulting in the release of fibrin degradation products of various molecular weights. When plasmin cleaves one of the α , β and γ chains, it exposes carboxyl-terminal basic residues providing binding sites for additional plasmin. This increases the local concentration of plasmin in these specific regions on fibrin allowing cleavage of the other chains in the vicinity. When TAFIa cleaves the carboxyl-terminal basic residues it prevents cooperative cleavage of fibrin by plasmin. TAFIa prevents the accumulation of plasmin on fibrin at specific sites and instead forces plasmin to cleave at additional random sites on the fibrin meshwork, making more cleavages than necessary to achieve fibrin dissolution [167, 168].

The TAFI pathway plays an important role in regulating fibrinolysis and it has been demonstrated through a threshold dependent mechanism [169, 170]. The basis of the threshold dependent mechanism is as long as the TAFIa concentration remains at or above the specific threshold value, fibrinolysis would be attenuated. When the TAFIa concentration drops below the threshold value, the number of carboxyl-terminal basic residues increases allowing the progression of fibrinolysis. The period of time TAFIa remains at or above the threshold is influenced by the rate of TAFIa formation and its intrinsic thermal instability [169, 170]. Mutagenesis studies have revealed that the maximum prolongation of fibrinolysis is proportional to the half-life of the TAFIa enzyme. Therefore, variants with an increased half-life possess a higher antifibrinolytic potential [149, 152].

1.8 The Physiological Role of TAFI

Numerous *in vitro* studies have established that the TAFI pathway regulates fibrinolysis [88, 90, 107, 171-174]. Studies utilizing *in vitro* clot lysis assays have shown with increasing concentrations of TAFI the time to lyse a clot increases and eventually reaches a plateau [89, 102]. The time to lyse a clot at saturation (10 nM TAFIa) is approximately three-fold longer compared to in the absence of TAFIa. In fact, the concentration of TAFIa needed to achieve the half-maximal prolongation of lysis time is 1 nM which represents less than 2% of the concentration of TAFI in plasma [89]. Thus, only a small fraction of the available TAFI needs to be activated to have a significant impact on fibrinolysis although, the degree at which TAFIa inhibits fibrinolysis is dictated by a threshold dependent mechanism as previously discussed. As well, *in vitro*

clot lysis assays where TAFI is depleted from plasma or addition of PTCl, a specific TAFIa inhibitor, showed substantial decreases in clot lysis time [175].

The TAFI pathway has also been shown to modulate fibrinolysis *in vivo* as demonstrated through many animal studies. One of the first studies that provided this evidence came from a canine model with electrically induced thrombosis of the coronary artery which showed an increase in TAFIa activity in plasma samples following thrombosis and thrombolytic therapy with tPA. A relationship was observed between the time to reperfusion and the concentration of TAFIa whereby the higher the concentration of TAFIa, the longer the time for tPA to establish blood flow [176]. Another study involving a rabbit thrombolysis model where factor XI or TAFI was inhibited resulted in an almost two-fold increase in endogenous lysis of jugular vein clots [111]. This study supports the *in vitro* studies indicating that an intact intrinsic pathway of coagulation is vital for generating the high concentrations of thrombin that are required for efficient TAFI activation which is involved in down-regulating fibrinolysis [106, 111, 177]. In addition, several animal models of arterial and venous thrombolysis in which TAFI was inhibited were shown to enhance thrombolysis by tPA and were able to reduce the amount of tPA required to attain a similar amount of lysis [111, 178-181]. These studies provide evidence for possible TAFIa inhibitors in enhancing the efficacy of tPA in thrombolytic therapies.

The coagulation pathway plays an important role in the formation of a fibrin clot as well as the stabilization of the clot through activation of TAFI. Any abnormalities in the coagulation pathway caused by deficiency of coagulation factors will affect the activation of TAFI and in turn the stabilization of the clot. For example, deficiency of coagulation

factors VIII or IX results in the serious bleeding disorders hemophilia A and B, respectively, and deficiency of factor XI causes a milder form of hemophilia. In these individuals, the initiation phase of coagulation is delayed at low tissue factor concentrations and the large burst in thrombin formation from the propagation phase is severely impaired [182, 183]. Furthermore, studies involving plasmas deficient in intrinsic pathway coagulation factors VIII, IX, X, XI have shown premature lysis compared to normal plasma and upon addition of TAFI, TM or the deficient factor the coagulation deficiency is corrected [182, 184]. Therefore, the reduction in thrombin formation through the intrinsic pathway results in a decrease in TAFI activation and a decrease in its ability to suppress fibrinolysis and stabilize the fibrin clot. In this regard, it has been proposed that the bleeding observed in hemophilia patients is the result of not only an inability to form the clot but also the failure of TAFI to stabilize the clot [177, 182, 184].

On the other hand, abnormalities in the coagulation pathway due to elevated coagulation factor concentrations are susceptible to an increased risk for thrombotic disorders. For example, individuals with high levels of intrinsic coagulation factors (FVIII, IX, XI) would lead to a prolonged and increased rate of thrombin formation and an increased activation of TAFI resulting in inappropriate down-regulation of fibrinolysis. Studies have shown that the risk of venous thrombosis is increased about two-fold with elevated levels of coagulation factors VIII, IX, XI [185-187]. As well, the Leiden Thrombophilia Study (LETS) showed that elevated plasma TAFI concentrations were associated with a mild risk for venous thrombosis [188]. Individuals with high TAFI levels compared to low TAFI levels were at a two-fold increased risk for recurrent venous

thromboembolism and patients with both high TAFI levels and one of the coagulation factors XI, VIII, and IX were at a three-fold increased risk [188, 189]. In addition, individuals with factor V Leiden suffer from higher thrombotic tendencies due to the increased formation of thrombin and increased TAFI activation. Factor V Leiden is a factor V Arg506Gln mutation in the APC cleavage site that decreases the rate of inactivation by APC. This results in increased thrombin formation and increased activation of TAFI and consequently down-regulation of fibrinolysis [190, 191]. Any defects in the coagulation system as described can lead to changes in the activation of TAFI and the regulation of the fibrinolytic system.

To understand the function of the TAFI pathway *in vivo*, mouse strains in which the TAFI gene has been inactivated by homologous recombination have been developed [192-195]. The TAFI-deficient mice were normal in terms of embryonic development and viability, fertility, and possessed no phenotypic abnormalities. In addition, haematological parameters of the mice were comparable with their wild-type littermates, although clots formed from the TAFI-deficient mice plasma rapidly lysed [192, 194]. The TAFI-deficient mice were subjected to acute haemostatic challenges to induce a phenotype including models of venous and arterial thrombosis, thrombin-induced acute thromboembolism, endotoxin-induced disseminated intravascular coagulation (DIC), tail bleeding, and kaolin-induced writhing response however no differences were observed compared to the wild-type mice [192]. However, after back-crossing TAFI-deficient mice into a heterozygous plasminogen background, a role of TAFI in regulation of plasminogen *in vivo* was observed in models of pulmonary embolism. In the mice with heterozygous plasminogen and partial TAFI deficiency (Plg^{+/-}, TAFI^{+/-}) and complete

TAFI deficiency (Plg^{+/-}, TAFI^{-/-}) fibrinolysis was enhanced in a pulmonary clot lysis model compared to wild-type (Plg^{+/-}, TAFI^{+/+}) [196]. Following this study, many reports have been published providing additional evidence for the role of TAFI in fibrinolysis *in vivo*. For example, in a batroxobin-induced pulmonary embolism model, TAFI-deficient mice exhibited lower fibrin retention in the lungs than wild-type mice [194]. In agreement, another report demonstrated that bleomycin-induced lung fibrosis in mice lacking TAFI resulted in significantly reduced fibrosis in the lungs suggesting that TAFI promotes lung fibrosis by modulating plasmin generation and decreasing fibrin degradation in the lungs [195]. In a FeCl₃-induced vena cava thrombosis model TAFI-deficient mice resulted in a reduced thrombus formation; note that these mice also exhibited an increase in tail bleeding time [197]. As well, an enhanced fibrinolytic capacity was observed in TAFI-deficient mice and TAFI/PAI-1 double-knockout mice compared to wild-type TAFI or PAI-1 mice in a mouse thromboembolism model which indicates the importance of TAFI deficiency on the fibrinolytic pathway [198]. Further investigation of the TAFI pathway in animal models will aid in understanding the role of TAFI in hematologic diseases.

Our understanding is limited in terms of how the TAFI pathway is regulated *in vivo*. Specifically, the relative contributions of thrombin, thrombin-TM and plasmin in regulating the TAFI pathway *in vivo* is unknown. Thus far, only two studies attempted to identify the physiological activator of TAFI *in vivo* [199, 200]. Bajzar and coworkers used a baboon model of *Escherichia coli*-induced sepsis to study TAFI activation *in vivo*. This study demonstrated that thrombin-TM is the predominant physiologic activator of TAFI in a setting of severe sepsis [199]. Another study by Gils and coworkers utilized an

antibody that inhibited plasmin-mediated TAFI activation *in vitro* and found that when injected into a mouse thromboembolism model it resulted in decreased fibrin deposition in the lungs indicating an acceleration of fibrinolysis. Therefore, in the thromboembolism model plasmin would be the predominant physiologic activator of TAFI [200]. Which activator predominates *in vivo* would depend on the complex interaction of a variety of different factors. For instance, different vascular compartments differ in their hemodynamic characteristics, phenotype of the endothelium, contribution of platelets, and ratio of the volume of blood to surface area of the endothelium [5, 8]. The activation of TAFI is likely a function of the site of thrombosis and the extent of activation of the coagulation and fibrinolytic cascades which would depend on the relative concentrations of different activators, inhibitors, and cofactors. As well, the regulation of TAFI activation could be altered in different physiological and pathophysiological circumstances such as in inflammatory disorders, coagulopathies, atherosclerotic disease, and tumour angiogenesis and metastasis.

1.9 Measurement of TAFI

Many methods are available for measurement of the basic carboxypeptidase activity of TAFI generated following activation. The small synthetic substrates hippuryl-arginine/hippuryl-lysine (Hipp-Arg/Hipp-Lys) can be monitored spectrophotometrically, by high-performance liquid chromatography (HPLC), and by a colorimetric analysis through chemical derivatization of the hippuric acid. While the detection limits for HPLC measurement of Hipp-Arg/Hipp-Lys are very low, it is a discontinuous assay and thus not suited to use in high-throughput settings. As well, the disadvantage of the colorimetric

analysis is its time-consuming nature [201]. Another class of small synthetic substrates, anisylazoformyl-arginine/anisylazoformyl-lysine (AAFR/AAFK), can be monitored spectrophotometrically and are able to quantify TAFIa levels in a purified system [102, 132, 152]. Unfortunately, due to their low sensitivity, these substrates cannot be used to detect TAFIa activity in plasma and would suffer from interference in detection due to the turbidity resulting from the formation of a fibrin clot. Alternatively, functional assays exist for the detection of TAFIa activity in plasma and involve its ability to remove carboxyl-terminal basic residues from soluble FDPs. The functional TAFIa assays measure TAFIa concentration indirectly through plasminogen activation or through the release of plasminogen bound to soluble FDPs [202, 203]. However, numerous components are required for the functional assay which can lead to variability. Moreover, none of the existing methods are suitable for the monitoring of TAFIa formation in real-time in the context of a clot. For this, a fluorogenic substrate for TAFIa is required, yet no such compound for TAFIa (or any other carboxypeptidase) exists.

1.10 Rationale, Hypothesis and Objectives

TAFI can be activated by thrombin, thrombin-TM and plasmin however, their relative contribution in regulating the TAFI pathway *in vivo* is unknown. In order to appreciate the physiological role of the TAFI pathway it is essential to understand how TAFI is activated. The regulation of TAFI activation is dependent a variety of variables including the site of thrombosis, the extent of activation of the coagulation and fibrinolytic cascades, the vascular compartment, and different physiological and pathophysiological conditions. The current studies were undertaken to examine the structural basis for the regulation of TAFI activation and function. The structural insight into the activation of TAFI will shed light on the molecular basis for its function *in vivo*.

The activation of TAFI by thrombin is greatly enhanced in the presence of TM. In fact, accumulating evidence from several studies suggests that TM directly binds to TAFI at sites remote from the activating cleavage site. At this point, however, it is not completely understood how TM promotes TAFI activation by thrombin. The elements of TAFI structure that allow accelerated activation of thrombin by TM have not been completely identified. Based on the results of Wu and coworkers [204], it appears that the residues on TAFI that displayed the strongest TM dependence are focused around the activation domain. Therefore, it is possible that the activation domain of TAFI allows for efficient interaction with TM to allow accelerated activation by thrombin.

Previous studies have shown that the presence of GAGs, such as heparin, is able to accelerate TAFI activation by plasmin and stabilize TAFIa. However, the structural basis for the effects of GAGs on plasmin-mediated TAFI activation or TAFIa stability is unknown.

It is apparent that the currently available methods to measure TAFIa activity and assess its activation in real-time in plasma are not adequate. Indeed, the existing methods are either not particularly sensitive or require numerous biological components which can lead to variability in the performance of the assay.

The hypotheses to be addressed are: 1) TAFI interacts with TM such that the enhancement of TAFI activation by the thrombin-TM complex is through binding at a site or sites remote from the activation site; 2) the activation domain of TAFI is responsible for the TM dependence of acceleration of activation by thrombin; 3) TAFI and TAFIa possess GAG binding site(s) that are important in increased TAFI activation by plasmin and/or increased thermal stability of TAFIa in the presence of heparin; and 4) a novel fluorescence assay for the measurement of TAFIa activity will have increased sensitivity compared to current methods and will allow for monitoring of TAFI activation in the clot in real-time.

The research objectives of the current studies are as follows:

- 1) To determine the structural basis for the enhancement of TAFI activation by thrombin in the presence of TM;
- 2) To evaluate the role of the activation domain of TAFI in activation by thrombin-TM;
- 3) To assess the structural basis and functional significance of TAFI binding to GAGs;
- 4) To develop a novel fluorescence assay to assess the function of activated TAFI in real-time.

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Chapter 2

Identification of a Thrombomodulin Interaction Site on Thrombin-Activatable Fibrinolysis Inhibitor that Mediates Accelerated Activation by Thrombin

2.1 Summary

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a human plasma zymogen that provides a molecular connection between coagulation and fibrinolysis. TAFI is activated through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin. Evidence from several studies suggests that TM and TAFI make direct contact at sites remote from the activating cleavage site to facilitate acceleration of thrombin-mediated TAFI activation. The elements of TAFI structure that allow accelerated activation of thrombin by TM are incompletely defined. *Objectives:* To identify TM interaction regions on TAFI that mediate acceleration of activation by thrombin and therefore indicate TM binding sites on TAFI. *Methods:* We mutated selected surface-exposed charged residues on TAFI to alanine in order to identify sites that mediate acceleration of activation by TM. The kinetics of activation of the mutants by thrombin in the presence or absence of TM as well as their thermal stabilities and antifibrinolytic potentials were determined. *Results:* TAFI variants R15A, E28A, K59A, D75A/E77A/D78A, E99A and E106A all exhibited moderately reduced catalytic efficiencies (k_{cat}/K_M) of activation by thrombin-TM. TAFI variants R377A and, particularly, R12A and R12A/R15A exhibited severely reduced activation by thrombin-TM that was not explained by differences in activation by thrombin alone. *Conclusions:* We have identified R12 and R377 as key residues for the activation of TAFI by thrombin-TM. R12 likely directly binds to TM while R377 may affect the thrombin-TAFI interaction specifically in the presence of TM.

2.2 Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) (also referred to as pro-CPU and encoded by the gene *CPB2*) is a human plasma zymogen that provides a molecular connection between the coagulation and fibrinolytic cascades [1, 2]. TAFI is activated through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin to generate the enzyme activated TAFI (TAFIa) [3-5]. TAFIa possesses basic carboxypeptidase activity that down-regulates fibrinolysis by removing carboxyl-terminal lysine and arginine residues from partially degraded fibrin thereby attenuating positive feedback in the fibrinolytic cascade [6, 7]. Thrombin and plasmin are relatively weak activators of TAFI, however, while binding of thrombin to TM increases the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of TAFI activation by 1250-fold compared to thrombin alone [4]. Structure/function studies of TAFI activation suggest that a TAFI-TM interaction accounts for this acceleration [8-11]. However, the elements of TAFI structure that allow accelerated activation of thrombin by TM have not been completely identified.

TM is an integral plasma membrane protein on endothelial cells consisting of an amino-terminal lectin-like domain, six epidermal growth factor (EGF)-like domains, a serine/threonine-rich domain, a transmembrane domain and a short cytoplasmic tail [12]. Binding of thrombin to TM alters the substrate specificity of thrombin from acting as a procoagulant enzyme to possessing anticoagulant and antifibrinolytic properties [12]. The only other known physiological substrate of the thrombin-TM complex is protein C (PC). While TAFI and PC are similar with respect to kinetics of activation by the thrombin-TM

complex [5], the structural requirements in TM differ between the two substrates. The smallest primary structure of TM capable of efficiently activating PC requires the residues of EGF-4 through EGF-6 plus the six residues connecting EGF-4 to EGF-3 whereas TAFI requires residues of the c-loop of EGF-3 through EGF-6 [8, 9]. The importance of the residues within the c-loop of EGF-3 is exemplified by a report using alanine scanning mutagenesis of TM showing that substitution of residues within this region resulted in a 90% or greater reduction in TAFI activation but no effect on PC activation [9].

It was previously thought that the accelerative effects of TM on PC activation were due to the ability of TM to allosterically alter the conformation of the thrombin active site in order to minimize the repulsive charge interactions that may exist between thrombin and the P3 and P3' residues of the PC scissile bond [13, 14]. However, crystallographic studies of the thrombin-TM complex revealed that TM binding to thrombin does not cause allosteric changes in its active site but instead suggested that TM may bind directly to PC in order to optimally present the scissile bond to the thrombin active site [15]. This mechanism likely applies also to TAFI since a study of the residues immediately surrounding the scissile bond in TAFI showed that they do not determine the TM dependence of TAFI activation [10].

Structural modeling of PC with the thrombin-TM complex revealed multiple interactions between PC and TM partly mediated by the three consecutive lysine residues in PC that enhance the binding to TM [15]. To determine whether a similar mechanism occurs in TAFI, Wu and coworkers mutated several basic residues on the surface of TAFI, including three consecutive lysine residues within the activation domain [11];

however, these mutations had only a small (8-fold decrease) effect on TM cofactor ability against TAFI compared to mutation of the analogous residues in PC (70 – 2000-fold decrease) [16]. Furthermore, when the crystal structure of TAFI was docked onto the thrombin-TM complex structure, the roles of specific residues on TAFI could not be completely assessed as the charged residues of the c-loop of TM-EGF-3, required for TAFI activation, are absent from this structure [11]. Most recently, Plug and coworkers demonstrated that mutation of arginine 12 in TAFI to glutamine reduced the catalytic efficiency of activation of TAFI by thrombin-TM by 54-fold [17]. Therefore, the residues on TAFI responsible for the acceleration of TAFI activation by TM remain to be completely characterized.

In the present study, mutation of selected surface-exposed charged residues on TAFI to alanine was used to identify sites that mediate acceleration of activation by TM, potentially indicating sites where TM binds to TAFI directly. The data identify two residues crucial for activation of TAFI by thrombin-TM.

2.3 Experimental Procedures

2.3.1 Materials

The synthetic carboxypeptidase substrate anisylazofornyl-arginine (AAFR) was obtained from Bachem Americas, Inc. (Torrance, CA, USA). Hippuryl-L-arginine, heparin sodium salt (from porcine intestinal mucosa), and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada). The plasmin inhibitor D-valylphenylalanyllysyl chloromethylketone (VFKck), the TAFIa inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (Plummer's inhibitor) and the thrombin inhibitor D-phenylalanylprolylarginyl chloromethylketone (PPack) were purchased from Calbiochem (San Diego, CA, USA). QuikChange® Site-directed Mutagenesis kit was from Stratagene (La Jolla, CA, USA). DNA restriction and modification enzymes (T4 DNA Ligase and Q5 High-Fidelity DNA Polymerase) were purchased from New England Biolabs (Mississauga, ON, Canada). pcDNA™4A/myc-His mammalian expression vector was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Oligonucleotides for mutagenesis were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Dulbecco's modified Eagle's medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, penicillin/streptomycin/fungizone (PSF) were obtained from Invitrogen. Newborn Calf Serum was obtained from Sigma-Aldrich. Effectene transfection reagent was purchased from QIAGEN, Inc. (Toronto, ON, Canada). Methotrexate was purchased from Mayne Pharma, Inc. and Zeocin™ was purchased from Invitrogen. Thrombin, rabbit-lung TM and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, USA).

Recombinant tPA (Alteplase) was purchased from Kingston General Hospital Pharmacy. Polyclonal sheep anti-human TAFI antibody was purchased from Affinity Biologicals (Ancaster, ON, Canada) and polyclonal rabbit anti-sheep antibody was purchased from ThermoFisher Scientific. (Ottawa, ON, Canada). The mouse-anti-human TAFI monoclonal antibody MA-T4E3 was obtained from Dr. Ann Gils (Katholieke Universiteit Leuven, Leuven, Belgium) and coupled to CNBr Activated Sepharose 4B (GE Healthcare Life Sciences, Mississauga, ON, Canada) according to manufacturer's instructions (2-6 mg antibody/mL resin). Ni Sepharose excel resin was purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). The phospholipids L- α -phosphatidylcholine from frozen egg yolk (Type XIII-E) and 1,2-diacyl-*sn*-glycero-3-phospho-L-serine from bovine brain were purchased from Sigma-Aldrich. Synthetic phosphatidylcholine/phosphatidylserine vesicles (PC/PS, 80:20) were prepared by modification of the methods of Barenholz *et al.* and Bloom *et al.* [18, 19].

2.3.2 Construction of TAFI Variants

Mutagenesis was carried out using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used are shown in the Supplementary Table 2-1. In all cases, the template for the mutagenesis was TAFI-pNUT, which contains human TAFI cDNA which contains threonine at positions 147 and 325 [20]. The presence of the mutations was verified by DNA sequence analysis. All variants were expressed from the pNUT plasmid except the R12A and R12A/R15A variants.

The TAFI-R12A and TAFI-R12A/R15A variants, as well as wild-type TAFI, were also inserted into the pcDNA4A/myc-His expression vector in order to generate

recombinant proteins with a carboxyl-terminal 6× His tag. The sequences of the primer pairs are follows: sense, 5'- AAA CTG CAG TTG GGA TGA AGC TTT GC -3' (*Pst*I site underlined) and anti-sense, 5'- GGA CCG GTA ACATTC CTA ATG ACATGC CAA G -3' (*Age*I site underlined). The cDNA wild-type TAFI, TAFI-R12A, and TAFI-R12A/R15A was amplified by polymerase chain reaction and inserted into pcDNA4A/myc-His digested with *Pst*I and *Age*I sites.

2.3.3 *Expression and Purification of Recombinant TAFI Variants*

Recombinant TAFI variants were purified from conditioned medium harvested from Baby hamster kidney (BHK) cell lines stably expressing the variants. Variants lacking the 6×His tag were purified by immunoaffinity chromatography as previously described [21]. Variants containing the 6×His tag were purified by affinity chromatography over Ni²⁺-Sephrose.

2.3.4 *Kinetics of activation of TAFI variants by thrombin-thrombomodulin complex*

Each TAFI variant at various concentrations (0.0156 – 1.0 μM) was incubated in the presence of CaCl₂ (5 mM), thrombin (1 nM) and rabbit-lung thrombomodulin (5 nM) in HBST (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween 80) for 10 minutes at 21°C, then reactions were stopped by the addition of a solution containing the thrombin inhibitor phenylalanylprolylarginyl chloromethylketone (PPack; 1 μM final concentration) and TAFIa substrate hippuryl-L-arginine (1.2 mM final concentration). Rates of substrate hydrolysis (measured by absorbance at 254 nm) were converted into rates of TAFIa formation, and the data were fit to the Michaelis-Menten equation as

previously described [10]. The kinetic constants obtained are considered “apparent” since they were determined at one concentration of the cofactor TM.

2.3.5 Kinetics of activation of TAFI variants by thrombin

Each TAFI variant at various concentrations (0.1 – 2.0 μM) was incubated in the presence of CaCl_2 (5 mM), thrombin (100 nM) and potato tuber carboxypeptidase inhibitor (PTCI) (20 $\mu\text{g/mL}$) in HBST for 15 minutes at 21°C. Thrombin activity was then quenched by the addition of PPAck. Samples were analyzed by SDS-PAGE and western blotting with a sheep anti-human TAFI polyclonal antibody. Blots containing various known concentrations of TAFIa (3.125 – 100 nM) were prepared and analyzed simultaneously with a given experiment to provide a standard curve. The TAFIa concentrations formed in each kinetic experiment were determined by interpolating from the TAFIa band densities on the standard curve. The standard curves were linear and the TAFIa band densities fell within the ranges of the standard curves. The concentration of TAFIa was then converted to the rate of TAFIa formation and the slope of the relationship between the rate and the TAFI concentration was determined to estimate the catalytic efficiency (k_{cat}/K_M) [21].

2.3.6 Activation of TAFI variants by plasmin

For activation of TAFI by plasmin, TAFI variants (200 nM) were incubated with plasmin (25 nM) and Plummer’s inhibitor (DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid; Calbiochem) (150 μM) in the presence or absence of heparin (0.2 U/ μL) at 21°C for 15 minutes. Plasmin activity was then quenched by the

addition of valylphenylalanyllysyl chloromethylketone (VFKck) (to 1 μ M). Samples were analyzed by SDS-PAGE and western blotting to give the rates of TAFIa formation as described above.

2.3.7 Thermal Stability Assay of the TAFIa variants

Purified recombinant TAFI variants (1 μ M) were activated by incubation with thrombin (25 nM), TM (100 nM), CaCl₂ (5 mM) in HBST at 21°C for 15 min. Thrombin activity was then quenched by the addition of PPAck (to 1 μ M). Activated TAFI was divided into aliquots corresponding to various time points and incubated at 37°C. At each time point, the corresponding aliquots were removed and placed on ice. Residual TAFIa activity was determined by measurement of the rate of hydrolysis of anisylazofornyl-arginine (AAFR) by monitoring the absorbance at 350 nm. Non-linear regression of the data to an equation describing first-order exponential decay was used to determine decay rate constants and thus half-lives of each TAFIa variant, as previously described [21].

2.3.8 Clot Lysis Assays

All clot lysis assays were performed in a final volume of 100 μ L in microtitre plates at 37°C. The TAFI deficient plasma (TdP), prepared from the plasma of healthy human volunteers as previously described [10, 22], was diluted 1:3 in HBST and supplemented with recombinant TAFI variants (50 nM) and synthetic phosphatidylcholine/phosphatidyl-serine (PC/PS) vesicles (20 μ M) in the presence of different concentrations of TM (0.02 – 2 nM, final) before clotting was initiated by addition of the assay mixture into the wells of microtitre plates containing small, separated aliquots of

thrombin (6 nM, final), CaCl₂ (5 mM, final) and tPA (2 nM, final). Clot lysis was monitored by the change in turbidity of each reaction at 405 nm in a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 37°C and the time to 50% lysis was determined graphically from the midpoint between maximum and minimum turbidities of the clots.

2.3.9 *Statistical Methods*

Statistical analyses were performed with the use of SPSS software, version 22.0 (SPSS Inc., Chicago, Illinois). Comparisons between samples were performed by one-way ANOVA using a Tukey post-hoc analysis. Statistical significance was assumed at $p < 0.05$.

2.4 Results

2.4.1 Construction, expression, and purification of TAFI variants

To determine the elements of TAFI structure that mediate acceleration of activation by TM we have mutated selected surface-exposed charged residues on TAFI to alanine (Figure 2-1). The crystal structure of human TAFI and the model of TAFI docked to the thrombin-TM complex guided the construction of these variants while we avoided lysine and arginine residues previously reported by Wu and coworkers to partially reduce the efficiency of TAFI activation [11, 23, 24].

Eleven mutants of human TAFI with alanine substituted for surface-exposed, charged residues were constructed (Figure 2-1). In some cases, charged residues located in close proximity were mutated in combination, yielding R12A/R15A, D54A/D56A, D75A/E77A/D78A, and E112A/E116A. With the exception of R12A and R12A/R15A, all variants including wild-type TAFI (T147/T325 isoform) were purified by immunoaffinity chromatography. The R12A and R12A/R15A variants contain a carboxyl-terminal 6×His tag for purification by affinity chromatography over Ni²⁺-Sepharose. As a control for possible effects of the affinity tag, we also expressed wild-type TAFI with the 6×His tag. All variants were purified to homogeneity from conditioned medium harvested from stably-expressing BHK cell lines (data not shown).

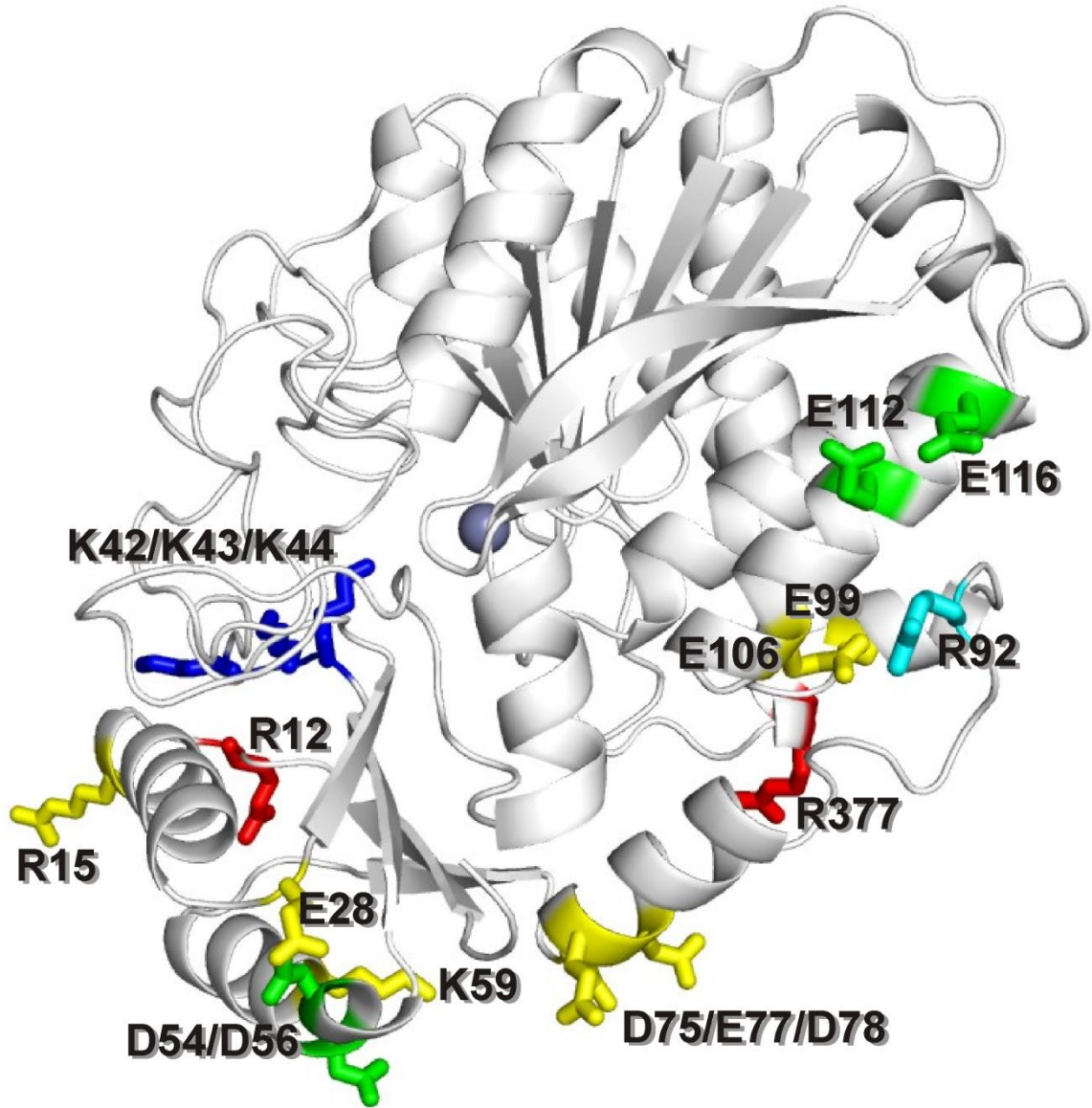


Figure 2-1. Mutations analyzed in this study. Image was drawn using Polyview-3D and the coordinates for chain B in pdb accession #3D66 [23]. The structure is rendered in approximately the same orientation as TAFI in the TAFI-thrombin-TM model of Wu and coworkers [11]. The arginine cleaved by thrombin and plasmin (R92) is shown in cyan. Residues that have little or no impact on activation of TAFI by thrombin-TM are colored green. Residues that have a moderate impact are colored yellow. Residues that have a large impact are colored red. The grey sphere is the active site Zn^{2+} atom. Also shown are K42/K43/K44, studied by Wu and coworkers [11], in blue.

2.4.2 Kinetics of activation of TAFI variants by thrombin in the presence of TM

The kinetics of activation of the TAFI variants by thrombin (1 nM) in the presence of TM (5 nM) were determined in order to examine whether any variants were resistant to the effects of TM (Figure 2-2). The estimated Michaelis-Menten kinetic parameters are summarized in Table 2-1, with k_{cat} and K_M cited as “apparent” because a single concentration of TM was employed. The variants fell into three broad categories: those whose activation by thrombin-TM was modestly affected, if at all (D54A/D56A and E112A/E116A; Figure 2-2A), those whose activation was moderately decreased (R15A, E28A, K59A, D75A/E77A/D78A, and E106A; Figure 2-2B), and those whose activation was severely impaired (R12A, R12A/R15A, R377A; Figure 2-2C). The apparent catalytic efficiency of wild-type TAFI and wild-type TAFI-6×His activation in the presence of thrombin-TM complex is $2.61 \mu\text{M}^{-1}\text{s}^{-1}$ and $1.22 \mu\text{M}^{-1}\text{s}^{-1}$, respectively, a difference arising because of a higher apparent K_M for the latter (Table 2-1). The variant R377A showed a dramatically lower catalytic efficiency in the presence of TM, compared to wild-type, an effect mostly attributable to large increase in apparent K_M . Under the conditions employed to assess the activation by thrombin-TM of all the other variants, activation of R12A and R12A/R15A could not be detected. Therefore, we used higher concentrations of thrombin and TM (100 nM each) specifically for these mutants to assess whether they are capable of being activated at all. Even under these conditions, the relationship between activation rate and TAFI concentration was linear (Figure 2-2C), and so only the $k_{\text{cat}(\text{app})}/K_{M(\text{app})}$ could be estimated (Table 2-1).

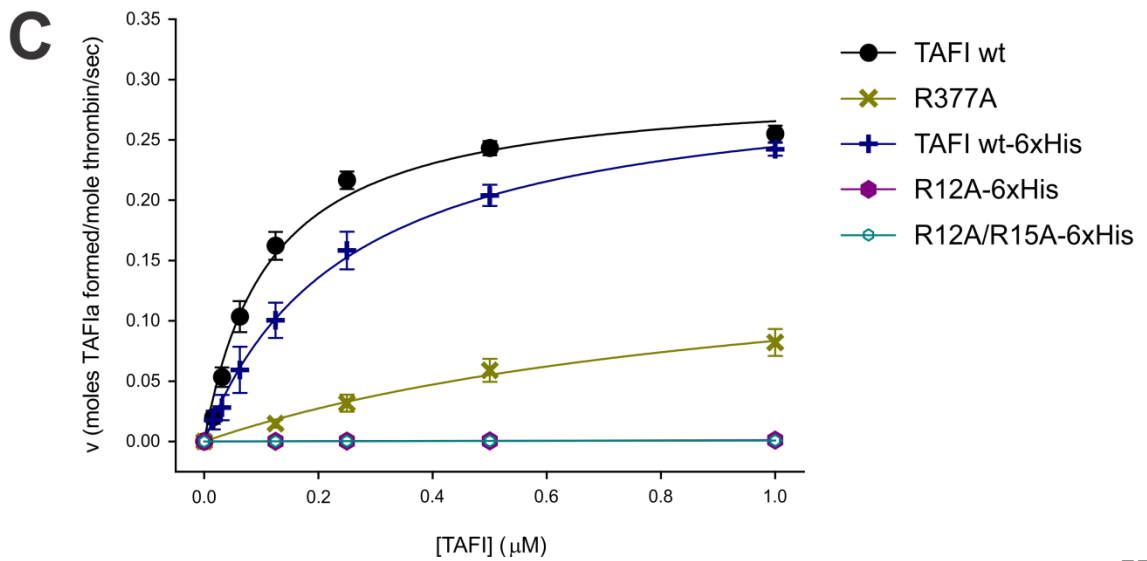
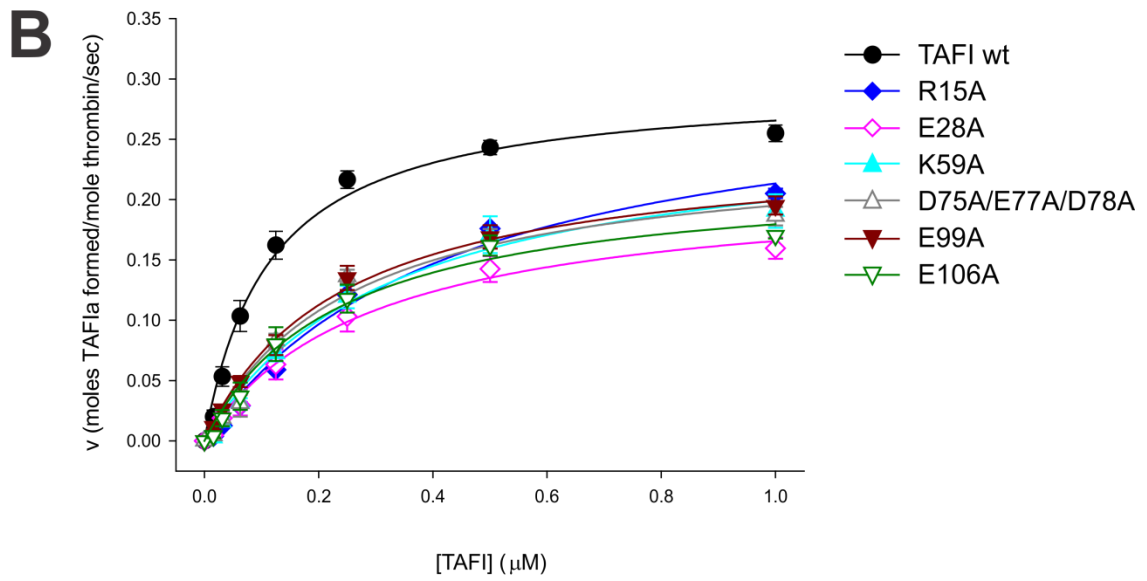
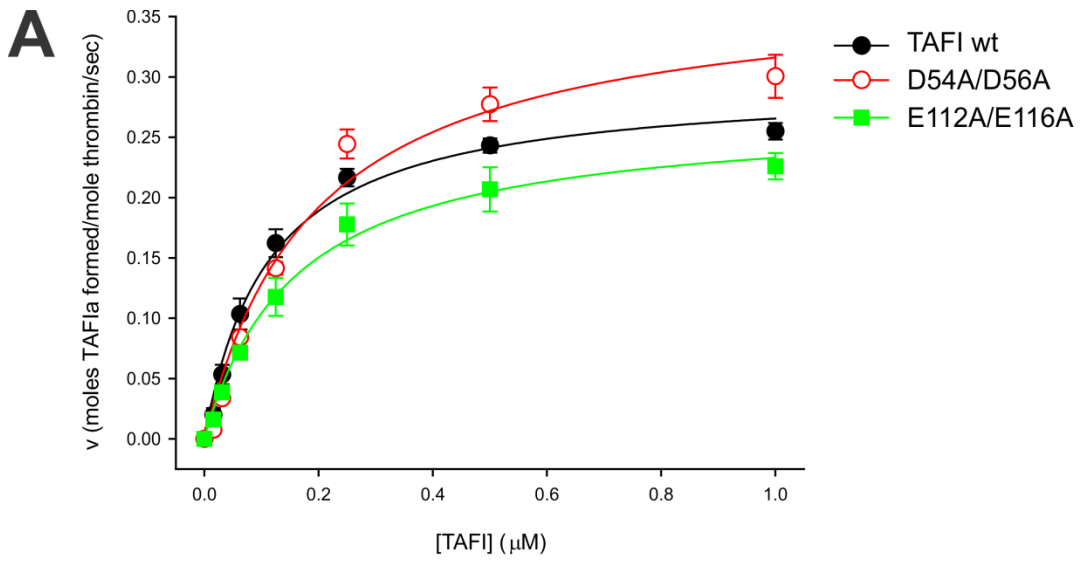


Figure 2-2. The kinetics of activation of TAFI variants by thrombin in the presence of TM. The variants were incubated at different concentrations with 1 nM IIa, 5 nM TM and 5 mM CaCl₂ for 10 min at room temperature. The rate of TAFIa formation was measured using the hydrolysis of the substrate hippuryl arginine (symbols). The data are the means ± standard errors of the mean of 3 – 9 independent experiments. The data were fit to the Michaelis-Menten equation by non-linear regression analysis; the lines are drawn based on the mean k_{cat} and K_M for each variant. A: Variants showing little or no difference in activation compared to wild-type TAFI. B: Variants showing moderate differences in activation. C: Variants showing large differences in activation. The data for wild-type TAFI (TAFI wt) are shown in each panel for comparison purposes.

Table 2-1. Kinetic Parameters for Activation of TAFI variants by thrombin-TM or thrombin alone^a

Variant	thrombin-TM				thrombin		Fold enhancement
	$k_{cat(app)}$ s^{-1}	$K_{M(app)}$ μM	$k_{cat(app)}/K_{M(app)}$ $\mu M^{-1}s^{-1}$	p value ^b	k_{cat}/K_M $(\mu M^{-1}s^{-1})$	p value	
TAFI wt	0.30 ± 0.009	0.11 ± 0.02	2.61 ± 0.47	-	0.00129 ± 0.00017	-	2021
D54A/D56A	0.38 ± 0.025	0.19 ± 0.01	1.95 ± 0.03	0.625	0.00131 ± 0.00019	1.000 ^b	1487
E112A/E116A	0.27 ± 0.016	0.16 ± 0.02	1.69 ± 0.20	0.041	0.00081 ± 0.00007	1.000 ^b	2098
R15A	0.31 ± 0.011	0.43 ± 0.02	0.71 ± 0.02	<0.001	0.00066 ± 0.00004	0.058 ^b	1068
E28A	0.21 ± 0.010	0.29 ± 0.07	0.73 ± 0.18	<0.001	0.00107 ± 0.00010	0.994 ^b	682
K59A	0.26 ± 0.022	0.33 ± 0.03	0.80 ± 0.08	<0.001	0.00070 ± 0.00011	0.029 ^b	1141
D75A/E77A/D78A	0.24 ± 0.012	0.25 ± 0.04	0.96 ± 0.12	0.003	0.00016 ± 0.00002	<0.001 ^b	6114
E99A	0.25 ± 0.010	0.24 ± 0.04	1.03 ± 0.13	<0.001	0.00102 ± 0.00011	0.804 ^b	1012
E106A	0.22 ± 0.012	0.24 ± 0.09	0.94 ± 0.22	0.001	0.00121 ± 0.00010	1.000 ^b	773
R377A	0.17 ± 0.010	1.05 ± 0.23	0.15 ± 0.04	<0.001	0.00076 ± 0.00005	0.052 ^b	214
TAFI wt-6×His	0.31 ± 0.021	0.25 ± 0.07	1.22 ± 0.10	0.019	0.00027 ± 0.00002	<0.001 ^b	4550
R12A-6×His	NA ^d	NA	0.00090 ± 0.0003 ^e	-	0.00031 ± 0.00007	0.886 ^c	3
R12A/R15A-6×His	NA	NA	0.000755 ± 0.0001 ^e	-	0.00039 ± 0.00003	0.454 ^c	2

^aData are the means ± standard errors of the mean of 3-9 independent experiments performed in triplicate

^bANOVA versus TAFI wt

^cANOVA versus TAFI wt-6×His

^dNA: no activation detected under these conditions

^eExperiment performed with higher concentrations of thrombin and TM

2.4.3 Kinetics of activation of TAFI variants by thrombin

We next assessed if changes in the rate of activation of the variants by thrombin-TM may be attributable to changes in their rate of activation by thrombin, by measurement of their kinetics of activation by thrombin in the absence of TM. The rate of activation was calculated following western blot analysis of the samples. The relationships between rate and TAFI concentration were linear, indicating high K_M values (Figure 2-3). Therefore, the slopes of the rate data were used to estimate the catalytic efficiencies (k_{cat}/K_M) of activation (Table 2-1). The catalytic efficiencies of activation of variants E28A, D54A/D56A, E99A, and E106A are all comparable to wild-type. On the other hand, the catalytic efficiencies of activation of variants R15A, K59A, E112A/E116A, R377A and, most dramatically, D75A/E77A/D78A, are decreased compared to wild-type. For all these variants, there is no apparent relationship between the effects of the mutations on activation by either thrombin-TM or thrombin alone. This is illustrated by comparing Figures 2-2 and 2-3 and Table 2-1, each of which are organized by the effects of the mutations on activation by thrombin-TM. Most interestingly, the catalytic efficiencies of R12A and R12A/R15A were similar to wild-type TAFI containing the 6×His tag (Figure 2-3), yet these variants are activated very slowly by thrombin-TM (Figure 2-2). Table 2-1 shows the impact of each mutation on the enhancement by TM of the catalytic efficiency of TAFI activation by thrombin.

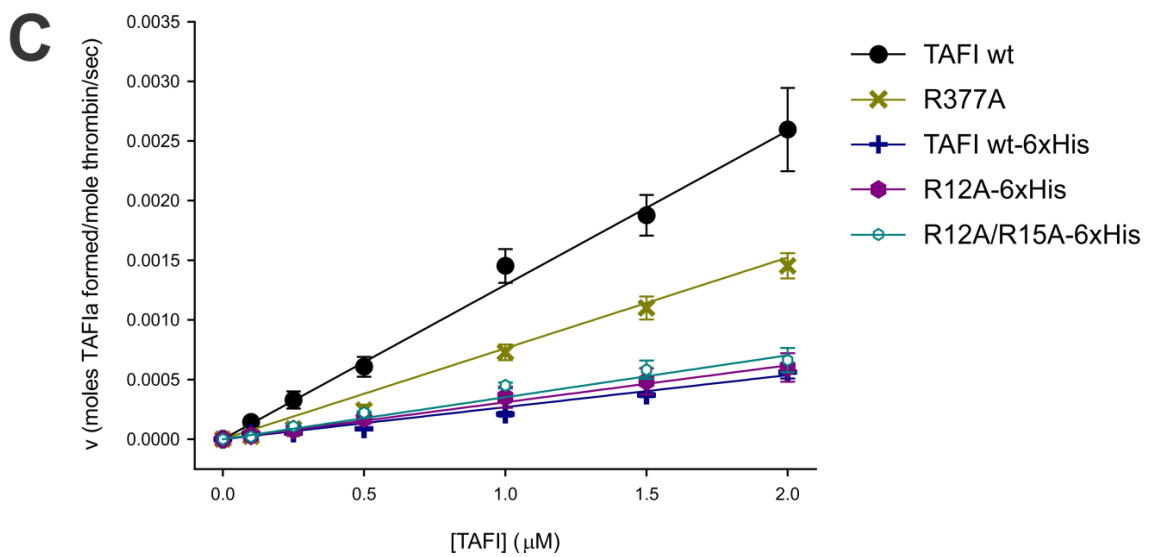
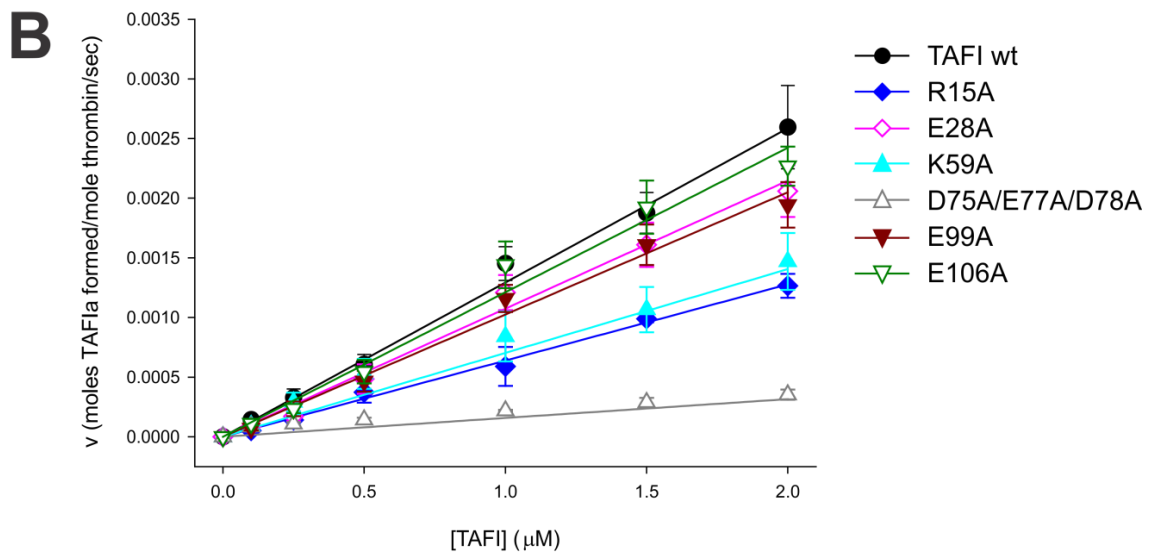
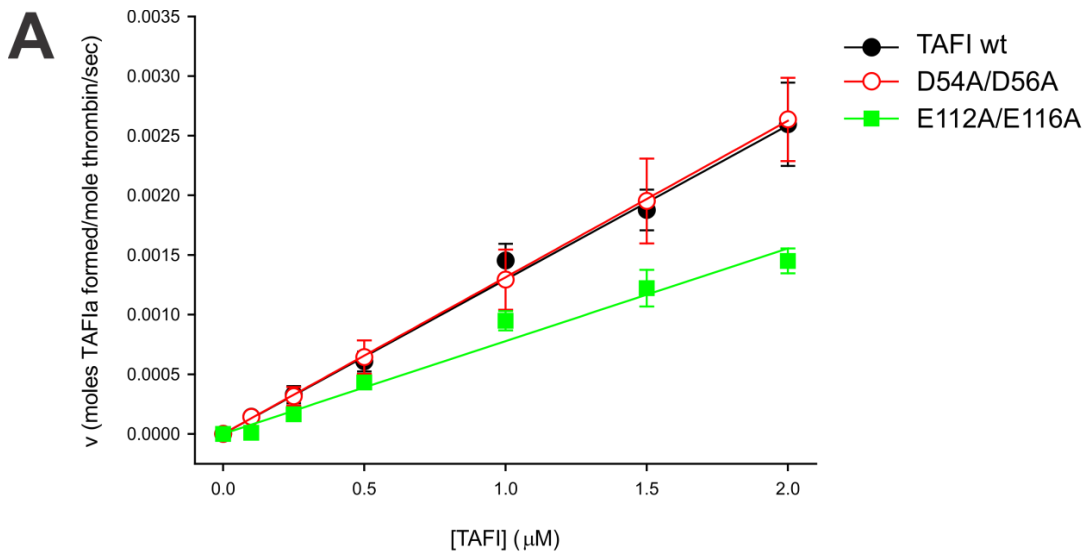


Figure 2-3. The kinetics of activation of TAFI variants by thrombin. The variants were incubated at different concentrations with 100 nM IIa, 20 $\mu\text{g/mL}$ PTCl and 5 mM CaCl_2 for 15 min at room temperature. The rates of TAFIa formation were calculated using western blot analysis (symbols). The data are the means \pm standard errors of the mean of 3 – 9 independent experiments. The lines represent the mean slope for each variant and are taken to be the catalytic efficiency (k_{cat}/K_M). A – C: Variants are organized by their effect on activation by thrombin-TM, as in Figure 2-2. The data for wild-type TAFI (TAFI wt) are shown in each panel for comparison purposes.

2.4.4 *Activation of TAFI variants by plasmin*

The activation of the TAFI variants by plasmin in the absence and presence of heparin was examined. Rates of activation at a single concentration of TAFI were measured by western blot analysis and were normalized to wild-type TAFI minus heparin (Figure 2-4). In general, heparin resulted in a 2 – 3-fold acceleration of plasmin-mediated TAFI activation. Only in the case of R377A was activation by plasmin in the presence of heparin significantly less than wild-type.

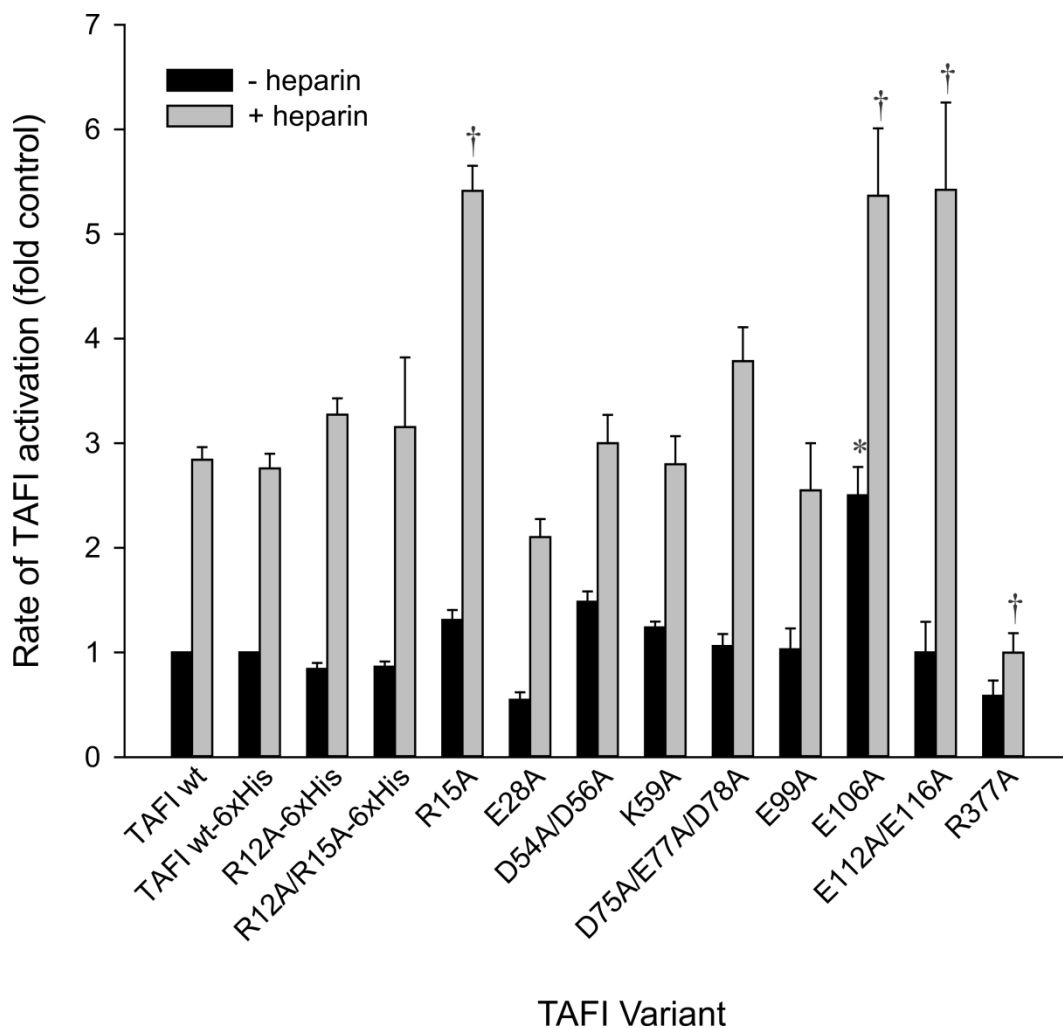


Figure 2-4. Plasmin-mediated activation of TAFI variants. The TAFI variants (200 nM) were incubated with 25 nM plasmin and 150 μ M Plummer's inhibitor in the presence or absence of 0.2 U/ μ L heparin for 15 minutes at room temperature. The extents of TAFI activation were measured using western blot analysis. The extent of TAFIa formation was normalized to wild-type TAFI in the absence of heparin. The data are the means \pm standard errors of the mean of 3 – 13 independent experiments.* and †: $p < 0.05$ versus wild-type in the absence or presence of heparin, respectively, by ANOVA.

2.4.5 *Thermal Stabilities of the TAFI variants*

The thermal stabilities of the TAFI(a) variants were determined by activation of the zymogen by the thrombin-TM complex, followed by incubation at 37°C and measurement of residual TAFIa activity by AAFR hydrolysis. Half-lives of each variant were calculated from the resultant first-order decay rate constants (Table 2-2). The half-lives of wild-type TAFI and wild-type TAFI-6×His were 7.0 ± 0.5 min and 6.9 ± 0.1 min, respectively. Compared to wild-type TAFI, all the variants exhibited comparatively small changes in their half-lives, as expected as all mutations are present either in the activation domain or remote from regions of the catalytic domain that dictate stability [23, 25, 26]. The exceptions are R12A and R12A/R15A, which showed apparently markedly greater half-lives. However, since the activation of these zymogens by thrombin-TM is extremely inefficient, the enzymatic activities as measured using the AAFR substrate are very close to background, likely affecting estimates of half-life. Moreover, we would not expect that mutations in the activation domain would affect the stability of the enzyme after activation.

Table 2-2. Intrinsic Stability of TAFI variants^a

Variant	Half-life (min)
TAFI wt	7.0 ± 0.2
TAFI wt-6×His	6.9 ± 0.1
R12A-6×His	10.2 ± 1.3
R12A/R15A-6×His	18.5 ± 2.8
R15A	6.8 ± 0.1
E28A	6.7 ± 0.3
D54A/D56A	6.6 ± 0.1
K59A	5.1 ± 0.4
D75A/E77A/D78A	5.1 ± 0.1
E99A	5.2 ± 0.2
E106A	7.9 ± 0.3
E112A/E116A	6.4 ± 0.1
R377A	6.4 ± 0.1

^aData are the means ± standard errors of the mean of 3-4 independent experiments performed in triplicate

2.4.6 Antifibrinolytic potential of TAFI variants

The antifibrinolytic potential of each variant using an *in vitro* plasma clot lysis assay was determined at a constant concentration of added TAFI (50 nM), titrating TM from 0.02 to 2 nM (Figure 2-5). As with the previous figures, we have grouped the variants according to the catalytic efficiency of their activation by thrombin-TM (see Figure 2-2, Table 2-1). In the presence of TM, the variants R12A and R12A/R15A showed massive decreases in antifibrinolytic activity, as expected since their activation by thrombin-TM is very slow. The other variant with substantially impaired activation by thrombin-TM, R377A, also showed a much reduced antifibrinolytic effect. For the remaining variants, there was a notable relationship between the catalytic efficiency of thrombin-TM activation and their antifibrinolytic potential in the presence of TM. The two variants activated similarly to wild-type by thrombin-TM (D54A/D56A and E112A/E116A) are very similar to wild-type in their antifibrinolytic activity in response to TM (compare Figures 2-2A and 2-5A), whereas the variants with moderately reduced activation by thrombin-TM are likewise observed to have moderately reduced antifibrinolytic potential (compare Figures 2-2B and 2-5B).

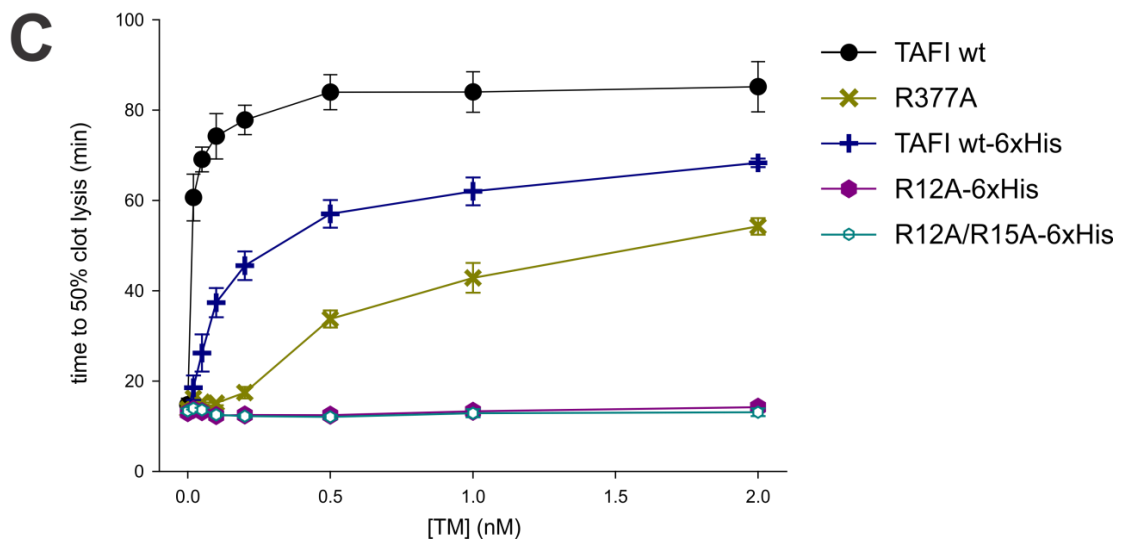
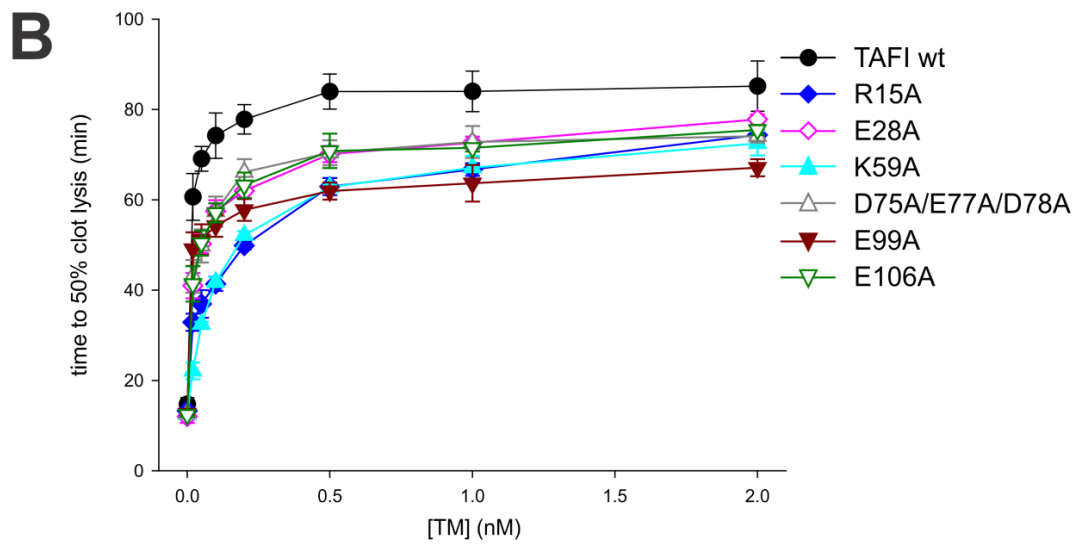
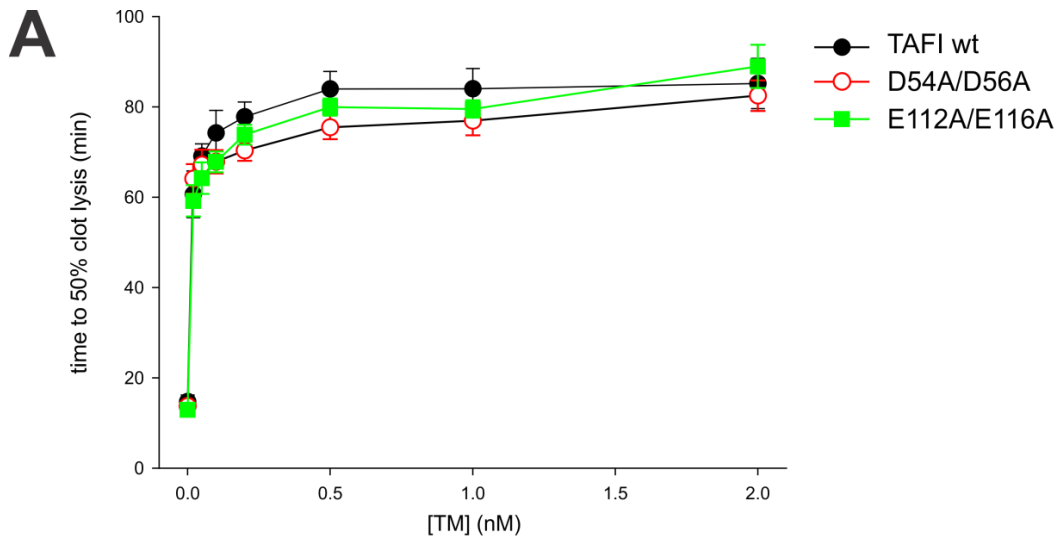


Figure 2-5. The antifibrinolytic potential of TAFI variants in the presence of TM. TAFI-deficient plasma was diluted 1:3 and supplemented with TAFI variants (50 nM) in the presence of 0.02 – 2 nM TM. The assay mixture was added into the wells of microtitre plates containing small, separated aliquots of thrombin, CaCl₂ and tPA to initiate clot formation and subsequent lysis. The change in turbidity of the clots was monitored at 405 nm at 37°C. The time to 50% lysis was determined graphically from the midpoint between maximum and minimum turbidities of the clots. Each data point represents the mean ± standard error of the mean of 3 – 4 independent experiments. A – C: Variants are organized by their effect on activation by thrombin-TM, as in Figure 2-2. The data for wild-type TAFI (TAFI wt) are shown in each panel for comparison purposes.

2.5 Discussion

Thrombin-mediated activation of TAFI is dramatically accelerated by TM: an over 1000-fold increase in catalytic efficiency (k_{cat}/K_M) has been reported [5]. The basis for this increase in catalytic efficiency is unclear as early measurements of the kinetics of TAFI activation by thrombin alone – showing a similar K_M for TAFI activation either in the presence or absence of TM – are likely incorrect [5]. In our hands and those of others [11, 17, 21], measurement of k_{cat} and K_M for thrombin activation is practically impossible as the relationship between TAFI concentration and rate of activation is linear over achievable concentrations of TAFI. It is clear from such plots, however, that TM results in a dramatic decrease in K_M , an effect that implies an enhanced interaction between substrate and cofactor-bound enzyme (compare Figures. 2-2 and 2-3, for example). Indeed, accumulating evidence from several studies suggests that TM and TAFI make direct contacts such that the enhancement of TAFI activation by TM results from binding at sites remote from the activating cleavage site (Arg92-Ala93) [9-11]. By analogy with the structural basis for the ability of TM to accelerate activation of protein C [15], TM and TAFI likely interact through exosite interactions that present TAFI optimally as a substrate for thrombin. However, the elements of TAFI structure that allow accelerated activation of thrombin by TM are incompletely defined. We have identified a novel residue (R377) that plays a key role in mediating enhanced activation of TAFI by TM, and have clarified the role of R12, previously identified by Plug and co-workers [17].

The lysine and arginine residues on the catalytic domain and the triple lysine residues within the activation domain reported by Wu and coworkers showed a reduced

catalytic efficiency of TAFI activation by the thrombin-TM complex [11]. The maximal decrease in catalytic efficiency was for mutation of K42/K43/K44: the observed 8-fold decrease is much less than the total increase attributable to TM. However, mutation of R377 results in a 17.4-fold decrease in catalytic efficiency (Figure 2-2; Table 2-1). Even more dramatically, mutation of R12 decreases the cofactor activity of TM to the extent that activation of TAFI is not even detectable at thrombin concentrations of 1 nM (data not shown). In order to more directly assess the ability of TM to accelerate activation of these mutants, we used 100 nM thrombin in the presence or absence of 100 nM TM. Under these conditions, only a 2- to 3-fold increase in k_{cat}/K_M was observed for R12A or R12A/R15A (Figure 2-2), strongly indicating that the R12A mutants are essentially resistant to the accelerative effects of TM. This is the first report of such a mutant version of TAFI.

R12 in TAFI is in the activation domain, close to K42/K43/K44 (Figure 2-1). Molecular modeling by Wu and coworkers where the TAFI structure was docked to the thrombin-TM EGF4-6 structure strongly indicated that the critical EGF-3 domain in TM would be located in this vicinity [11]. It is tempting to speculate that R12 in TAFI contacts key acidic residues in the EGF-3 domain, such as D341 or D343 located in the c-loop of EGF-3; mutation of these residues abolishes the cofactor activity of TM [9]. A very recent report by Plug and coworkers also identified R12 as a key residue mediating TM-dependent activation of TAFI [17]. Mutation of R12 to glutamine resulted in a 54-fold decrease in catalytic efficiency. In this work, R12 was also identified as a thrombin cleavage site [17]; Plug and coworkers were thus left to speculate that either R12 is involved in exosite interactions with TM that accelerate TAFI activation or prior cleavage

at R12 accelerated thrombin-TM-mediated TAFI activation. Taking their data and our data together, it is clear that the former explanation is most likely true. First, the kinetics of cleavage at R12 are too slow to account for the ability of TM to accelerate TAFI activation (see Figure 2 of [17]). Second, substitution of R12 for glutamine [17] or alanine (current study) results in dramatically different activation kinetics by thrombin-TM. Under conditions where activation of R12Q is detectable (1 nM thrombin, 1 – 8 nM TM), we see no activation of R12A (Figure 2-2). If a lack of cleavage at R12 is the key factor, we would not expect such a difference. It is possible that the glutamine residue at position 12 is still capable of making some productive contacts with EGF-3 of TM.

As with purification of K42A/K43A/K44A with monoclonal antibody MA-T4E3 [11], variants R12A and R12A/R15A also could not be isolated using monoclonal antibody MA-T4E3. This can be rationalized by examining the crystal structure of TAFI [23], since residue R12 is located on a loop directly below and in close proximity to the triple lysine residues which were not recognized by the antibody. Interestingly, another monoclonal antibody (MA-T12D11) has been previously shown to prevent TAFI activation by the thrombin-TM complex but not thrombin alone [27]. The key residue for binding this antibody is G66, which is located on a loop in the immediate vicinity of R12 [23], again indicating that this region on the TAFI structure may be important for TM binding. It is notable that the presence of the carboxyl-terminal histidine tag decreased the activatability and antifibrinolytic activity of TAFI, as can be observed by comparison of TAFI_{wt} and TAFI_{wt}-6×His. This finding is in agreement with previous results showing that a nanobody directed against residues in this region inhibits thrombin-TM-mediated activation of TAFI [28]. However, since we compared the effects of R12A mutations to

TAFIwt-6×His, it is clear that the mere presence of the histidine tag does not explain resistance of the R12A mutant to acceleration of thrombin activation by TM.

Mutation of additional surface-exposed charged residues had either little or no effect on thrombin-TM-mediated activation of TAFI (D54A/D56A and E112A/E116A) or decreased activation to a moderate extent (R15A, E28A, K59A, D75A/E77A/D78A, E99A, E106A) (Table 2-1). It is notable that several of the latter category of mutations are of negatively-charged residues, even though many of the residues on TM shown to be important for TAFI activation are themselves negatively-charged [9]. We speculate that these additional residues which we have identified map to a surface on TAFI that is important for interaction with the thrombin-TM complex. Moreover, as R15A, E28A, E99A and E106A show no significant difference in catalytic efficiency of activation by thrombin alone (Table 2-1), these residues may specifically be involved in interaction with TM.

The second mutation with dramatic effects on TAFI activation was R377A. This residue is located in a completely different region of TAFI (Figure 2-1) where TM is not anticipated to bind [11]. The R377A mutation decreased the catalytic efficiency of activation by thrombin alone by less than two-fold but decreased that of thrombin-TM by approximately 17-fold (Table 2-1). Upon examination of the model prepared by Wu and coworkers, it is apparent that R377 is located approximately where the 149 loop of thrombin contacts TAFI [11]. The 149 loop is one of two surface loops, the other being the 60D loop, that reside on either side of the active site groove [29]. While the 60D loop shows some structural alterations when bound by TM, the 149 loop is disordered in the presence of TM (as it is in many other thrombin structures) while being in close

proximity to TM EGF-5 [15]. It is possible that the 149 loop is positioned in such a way by TM that brings it into productive contact with the surface helix in TAFI containing R377.

In conclusion, we have identified two residues in TAFI, R12 and R377, that are crucial for the ability of TM to accelerate TAFI activation by thrombin. R12 likely directly contacts EGF-3 of thrombomodulin while R377 may be important for the TAFI-thrombin interaction in the context of TM. The R12 mutation may be a useful tool to probe the importance of TM in activation of TAFI both in *in vitro* and *in vivo* experiments.

2.6 Acknowledgements

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2.7 Addendum

T.M. designed the research, performed the research, analyzed the data, and wrote the manuscript. M.B.B. designed the research, analyzed the data, and wrote the manuscript.

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2.9 Supporting information

Supplementary Table 2-1. Primer sequences for construction of TAFI Variants^a

Variant	Primer Sequence
TAFI-R12A	5'-AGTTCTAGCTGCTCTTCCT <u>GCA</u> ACCTCTAGGCAAGTTCAA-3'
TAFI-R15A	5'-TAGCTGCTCTTCCTAGAACCTCT <u>GCG</u> CAAGTTCAAGTTCT-3'
TAFI-R12A/R15A	5'-GGCCAAGTTCTAGCTGCTCTTCCT <u>GCA</u> ACCTCT <u>GCG</u> CAAGTTCAAGTTCTAC-3'
TAFI-E28A	5'-CTTACTACAACATAT <u>GCG</u> ATTGTTCTCTGGC-3'
TAFI-D54A/D56A	5'-CATTTTTTTGTAAATGCATCT <u>GCT</u> GTC <u>GCA</u> ATGTGAAAGCCC-3'
TAFI-K59A	5'-AAATGCATCTGATGTCGACAATGTG <u>GCA</u> GCCCATTTAAATGTGAGC-3'
TAFI-D75A/E77A/D78A	5'-GTGTCTTGCTGGCAG <u>CCG</u> TGG <u>CAGCT</u> CTTATTCAACAGCAG-3'
TAFI-E99A	5'-CTCCGCATCGTACTAT <u>GCA</u> CAGTATCCTACTCACTAAATG-3'
TAFI-E106A	5'-GAACAGTATCACTCACTAAAT <u>GCA</u> ATCTATTCTTGGATAGAATTTATAAC-3'
TAFI-E112A/E116A	5'-CTATTCTTGGATAG <u>CA</u> TTTATAACT <u>GCG</u> AGGCATCCTGATATGCTTA-3'
TAFI-E116G	5'-CTTGGATAGAATTTATAACT <u>GGC</u> AGGCATCCTGATATGCTTACAAAAATCCAC-3'
TAFI-R377A	5'-CATACGGATTCTTGCTGCCGGAG <u>GCC</u> TACATCAAACCCACC-3'

^aSense strand sequences only are shown. Ala/Gly codons are double underlined; mutated nucleotides are in boldface type.

Chapter 3

**Role of residues 1 through 12 of the activation domain of TAFI
in activation of TAFI by thrombin-thrombomodulin**

3.1 Summary

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that functions as a link between the coagulation and fibrinolysis pathways. TAFI is activated through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin. Several studies suggest that the activation domain of TAFI plays an important role in its activation by the thrombin-TM complex. One report has suggested that cleavage of TAFI after arginine-12 by thrombin accelerates activation of TAFI by thrombin-TM. *Objectives:* To determine the effect of amino-terminal truncation of the activation domain on activation by thrombin in the presence of TM. *Methods:* We generated several amino-terminal deletions of the activation domain of TAFI including TAFI $_{\Delta 1-6}$, TAFI $_{\Delta 1-6, R12A}$, TAFI $_{\Delta 1-11}$, and TAFI $_{\Delta 1-12}$ in order to identify the minimum length of the activation domain that is required to mediate acceleration of activation by TM. The activation of the mutants by thrombin in the presence or absence of TM was determined. *Results:* The variants were not detectably secreted from mammalian cells but were all found to be expressed intracellularly. Activation of the intracellular forms of the mutants could not be reliably detected. *Conclusions:* The results from this study are inconclusive and further studies need to be performed to determine the functionality of the amino-terminally truncated TAFI activation domain variants.

3.2 Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that functions as an important regulator of the fibrinolytic system upon activation [1, 2]. The mature TAFI protein is a 60 kDa procarboxypeptidase that consists of a 92-amino acid activation domain (Phe1–Arg92) which is *N*-glycosylated at four sites (Asn22, Asn51, Asn63, Asn86) and a 309-amino acid catalytic domain (Ala93–Val401) [3, 4]. TAFI is activated through proteolytic cleavage at Arg92–Ala93 to generate the enzyme activated TAFI (TAFIa) [5-7]. TAFIa possesses basic carboxypeptidase activity that efficiently cleaves the carboxyl-terminal lysine and arginine residues from partially-degraded fibrin, thus yielding a form of fibrin that possesses almost no cofactor activity [8]. Thus, the TAFI pathway attenuates fibrinolysis by interfering with positive feedback in the fibrinolytic cascade that is essential for plasminogen activation [9, 10]. Thrombin and plasmin alone are relatively weak activators of TAFI however, when thrombin binds to the endothelial cell cofactor TM, the catalytic efficiency (k_{cat}/K_M) of TAFI activation is accelerated by over 1000-fold compared to thrombin alone [6, 7].

Several studies have revealed clues as to the structural basis for the acceleration of TAFI activation by thrombin in the presence of TM. A study by Wu and coworkers showed that substitution of the consecutive lysine residues in the activation domain of TAFI, Lys42/Lys43/Lys44, with alanine exhibited an 8-fold reduced catalytic efficiency of TAFI activation by the thrombin-TM complex [11]. In addition, Plug and coworkers identified Arg12 in the activation domain as an important residue in TM-mediated activation of TAFI. When Arg12 was mutated to a glutamine a 54-fold decrease in

catalytic efficiency was observed [12]. However, in a recent study by our group we have found that mutation of Arg12 with alanine resulted in a mutant form of TAFI that is essentially resistant to the accelerative effects of TM [13]. A deletion mutant of TAFI constructed by Zhou and coworkers where the first 73 amino acids of the activation domain are absent failed to exhibit full cleavage by thrombin-TM even following extended incubation periods [14]. Notably, all these studies have emphasized the important role of the activation domain of TAFI in its activation by the thrombin-TM complex. Therefore, we hypothesize that the activation domain of TAFI makes contacts with TM, therefore constituting an exosite which affects the rate of cleavage. Moreover, Arg12 is a key residue in this exosite.

Interestingly, Plug and coworkers found that Arg12 is a novel cleavage site recognized by thrombin and plasmin [12]. That the Gln12 variant was more slowly activated by thrombin-TM prompted them to speculate that cleavage after Arg12 results in a form of TAFI that is more efficiently activated by thrombin-TM. On the other hand, their work also showed that the kinetics of cleavage at Arg12 are too slow to account for the effect of TM on thrombin cleavage at Arg92 [12]. In order to address this question in a more definitive way, we have constructed and expressed several amino-terminal deletions of the activation domain of TAFI including TAFI $_{\Delta 1-6}$, TAFI $_{\Delta 1-6, R12A}$, TAFI $_{\Delta 1-11}$, and TAFI $_{\Delta 1-12}$. The activation of the mutants by thrombin in the presence or absence of TM was determined.

3.3 Experimental Procedures

3.3.1 Materials

DNA restriction and modification enzymes (T4 DNA Polymerase and T4 DNA Ligase) were purchased from New England Biolabs (Mississauga, ON, Canada). pcDNATM4A/myc-His mammalian expression vector was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Dulbecco's modified Eagle's medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, penicillin/streptomycin/fungizone (PSF) were obtained from Invitrogen. Newborn Calf Serum was obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada). MegaTran 1.0 transfection reagent was purchased from Origene (Atlanta, GA, USA). RNeasy Plus Midi Kit was purchased from QIAGEN, Inc. (Toronto, ON, Canada). RQ1 (RNA Qualified) RNase-Free DNase was purchased from Promega (Madison, WI, USA). Diethyl pyrocarbonate (DEPC) was obtained from Sigma-Aldrich. iTaq Universal SYBR Green One-Step Kit was purchased from BioRad (Mississauga, ON, Canada). Thrombin and rabbit-lung thrombomodulin was obtained from Haematologic Technologies (Essex Junction, VT, USA). The thrombin inhibitor D-phenylalanylprolylarginyl chloromethylketone (PPack) was purchased from Calbiochem (San Diego, CA, USA). Polyclonal sheep anti-human TAFI antibody was purchased from Affinity Biologicals (Ancaster, ON, Canada) and polyclonal rabbit anti-sheep antibody was purchased from ThermoFisher Scientific (Ottawa, ON, Canada).

3.3.2 Construction of TAFI Activation Domain Deletion Mutants

The activation domain deletion mutants TAFI $_{\Delta 1-6}$, TAFI $_{\Delta 1-6, R12A}$, TAFI $_{\Delta 1-11}$, and TAFI $_{\Delta 1-12}$ were generated in the wild-type TAFI- pcDNA4A/myc-His vector. cDNA sequences encoding the activation domain deletion mutants were obtained from GenScript custom gene synthesis services in the pUC57 vector (Piscataway Township, NJ, USA) such that sequences encoding the native TAFI secretion peptide were fused with sequences encoding the amino-terminally truncated activation domain.

These fragments were then transferred into the wild-type TAFI- pcDNA4A/myc-His expression vector, which was previously described and contains human TAFI cDNA containing threonine at positions 147 and 325 and encodes a carboxyl-terminal 6 \times -His tag [4, 13]. The wild-type TAFI- pcDNA4A/myc-His vector contains two *Bgl*III restriction sites, one in the vector backbone and one within the TAFI coding sequence. To allow utilization of *Bgl*III in the cloning of the deletion mutants into the wild-type TAFI- pcDNA4A/myc-His vector the *Bgl*III site in the vector backbone was removed. This was done by limiting digestion of the vector through titration of the *Bgl*III enzyme. The digestion products were resolved on an agarose gel, and the singly-digested vector fragment was excised from the gel. The *Bgl*III ends were made blunt using the T4 DNA Polymerase and ligated, since *Bgl*III (recognition sequence AGATCT) leaves a 5'-overhang, the ligation products (AGATCGATCT) would not be able to be digested by this enzyme. To determine which of the *Bgl*III sites was eliminated by this maneuver, clones were digested with *Pst*I and *Bgl*III and those yielding ~5.95 kb and 300 bp bands (reflecting cleavage of the *Bgl*III site in the TAFI cDNA) were selected. The resulting wild-type TAFI- pcDNA4A/myc-His $_{\Delta BglIII}$ vector was digested with *Pst*I and *Bgl*III, and

the respective truncation mutant fragments that had been excised from pUC57 were inserted. The presence of the truncation mutations was verified by DNA sequence analysis.

The activation domain deletion mutants TAFI Δ 1-6, TAFI Δ 1-6, R12A, TAFI Δ 1-11, and TAFI Δ 1-12 were also generated in the context of the TAFI-CIIYQ variant. Each truncation mutant expression vector was digested with *Bgl*III and *Age*I and the wild-type fragment was substituted with the corresponding fragment encoding the CIIYQ mutation as well as the carboxyl-terminal 6 \times -His tag, which had been excised from the TAFI-CIIYQ-pcDNA4A/myc-His expression vector digested with these enzymes.

3.3.3 *Transient Transfection of TAFI Activation Domain Deletion Mutants*

Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (1:1) (DMEM/F-12) containing 5% (v/v) newborn calf serum (NCS) and 1% (v/v) PSF in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere. Expression plasmids encoding the respective TAFI mutants were transfected into cells using MegaTran 1.0 transfection reagent (10 μ g plasmid DNA in a 100 mm plate of cells, 0.75 μ g plasmid DNA in a 6-well plate of cells) with a 3:1 ratio of reagent to DNA as per the manufacturer's instructions. After 24 hour incubation with the transfection mixture, the media was removed and the transiently expressing lines were cultured in Opti-MEM containing 1% (v/v) PSF. Conditioned medium was collected at 72 hour post-transfection and the cells in the 100 mm plate were then lysed with 1 mL lysis buffer (50 mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% SDS). Harvested conditioned medium was centrifuged at 3000 \times g for 5

minutes, supplemented with Tris-HCl, pH 8.0 (to 5 mM) and phenylmethanesulphonyl fluoride (PMSF) to (2 μ M) and stored at -20°C.

3.3.4 Activation of TAFI Mutants and Western blot analysis

For activation of TAFI, 75 μ L of conditioned medium or lysate corresponding to each TAFI variant was incubated in the presence of CaCl₂ (5 mM) and thrombin (25 nM) in the presence or absence of rabbit-lung thrombomodulin (100 nM) in HBST for a final volume of 100 μ L for 10 minutes at 21°C. Thrombin activity was then quenched by the addition of PPAck (to 1 μ M) before the mixture was placed on ice. SDS-PAGE sample buffer (0.2 M Tris-HCl, 8% SDS, 40% Glycerol, 0.08% bromophenol blue) was added to the samples. Samples were analyzed by SDS-PAGE and western blotting analysis.

Following SDS-PAGE on 12% polyacrylamide gels, proteins were transferred onto a an Immobilon-P transfer membrane (Millipore) and blocked in blocking buffer consisting of 6% (w/v) non-fat milk in 1 \times NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, and 0.05% Triton X-100) for at least 1 hour at room temperature. The blots were then incubated overnight at 4°C with the sheep anti-human TAFI polyclonal antibody (Affinity Biologicals) (1:5,000 dilution) in blocking buffer containing 3% (v/w) non-fat milk. The blots were washed with 1 \times NET (three times for 10 minutes each) then incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG secondary antibody (Pierce Biochemical) (1:15,000 dilution) in blocking buffer containing 3% (v/w) non-fat milk at room temperature for 1 hour. The membranes were then washed with 1 \times NET as described above. The immunoreactive bands were visualized using SuperSignal

West Femto Maximum Sensitivity Substrate (Pierce Biochemical) and a FluorChem Q Gel Imaging System (Alpha Innotech).

3.3.5 RNA Extraction and DNase Treatment of RNA

Total RNA was extracted from transiently transfected cells (grown in 6-well plates) using the RNeasy Plus Midi Kit according to the manufacturer's protocol. Subsequently, RNA was DNase treated using RQ1 RNase-free DNase for 2 hours at 37°C. Following DNase treatment, the RNA samples were phenol/chloroform extracted, precipitated, and re-suspended in DEPC-treated H₂O. RNA concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

3.3.6 Real-time quantitative RT-PCR

Determination of mRNA expression corresponding to the recombinant variants was assessed through iTaq™ Universal SYBR® Green One-Step kit (Bio-Rad) on a BioRad CFX96 Real-Time thermal cycler system. The following primers were used: TAFI forward 5' -GCT GCC GGA GCG TTA CAT- 3'; TAFI reverse 5' -CAT TCC TAA TGA CAT GCC AAG CT- 3'; GAPDH forward 5' -GGA GCC AAA AGG GTC ATC ATC- 3'; GAPDH reverse 5' -GTT CAC ACC CAT GAC GAA CAT G- 3'. Thermocycling conditions used for all samples were as follows: reverse transcription at 50°C for 15 minutes, activation of *Taq* polymerase at 95°C for 7 minutes, 42 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 40 seconds. Relative quantification of mRNA encoding the variants was performed by normalization of the

threshold cycle for each sample with that of GAPDH from the same reaction well using Bio-Rad CFX Manager software version 3.1 (Bio-Rad).

3.4 Results

3.4.1 *Expression of N-terminal deletion TAFI variants*

Amino-terminal truncation mutants of TAFI (Figure 3-1) were expressed both in the context of the wild-type TAFI amino acid sequence as well as the previously reported stable TAFI variant, TAFI-S305C-T325I-T329I-H333Y-H335Q (TAFI-CIIYQ). This variant contains mutations in the dynamic flap region that stabilize the region and increase the half-life of TAFIa approximately 180-fold [15]. The expression and activation of the truncated activation domain TAFI protein variants was examined using western blotting analysis. Expression vectors encoding the amino-terminal activation domain deletion variants as well as the full-length wild-type TAFI and TAFI-CIIYQ forms and the control empty pcDNA4a vector were transiently transfected into BHK cells. The conditioned medium was collected from each variant and incubated with thrombin alone and thrombin-TM (Figure 3-2A). Immunoreactive bands were observed in the conditioned medium of cells expressing the full-length variants. The full-length TAFI variants migrated at approximately 65 kDa and were activated by thrombin-TM, indicated by the 35 kDa band, as expected. Of particular note, the sizes of the zymogen bands are slightly larger than expected, likely due to the presence of the 6× His tag on the carboxyl-terminus. Activation of wild-type TAFI by thrombin alone was not detectable since a low concentration of thrombin was used (25 nM) and the activation period was short (10 minutes). In contrast, none of the truncation mutants, either in the context of wild-type TAFI or TAFI-CIIYQ, were detectable in the conditioned media.

The lysates from the transient transfections were also collected and incubated with thrombin alone and thrombin-TM (Figure 3-2B). Immunoreactive bands were observed on western blot analysis of the lysates at approximately 45-48 kDa for all variants tested (Figure 3-2C), although those for TAFI $_{\Delta 1-6, R12A}$ and all the truncation mutants in the context of TAFI-CIIYQ were considerably less abundant. Immunoreactive bands of 65 kDa were observed for full-length wild-type TAFI and TAFI-CIIYQ. The wild-type TAFI and TAFI-CIIYQ present in lysate was activated by thrombin-TM shown by the appearance of a faint 35 kDa band and the diminution or disappearance of the 65 kDa and 45-48 kDa bands (Figure 3-2B). Interestingly, the 45-48 kDa form of TAFI-CIIYQ appeared to be activated more efficiently by thrombin-TM than the 45-48 kDa form of wild-type TAFI (Figure 3-2B). As the abundance of the truncation variants in the lysates is low, activation of these variants could not be reliably ascertained from the western blot analysis (Figure 3-2B).

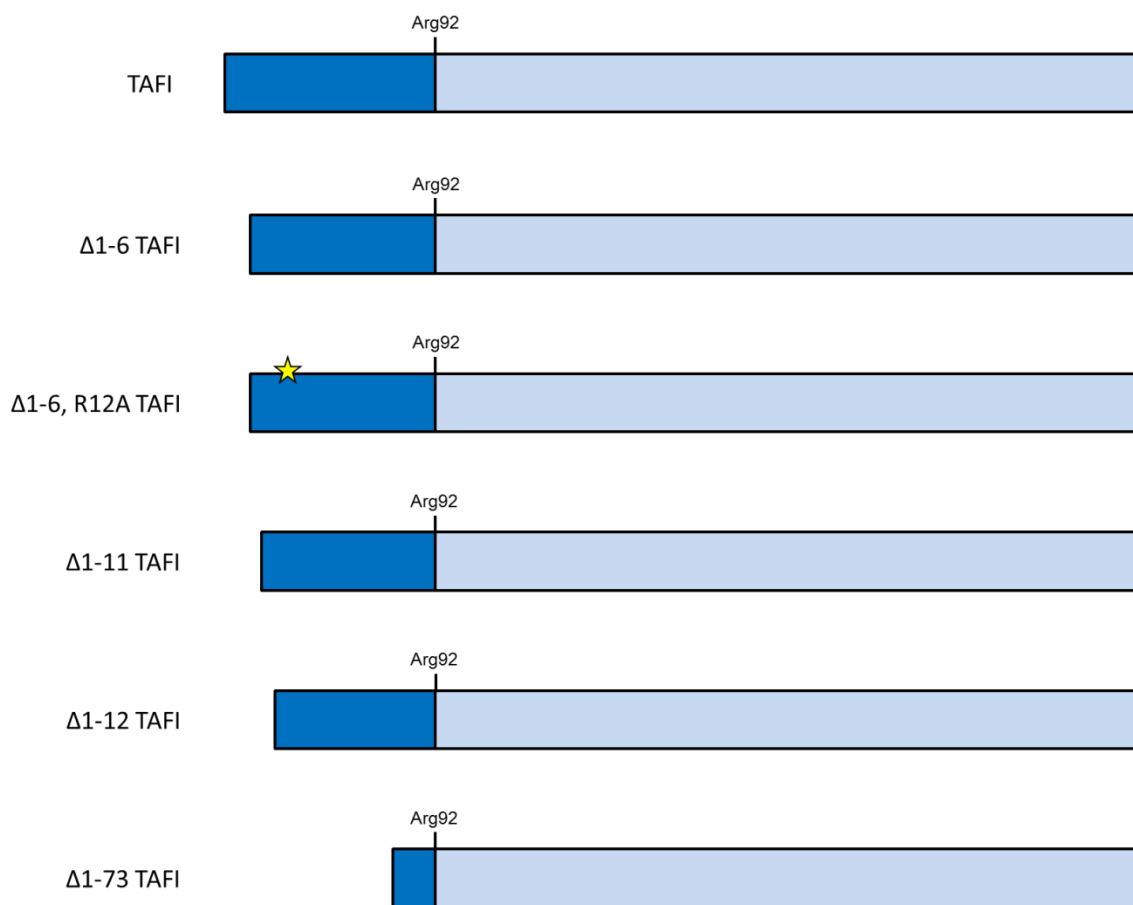
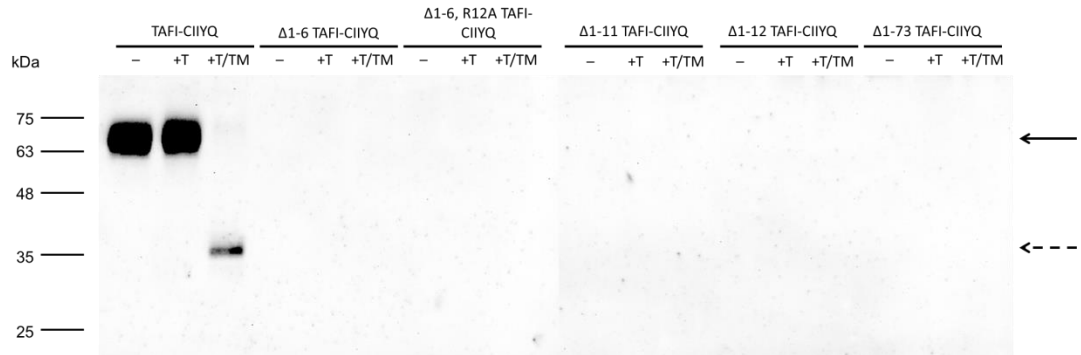
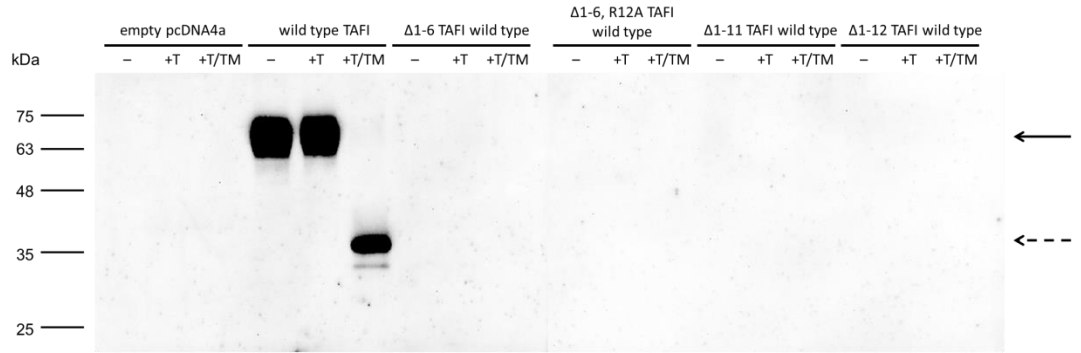
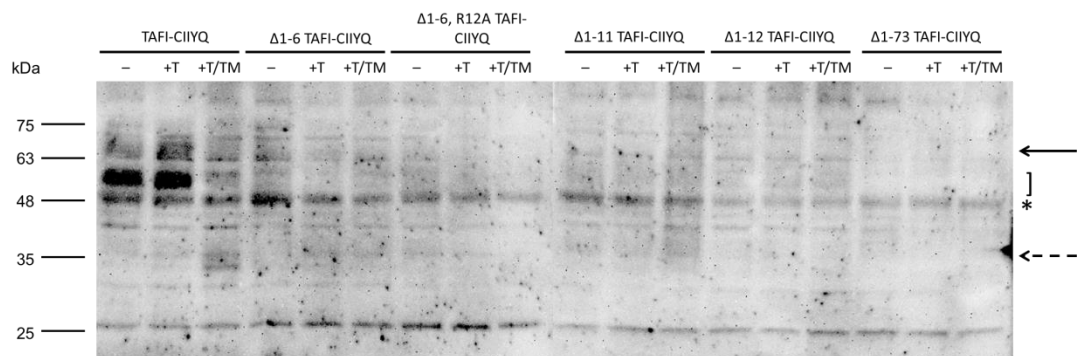
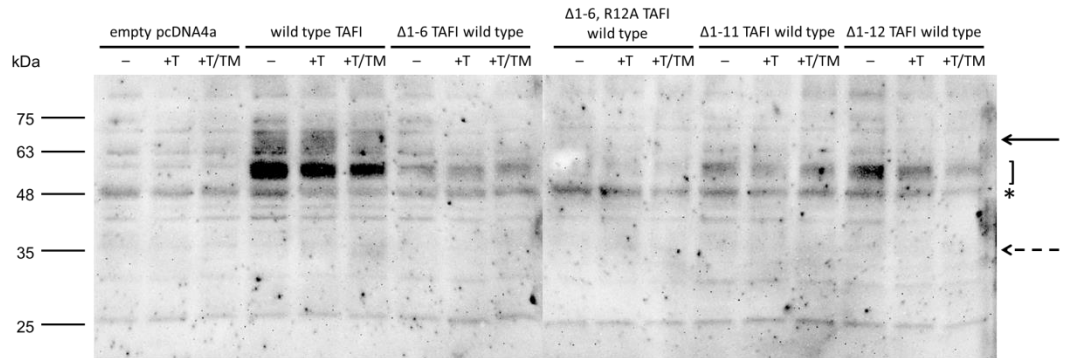


Figure 3-1. TAFI amino-terminal deletion mutants. The full length TAFI protein consists of a 92-amino acid activation domain (dark blue) and a 309-amino acid catalytic domain (light blue). The TAFI amino-terminal deletion mutants constructed are: TAFI_{Δ1-6}, TAFI_{Δ1-6, R12A}, TAFI_{Δ1-11}, and TAFI_{Δ1-12} which were made in the context of wild-type and CIYQ. The variant TAFI_{Δ1-73} was only made in the context of CIYQ. The location of residue R12 is indicated by the yellow star.

A**B**

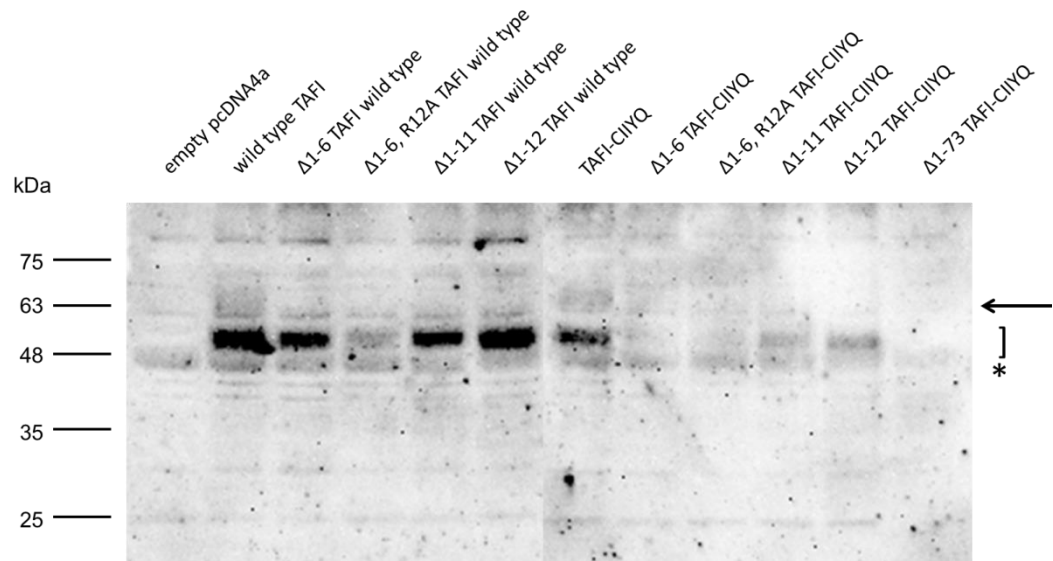
C

Figure 3-2. Activation of TAFI amino-terminal deletion mutants. BHK cells were transiently transfected with the expression plasmids empty pcDNA4a, wild-type TAFI, and TAFI-CIIYQ and the following variants in the context of wild-type and CIIYQ: TAFI $_{\Delta 1-6}$, TAFI $_{\Delta 1-6, R12A}$, TAFI $_{\Delta 1-11}$, and TAFI $_{\Delta 1-12}$ and the variant TAFI-CIIYQ $_{\Delta 1-73}$. A) Conditioned media and B) lysate samples were collected from the mutants. The samples were activated by thrombin alone (25 nM) and thrombin-TM (25 nM, 100 nM, respectively) as indicated and subjected to western blot analysis. C) Non-activated lysate samples for all variants. The position of TAFI (solid) and TAFIa (dashed) are indicated by arrow heads, non-specific bands are indicated by asterisks, and intracellular forms of TAFI are indicated by square brackets.

The expression of the truncated activation domain TAFI variants was also examined through measurement of their mRNA and was assessed using qRT-PCR (Figure 3-3). The results show that all the TAFI variants were expressed in BHK cells. TAFI-CIIYQ showed the highest mRNA expression of CPB2 being approximately 2- to 70- fold higher compared to the rest of the variants. The next highest expression is from TAFI Δ 1-12 CIIYQ followed by wild-type TAFI each with comparable expression being approximately 1-fold less than TAFI-CIIYQ. The variants TAFI Δ 1-6 wild type, TAFI Δ 1-11 wild type, and TAFI Δ 1-11 CIIYQ all have similar magnitude of expression with 2-fold less expression compared to wild-type TAFI while these variants TAFI Δ 1-6 CIIYQ, TAFI Δ 1-6, R12A CIIYQ, and TAFI Δ 1-12 wild-type are 3-fold less. The lowest expressed variants are TAFI Δ 1-6, R12A wild-type possessing 7-fold less expression and variant TAFI Δ 1-73 CIIYQ with 34-fold less expression compared to wild-type TAFI.

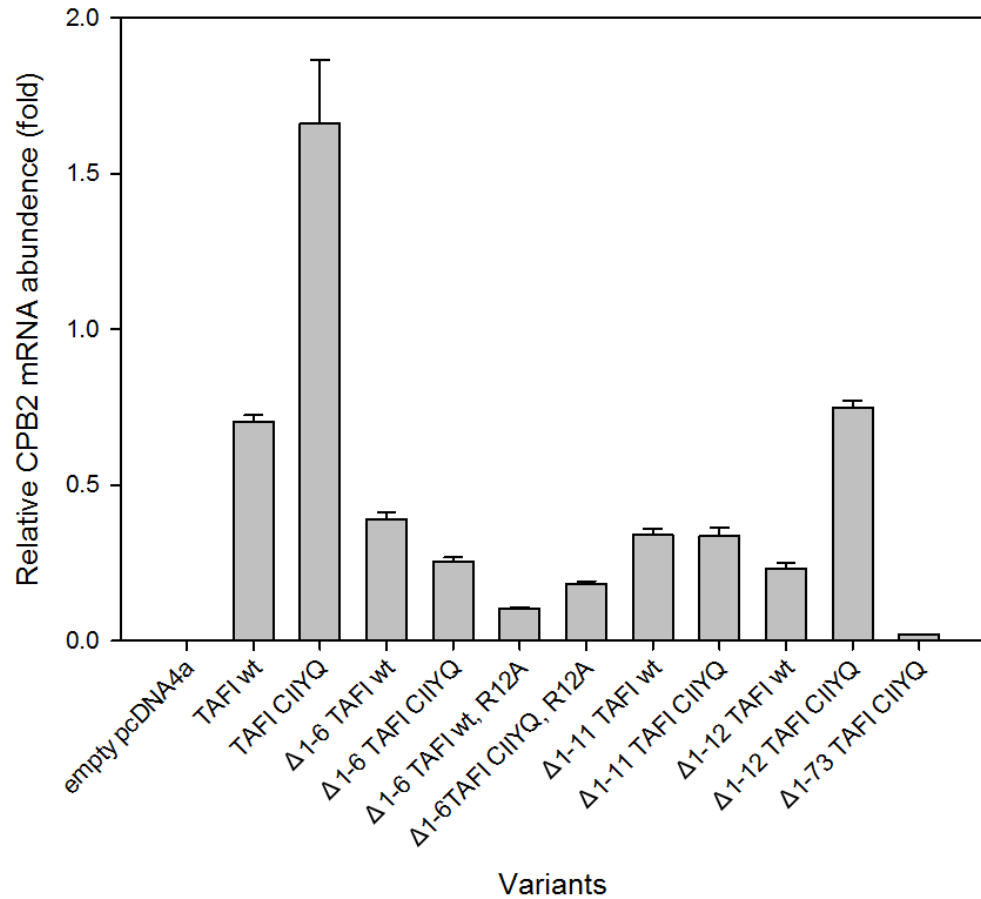


Figure 3-3. Quantification of amino-terminal deletion mutants expression. BHK cells were transiently transfected with the expression plasmids empty pcDNA4a, wild-type TAFI, and TAFI-CIIYQ and the following variants in the context of wild-type and CIIYQ: TAFI $_{\Delta 1-6}$, TAFI $_{\Delta 1-6, R12A}$, TAFI $_{\Delta 1-11}$, and TAFI $_{\Delta 1-12}$ and the variant TAFI-CIIYQ $_{\Delta 1-73}$. The expression of the respective mRNAs was analyzed by quantitative real-time RT-PCR. The data shown are the mean of one experiment performed in triplicate. The error bars represent the standard errors of the mean.

3.5 Discussion

The truncation mutants of the activation domain of TAFI were constructed to address the possibility that cleavage of TAFI by thrombin or plasmin at Arg12 might accelerate activation of TAFI by thrombin-TM, as recently suggested by Plug and coworkers [16]. Evidence from several studies suggests the importance of the activation domain of TAFI in its activation by the thrombin-TM complex. Plug and coworkers demonstrated that mutation of Arg12 to Gln residue resulted in a 54-fold decrease in catalytic efficiency (k_{cat}/K_M) of TAFI activation by thrombin-TM [12] while a recent study from our group showed that mutation of Arg12 to an Ala resulted in almost complete resistance of acceleration of TAFI activation by thrombin in the presence of TM. Furthermore, mutations within the activation domain caused a decrease in the catalytic efficiency of activation in the presence of TM [13]. Adding to the evidence is the study by Zhou and coworkers showing that a mutant version of TAFI-CIIYQ where the first 73 residues of the activation domain are absent was unable to be cleaved by thrombin-TM despite the extended activation periods [14]. Molecular modeling of the interaction of TAFI with thrombin-TM suggests that the key structure in TM for promoting TAFI activation – epidermal growth factor-like domain 3 – is likely to contact TAFI in the activation domain in the vicinity of Arg12. Clearly, Arg12 is an important residue in the activation of TAFI by thrombin-TM, but the question remains to be addressed if removal of residues 1 through 12 influences TAFI activation by thrombin-TM.

In the current study we have attempted to address this question by constructing mutant variants of TAFI with N-terminal deletions of the activation domain of TAFI including TAFI Δ 1-6, TAFI Δ 1-6, R12A, TAFI Δ 1-11, and TAFI Δ 1-12. We initially constructed these activation domain truncated mutants of TAFI in the context of wild-type TAFI. However, preliminary studies showed that the truncation variants were not secreted into the medium of transfected cells. Therefore, we also constructed the truncation variants in the context of the stable TAFI-CIIYQ variant. The rationale for this is that Zhou and coworkers expressed the more extensive truncation variant TAFI Δ 1-73 in this context [16]. It has been postulated that the activation domain contributes to the stabilization of the dynamic flap in the TAFI zymogen therefore, it is possible that the truncated forms of activation domain of TAFI do not support stabilization of the enzyme domain. The CIIYQ mutations in the dynamic flap stabilize the enzyme domain [15].

Expression plasmids encoding all of the truncation variants as well as full-length wild-type TAFI and TAFI-CIIYQ were transiently transfected into BHK cells and activation of the TAFI protein in the conditioned medium was assessed through western blotting analysis. Our results show that wild-type and TAFI-CIIYQ were able to be secreted and activated by thrombin-TM indicating successful transfection. However, none of the variants, either in the wild-type or CIIYQ form, were secreted from cells.

At this time it is not fully clear why the mutants were not secreted. The study by Zhou and coworkers demonstrated the Δ 1-73 TAFI-CIIYQ mutant is able to be secreted, with and without an amino-terminal 6 \times -His tag. Our mutants are in-frame (as verified by sequence analysis) and each contained an intact signal peptide. One difference between the studies of Zhou and the current work is the mammalian expression system utilized;

Zhou and coworkers utilized, human embryonic kidney (HEK293) cells versus the BHK cells used here. However, both BHK cells and HEK293 cells have been used to express recombinant TAFI [4, 11, 14, 17-19]. The most likely explanation is the presence of the carboxyl-terminal 6×-His tag in this study. It is notable that our previous work has shown the carboxyl-terminal 6×-His tag does materially affect the functional properties of TAFI, including its activation and antifibrinolytic effect [13]. How the presence of the tag affects the folding and ultimate secretion of the truncation variants is unclear, but certainly this could be overcome by constructing and expressing a new generation of truncation variants with an amino-terminal 6×His tag, as was done by Zhou and coworkers [14] and also by Marx and coworkers for the purpose of producing recombinant TAFI for crystallization [20].

The lysates from the transient transfections were also analyzed and a species presumably corresponding to a hypoglycosylated, intracellular form of TAFI (approximately 45-48 kDa) was detectable for all the variants tested (Figure 3-2C), albeit in greatest abundance for the secretable full-length forms of TAFI. We speculate that these forms represent incompletely folded and/or glycosylated forms of TAFI that are present in the endoplasmic reticulum. In the case of the truncated variants, the protein is never properly folded and/or glycosylated and so is unable to exit the endoplasmic reticulum and proceed through the secretory pathway. In this respect, they are analogous to lower molecular-weight forms of TAFI expressed from naturally-occurring alternatively spliced CPB2 transcript [21]. It is notable that the TAFI-CIIYQ intracellular form is efficiently activated by thrombin-TM while the corresponding form of wild-type

TAFI is not (Figure 3-2B). This may relate to the greater stability, and hence more efficient folding, of the CIIYQ form.

Using qRT-PCR analysis, we did verify that each of the expression plasmids was transfected into the BHK cells and mRNA corresponding to the variants expressed. Although there were notable differences in the abundance of these mRNAs, they do not fully explain the differences in the intracellular or extracellular abundance of the respective TAFI variants.

At this time, it is not possible to definitively conclude whether amino-terminal truncation of the TAFI activation domain affects cleavage of TAFI by thrombin-TM. However, it is likely that loss of a significant number of residues in the 1-12 sequences will profoundly affect the conformation of the activation domain, and may even have affected the ability of the truncation variants to be expressed. Residues 1-12 form a β -strand that is an internal strand in a four-strand antiparallel β -sheet that is a key part of the open-faced α/β -sandwich activation domain tertiary structure (Figure 3-4). Loss of these residues is likely to negatively impact the thermodynamic stability of this β -sheet and hence of the activation domain itself. Loss of proper folding of the activation domain would likely prevent secretion of the protein. Interestingly, the TAFI Δ 1-73 variant completely lacks the open-faced α/β -sandwich moiety, perhaps accounting for the secretability of this variant. Construction of variants without the carboxyl-terminal 6 \times -His tag will likely shed light on the importance of the activation domain not only for activation of TAFI by thrombin-TM but also folding and secretion of TAFI.

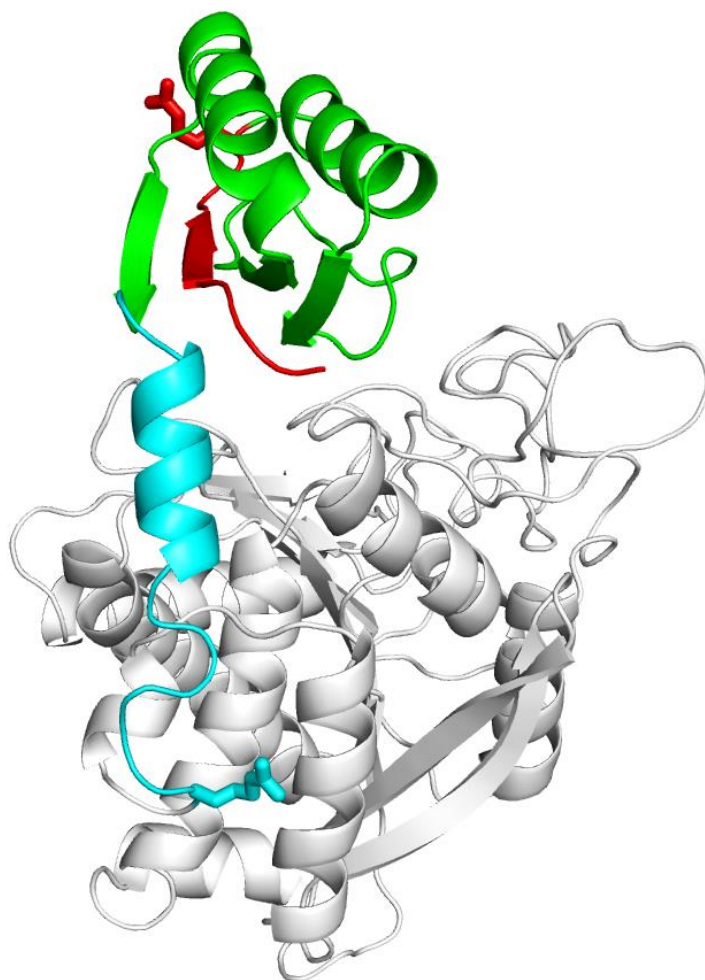


Figure 3-4. Structure of the activation domain of TAFI. This image was rendered using Polyview (<http://polyview.cchmc.org/polyview3d.html>), using the coordinates of the crystal structure of human recombinant TAFI (PDB ID 3D66; chain B) [19]. The activation domain (Residues 1-92 is coloured: Residues 1-12 are in red (with the side chain of Arg12 shown in red), residues 13-73 are in green, and residues 74-92 are in cyan (with the side chain of Arg92 shown in cyan).

3.6 References

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Chapter 4

Identification of Heparin Interaction Sites on Thrombin- Activatable Fibrinolysis Inhibitor that Modulate Plasmin- mediated Activation, Thermal Stability and Antifibrinolytic potential

4.1 Summary

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a human plasma zymogen that provides a molecular link between coagulation and fibrinolysis. TAFI is activated through cleavage at Arg92 by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin. Studies have shown that the presence of glycosaminoglycans (GAGs) is able to accelerate TAFI activation by plasmin and stabilize TAFIa. The elements of TAFI structure that allow these effects are unknown. *Objectives:* To identify heparin interaction regions on TAFI that mediate acceleration of activation by plasmin and contribute to stabilization of TAFIa. *Methods:* Based on crystallographic studies and homology to heparin-binding proteins, we performed mutagenesis of surface-exposed charged residues on TAFI that putatively constitute heparin-binding sites. The ability of the mutants to bind to heparin, kinetics of activation by plasmin in the presence or absence of heparin, the thermal stability and antifibrinolytic potential of each variant was determined. *Results:* Mutagenesis of Lys211 and Lys212 did not impair heparin binding or the effects of heparin on activation by plasmin and TAFIa stability but had a general effect on the ability of TAFI to be activated by plasmin. Mutagenesis of Lys306 did not impair heparin binding or any of the effects of heparin on TAFI/TAFIa function. Mutation of His308 did not impair heparin binding but this mutation generally had a severe negative effect on TAFI/TAFIa function. Mutation of Arg320 and Lys324 in combination markedly decreased heparin binding but had no effect on heparin-mediated acceleration of TAFI activation by plasmin while somewhat decreasing TAFIa stabilization by heparin. Finally, mutagenesis of Lys327 and Arg330 decreased (but did not eliminate) heparin binding while decreasing the ability of heparin

to accelerate plasmin-mediated TAFI activation, to stabilize TAFIa, and to increase the antifibrinolytic ability of TAFI/TAFIa. *Conclusion:* We conclude that Lys327 and Arg330 define a functionally-relevant heparin binding site, but that additional sites may be present on the TAFI structure.

4.2 Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that provides a molecular link between the coagulation and fibrinolytic cascades [1, 2] as well as between coagulation and inflammation [3]. Activated TAFI (TAFIa) possesses basic carboxypeptidase activity: the ability to remove carboxyl-terminal lysine and arginine residues from protein and peptide substrates. Accordingly, TAFIa down-regulates fibrinolysis by removing carboxyl-terminal lysine and arginine residues from partially degraded fibrin thereby attenuating positive feedback in the fibrinolytic cascade [4, 5]. TAFIa can also regulate pericellular plasmin formation by removal of carboxyl-terminal lysine residues from cellular plasminogen receptors [6], thereby regulating cell migration [7] and possibly other inflammatory responses. Indeed, deficiency of TAFI in mouse models influences a wide spectrum of inflammatory and fibrotic disorders [3, 8].

Two mechanisms exist to regulate the elaboration of active TAFI (TAFIa): control of the rate of TAFI activation and control of the rate of TAFIa inactivation. TAFIa is formed through cleavage at Arg92 by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin [9-11]. Thrombin and plasmin alone are relatively poor activators of TAFI but binding of thrombin to TM increases the catalytic efficiency (k_{cat}/K_M) of TAFI activation by 1250-fold [10]. It has been shown that the presence of glycosaminoglycans (GAGs), such as heparin, is able to accelerate TAFI activation by plasmin [11] such that the catalytic efficiency of the reaction is only ~10-fold lower than that exhibited by thrombin-TM. With respect to TAFIa inactivation, the enzyme has the unusual property of being metastable [12]: its activity spontaneously

decays with a half-life of 8 – 15 minutes at body temperature in the absence of any further proteolytic cleavage [13]. Biophysical and structural studies have attributed the loss of activity to profound structural changes in the enzyme [13-16]. Interestingly, GAGs are able to stabilize TAFIa, causing a 2.3-fold increase in its half-life at 25°C from 74 to 170 min [11]. However, the elements of TAFI structure that mediate the effects of GAGs on TAFI activation and TAFIa stability are unknown.

In a report on the crystal structure of bovine TAFI, Anand and coworkers described 22 bound sulfate molecules in the model [16]. These bound sulfates may represent potential GAG-binding sites and were clustered in three regions: near the so-called “dynamic flap” on the enzyme domain where it interfaces with the activation domain, at the top of the activation domain, and within the active site cleft. Only the first potential site seemed a reasonable possibility to account for the effects of GAGs: following activation of TAFI, heparin bound to the activation domain would not affect the stability of TAFIa, heparin bound in the active site cleft would prevent catalytic activity by blocking substrate access [16]. A putative GAG-binding site was identified by Mao and coworkers through sequence homology with heparin-binding sequences in GAG-binding proteins. A region in TAFI spanning Trp210 through Ser221 (WKKMRMWRKNRS, where the underlined residues are basic) is homologous to the heparin-binding sequence motif ($-XBBXB-$, where B and X represent basic amino acids and hydrophobic acids, respectively) in GAG-binding proteins [11].

Although plasmin is a weak activator of TAFI, it is a relevant activator. Studies by Gils and coworkers *in vitro* using monoclonal antibodies that inhibit plasmin-mediated TAFI activation showed that plasmin is a relevant TAFI activator during clot formation

and lysis in the context of plasma clot lysis assays [17]. Furthermore, when the monoclonal antibody MA-TCK26D6 – which inhibits TAFI activation specifically by plasmin – was injected into a mouse thromboembolism model, decreased fibrin deposition in the lungs was observed indicating an acceleration of fibrinolysis [18]. Plasmin-mediated TAFI activation would dominate at sites of vascular injury which expose an abundance of GAGs present in the extracellular matrix near the fibrin rich clot [11]. Additionally, as these injury sites would lack thrombomodulin, in the presence of GAGs TAFI activation by plasmin may therefore play a significant role in stabilizing the clot [11].

In this study, we constructed recombinant TAFI variants containing mutations in the “dynamic flap” instability region as well as the region spanning Trp210-Ser221 which are two sites predicted to interact with heparin. We assessed the ability of these variants to bind heparin, measured their kinetics of activation by plasmin in the presence or absence of heparin, and determined their thermal stabilities and antifibrinolytic potential.

4.3 Experimental Procedures

4.3.1 Materials

The synthetic carboxypeptidase substrate anisylazofornyl-arginine (AAFR) was obtained from Bachem Americas, Inc. (Torrance, CA, USA). Heparin sodium salt and heparin-agarose type I saline suspension (from porcine intestinal mucosa), and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada). The plasmin inhibitor D-valylphenylalanyllysyl chloromethylketone (VFKck), the TAFIa inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (Plummer's inhibitor) and the thrombin inhibitor D-phenylalanylprolylarginyl chloromethylketone (PPAck) were purchased from Calbiochem (San Diego, CA, USA). QuikChange® Site-directed Mutagenesis kit was from Stratagene (La Jolla, CA). DNA restriction and modification enzymes were purchased from New England Biolabs (Mississauga, ON, Canada). Oligonucleotides for mutagenesis were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Dulbecco's modified Eagle's medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, penicillin/streptomycin/fungizone (PSF) were obtained from Invitrogen. Newborn Calf Serum was obtained from Sigma-Aldrich. Effectene transfection reagent was purchased from QIAGEN, Inc. (Toronto, ON, Canada). Methotrexate was purchased from Mayne Pharma, Inc. Thrombin, rabbit-lung thrombomodulin and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, USA), Inc. Recombinant batroxobin was purchased from Cedarlane (Burlington, ON, Canada). Recombinant tPA (Alteplase) was purchased from Kingston General Hospital Pharmacy. Polyclonal sheep anti-human TAFI antibody was purchased from Affinity Biologicals (Ancaster, ON, Canada) and

polyclonal rabbit anti-sheep antibody was purchased from ThermoFisher Scientific. (Ottawa, ON, Canada). The mouse-anti-human TAFI monoclonal antibody MA-T4E3 was obtained from Dr. Ann Gils (Katholieke Universiteit Leuven, Leuven, Belgium) and coupled to CNBr Activated Sepharose 4B (GE Healthcare Life Sciences, Mississauga, ON, Canada) according to manufacturer's instructions (2-6 mg antibody/mL resin). The phospholipids L- α -phosphatidylcholine from frozen egg yolk (Type XIII-E) and 1,2-diacyl-*sn*-glycero-3-phospho-L-serine from bovine brain were purchased from Sigma-Aldrich. Synthetic phosphatidylcholine/phosphatidylserine vesicles (PC/PS, 80:20) were prepared by modification of the methods of Barenholz *et al.* and Bloom *et al.* [19, 20].

4.3.2 Construction of TAFI Variants

Mutagenesis was carried out using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutagenic primers used are shown in Table 4-1. In all cases, the template for the mutagenesis was TAFI-pNUT, which contains human TAFI cDNA which contains threonine at positions 147 and 325 [13]. The presence of the mutations was verified by DNA sequence analysis.

Table 4-1. Primer sequences for mutagenesis of TAFI^a

Variant	Primer Sequence
TAFI-K306A	5'-CCTATACACGAAGTAAAAGC GCA GACCATGAGGAACTGTCTC-3'
TAFI-H308F	5'-ACACGAAGTAAAAGCAAAGACT TTT GAGGAACTGTCTCTAGTAGC-3'
TAFI-K212A	5'-GTTATGACTACTCATGGAAA GCG AATCGAATGTGGAGAAAAG-3'
TAFI-K211Q/K212Q	5'-GTGGACGGTTATGACTACTCATGG CAACAG AATCGAATGTGGAGAAAAGAACCG-3'
TAFI-R320A/K324A	5'-ACTGTCTCTAGTAGCCAGTGAAGCAGTT GCT GCTATTGAG GCA ACTAGTAAAAATACC-3'
TAFI-K327A	5'-TTCGTGCTATTGAGAAAAGTAGT GCA AATACCAGGTATACACATGGCC-3'
TAFI-K327A/R330A	5'-TTCGTGCTATTGAGAAAAGTAGT GCA AATACC GCG TATACACATGGCCATGGCTCAG-3'

^aSense strand sequences only are shown. Ala/Phe/Gln codons are double underlined; mutated nucleotides are in boldface type

4.3.3 *Expression and purification of recombinant TAFI variants*

Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (1:1) (DMEM/F-12) containing 5% (v/v) newborn calf serum (NCS) and 1% (v/v) PSF in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere. Expression plasmids encoding the respective TAFI variants were transfected into cells using Effectene transfection reagent (2 µg plasmid DNA and 60 µL of transfection reagent per 100 mm plate of cells). After 24 hour incubation with the transfection mixture, the cells were then cultured in DMEM/F-12 containing 5% NCS and 1% PSF supplemented with 400 µM methotrexate for approximately 2 weeks, with the medium changed every other day, for the selection of stable cell lines expressing the respective recombinant TAFI variants. For recombinant TAFI production, stably expressing lines were cultured in triple flasks (500 cm²; Nunc) in Opti-MEM containing 1% (v/v) PSF and 40 µM ZnCl₂. Conditioned medium was collected at 48 hour intervals and replaced with fresh medium. Harvested conditioned medium was centrifuged at 3000 × g for 5 minutes, supplemented with Tris-HCl, pH 8.0 (to 5 mM), reduced glutathione (to 0.5 mM) and phenylmethanesulphonyl fluoride (PMSF) (to 2 µM) and stored at -20°C.

To purify the recombinant TAFI variants, conditioned medium was passed through a 0.22-µm filter prior to affinity chromatography over a 1-mL MA-T4E3-Sepharose column at 4°C as previously described [21]. Protein containing fractions were pooled and then were concentrated and exchanged into HBST (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween 80) using Amicon ultra-4 centrifugal filter units,

MWCO 10 kDa. Purified TAFI was quantified by measurement of absorbance at 280 nm and 320 nm ($\epsilon_{1\%, 280} = 26.4$; $M_r = 60,000$), aliquoted and stored at -70°C .

4.3.4 Binding of TAFI to heparin

A 1-mL heparin-agarose column was prepared by equilibrating the column with 10 column volumes of HBST. TAFI (0.25 μg) was applied to the column, after which a wash was performed (0.2 column volumes of HBST \times 12) followed by elution (0.2 column volumes of HBST containing 193 U/mL heparin). The TAFI content in each fraction was analyzed by SDS-PAGE and western blotting using a polyclonal antibody raised against human TAFI.

4.3.5 Kinetics of activation of TAFI variants by plasmin

Each TAFI variant at various concentrations (0.01 – 0.1 μM) was incubated with plasmin (25 nM), and Plummer's inhibitor (150 μM) in the presence or absence of heparin (200 U/mL) at 21°C for 12 minutes. Plasmin activity was then quenched by the addition of VFKck (to 1 μM) before the mixture was placed on ice. SDS-PAGE sample buffer (0.2 M Tris-HCl, 8% SDS, 40% Glycerol, 0.08% bromophenol blue) was added to the samples. Samples were analyzed by SDS-PAGE and western blotting with the sheep anti-human TAFI polyclonal antibody. A TAFIa standard was prepared along with each kinetic experiment. The TAFIa standard was prepared by activating wild-type TAFI (1 μM) by incubating with thrombin (25 nM), thrombomodulin (100 nM) and CaCl_2 (5 mM) at 21°C for 10 minutes and then placed on ice. Known amounts of TAFIa (3 – 37.5 nM) were subjected to SDS-PAGE and western blotting along with the kinetic samples.

For the poorly activated TAFI variants H308F, K211Q/K212Q, and K327A/R330A, a smaller TAFIa standard range was used (0.2 – 9.6 nM). For a given experiment, all blots containing standards and samples were processed simultaneously for visualization of immunoreactive bands. The density of the 35 kDa TAFIa and 25 kDa TAFIai bands were quantified using AlphaView SA software. The standard was constructed by plotting the density of each standard TAFIa sample *versus* concentration of TAFIa. The TAFIa concentrations formed in each kinetic experiment were determined by interpolating from the densities on the standard curve. The concentration of TAFIa was then converted to the rate of TAFIa formation, v (mol of TAFIa formed /mol of plasmin /second). The data were fit to the Michaelis-Menten equation (Sigmaplot 11.0, SPSS Inc., Chicago, IL).

4.3.6 *Thermal stability of the TAFIa variants*

Purified recombinant TAFI variants (1 μ M) were activated by incubation with thrombin (25 nM), TM (100 nM), CaCl₂ (5 mM) in HBST at 21°C for 15 min. Thrombin activity was then quenched by the addition of PPAck (to 1 μ M) before the mixture was placed on ice. The activated TAFI was dispensed into aliquots corresponding to various time points in the presence or absence of heparin (312 U/mL final) and incubated at 37°C. Following the final time point, 15 μ L of each aliquot was mixed with 35 μ L HBST and 50 μ L of 240 μ M synthetic carboxypeptidase substrate AAFR (120 μ M, final) in a microtiter plate which had been pre-treated (overnight) with HBS/1% Tween 80 and thoroughly rinsed with deionized distilled water. The initial rates of AAFR hydrolysis were measured at 350 nm in a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA). The amount of residual TAFIa activity relative to the initial residual TAFIa

activity was plotted as a function of time, and the data were fit by non-linear regression to the first-order exponential decay equation (SigmaPlot 11.0, SPSS Inc., Chicago, IL). The first-order decay constant k was obtained and used to determine the half-life of each TAFI variant.

4.3.7 Barium Adsorbed TAFI-Deficient Plasma

Barium adsorbed plasma (BAP) was prepared as described previously [9] and was subsequently made TAFI-deficient (TdBAP). To prepare TAFI-deficient plasma, 50 mL of BAP was passed over a 1-mL MA-T4E3-Sepharose column at room temperature. The plasma was passed over the column repeatedly, and between each pass the column was washed with HBST. After each pass, TAFI was eluted from the column with 0.2 M glycine, pH 3. An aliquot of the plasma was then used for an *in vitro* clot lysis assay (in the absence of added TAFI) in the presence or absence of 10 nM TM [22]. The plasma was considered to be TAFI-deficient when no difference was observed between clot lysis times in the presence or absence of TM.

4.3.8 Clot Lysis Assays

All clot lysis assays were performed in a final volume of 100 μ L in microtitre plates at 37°C. The TdBAP was diluted 1:3 in HBST and supplemented with synthetic phosphatidylcholine/phosphatidylserine vesicles (PC/PS) (20 μ M) as well as different concentrations of TAFI variants either as zymogen (0 – 100 nM) or as TAFIa (0 – 50 nM; following activation by thrombin-TM as described above). The mixtures also contained no heparin or one of two difference concentrations (25 or 100 U/mL) of heparin. Clotting

was initiated by addition of the assay mixtures into the wells of microtitre plates containing small, separated aliquots of batroxobin (0.5 ug/mL), CaCl₂ (5 mM) and tPA (2 nM). Clot lysis was monitored by the change in turbidity of each reaction at 405 nm in a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA) and the time to 50% lysis was determined graphically from the midpoint between maximum and minimum turbidities of the clots.

4.3.9 Statistical Methods

Statistical analyses were performed with the use of SPSS software, version 22.0 (SPSS Inc., Chicago, Illinois). Comparisons between different variants for a given assay parameter were performed by one-way ANOVA using a Tukey post-hoc analysis. Statistical significance was assumed at $p < 0.05$. Differences between individual variants in the absence or presence of heparin were determined using an unpaired Student's t-test.

4.4 Results

4.4.1 Construction, expression, and purification of TAFI variants

To identify the GAG interaction site(s) on TAFI we have expressed TAFI variants containing mutations of residues that are predicted to interact with heparin in the instability region as well as in the region spanning Trp210-Ser221 (Figure 4-1). The latter region was hypothesized to function as a heparin-binding sequence based on homology with heparin-binding proteins [11]. Specifically, this region contains two repeats of the –*XBBXB*X– consensus, where *B* = Lys or Arg and *X* = hydrophobic amino acids. In the bovine crystal structure of TAFI, however, only one out of 22 sulfate molecules were bound to the Trp210-Ser221 region: at Lys212 [16]. We therefore mutated Lys212 and the neighbouring Lys211. None of the other basic residues in this region are surface-exposed (Figure 4-2). The variant with both lysines mutated to Ala did not express, and so the two lysines were together mutated to Gln.

In the instability region, there are three sulfates bound in the bovine crystal structure [16]. One is bound at His308, Ser312 and Arg313; of these, only His308 is conserved in human; we therefore mutated this residue as well as the spatially close Lys306 residue. When His308 was mutated to Ala, the protein did not express so we mutated this residue to Phe. Another sulfate is bound to His326 and Arg327, neither of which is conserved in human. Therefore we mutated the Lys at position 324 and the spatially close Arg320 residue (Figure 4-2). The final sulfate is bound at Arg330. We mutated this residue along with the spatially close Lys327 residue (Figure 4-2). Altogether, seven variants were constructed (Figure 4-2).

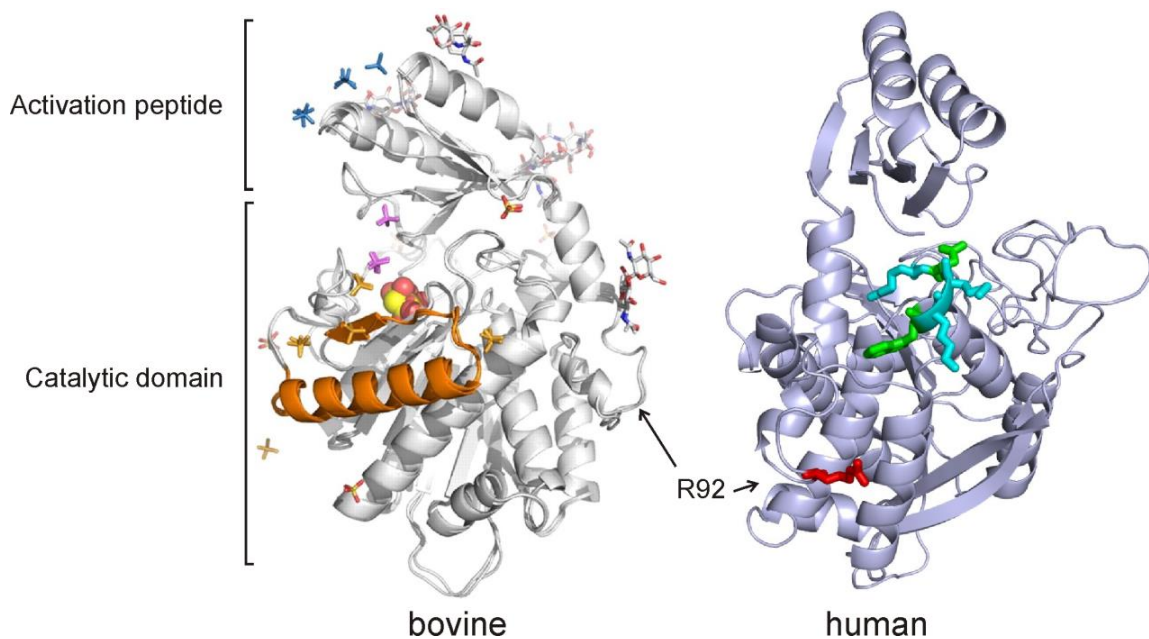


Figure 4-1. Putative glycosaminoglycan binding sites on TAFI. The ribbon diagram of bovine TAFI [16] on the left (superposition of three independent TAFI molecules) shows both the activation domain and the catalytic domain and four *N*-linked glycans. The bound sulfates on the structure represent potential heparin-binding sites and are clustered in three regions: near the dynamic flap (gold) where it interfaces with the activation domain (orange sulfates), at the prodomain (blue sulfates), and within the active site cleft (pink sulfates). The last set of sulfates is unlikely to represent a relevant heparin-binding site as it would block substrate access to the active site. The ribbon representation on the right of human TAFI, prepared from the crystallographic data from [15] (PDB accession #3D66), indicates the position Arg92 (side chain in red) and a putative GAG-binding site identified by through sequence homology with heparin-binding proteins. The region spanning Trp210 through Ser221 contains the heparin-binding sequence motif – *XBBXB*X–, where *B* and *X* represent basic amino acids (side chains in cyan) and hydrophobic acids (side chains in green), respectively.

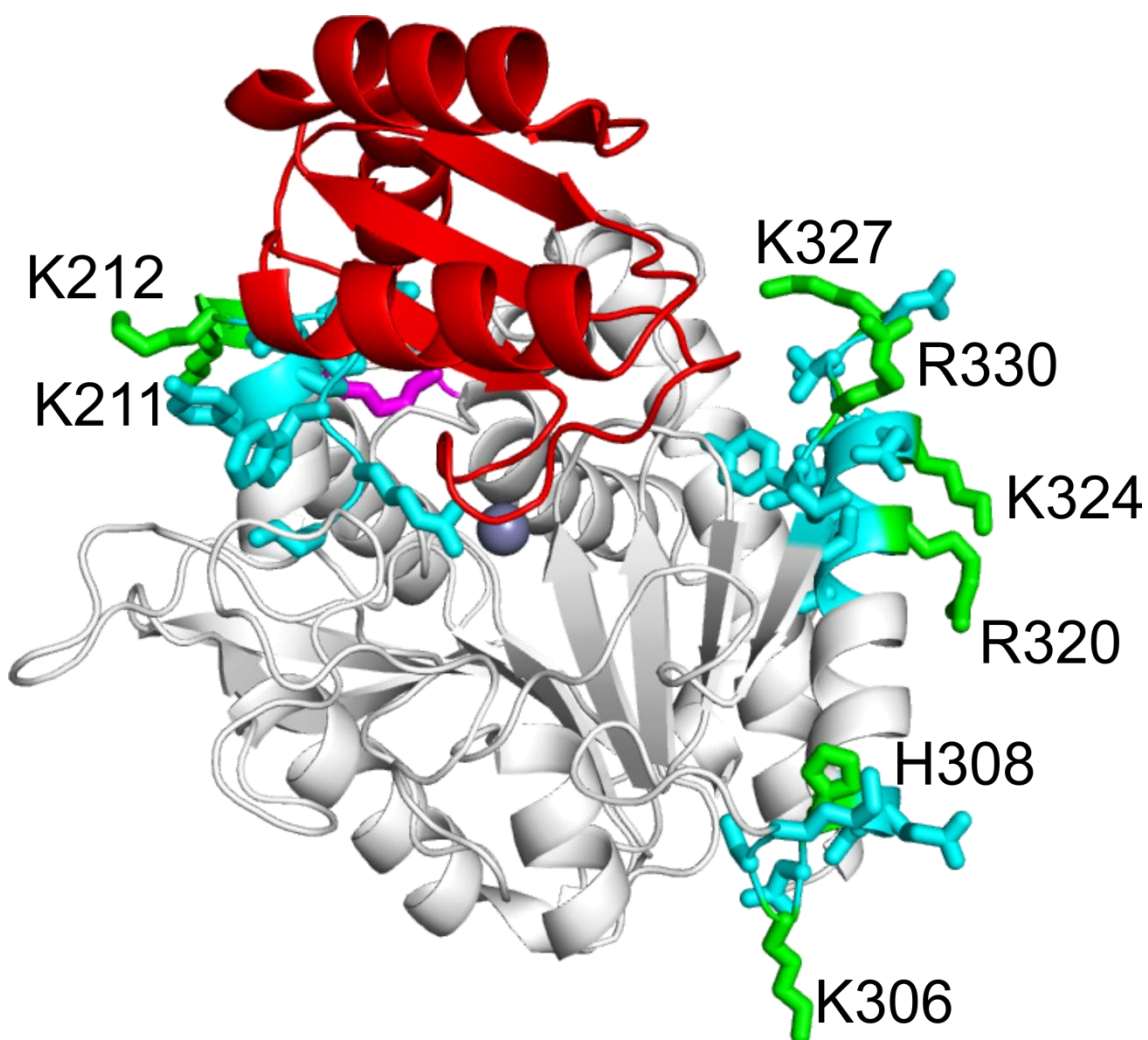


Figure 4-2. Mutations of Potential heparin binding sites on TAFI. The crystal structure of TAFI is rendered based on PDB #3D66 [15] using Polyview (<http://polyview.cchmc.org/polyview3d.html>). The activation domain is in red and the side chain of Arg92 is magenta. Side chains of residues that were mutated are labeled and shown in green. Surrounding side chains of note are also shown in cyan (Tyr208 – Lys218; Lys304 – Glu310; Ala318 – Thr332). The active site Zn²⁺ is shown as a sphere.

4.4.2 *Binding of TAFI variants to heparin-agarose*

We examined the ability of the variants to bind GAGs by utilizing a heparin-agarose column. The protein was applied to the column and the column was then given a buffer wash followed by elution using buffer containing heparin. The TAFI content in the fractions was evaluated using western blot analysis. Wild-type TAFI was eluted almost exclusively in the heparin-containing fractions (Figure 4-3), demonstrating specific binding of TAFI to heparin immobilized on the column. In a control experiment using an agarose column lacking covalently linked heparin, TAFI eluted completely in the buffer wash (Figure 4-3), again consistent with specific binding between TAFI and heparin on the column.

Variants H308F and K212A displayed similar elution profiles to wild-type whereas variants K306A and K211Q/K212Q were not as tightly bound compared to wild-type, indicated by the fact that slightly more of the protein was eluted in the wash (Figure 4-3). The variants R320A/K324A, K327A, and K327A/R330A exhibited the lowest affinity for the heparin-agarose; the majority of R320A/K324A and K327A eluted in the wash fractions while for K327A/R330A, an approximately equal amount of protein was present in the wash and elution fractions (Figure 4-3).

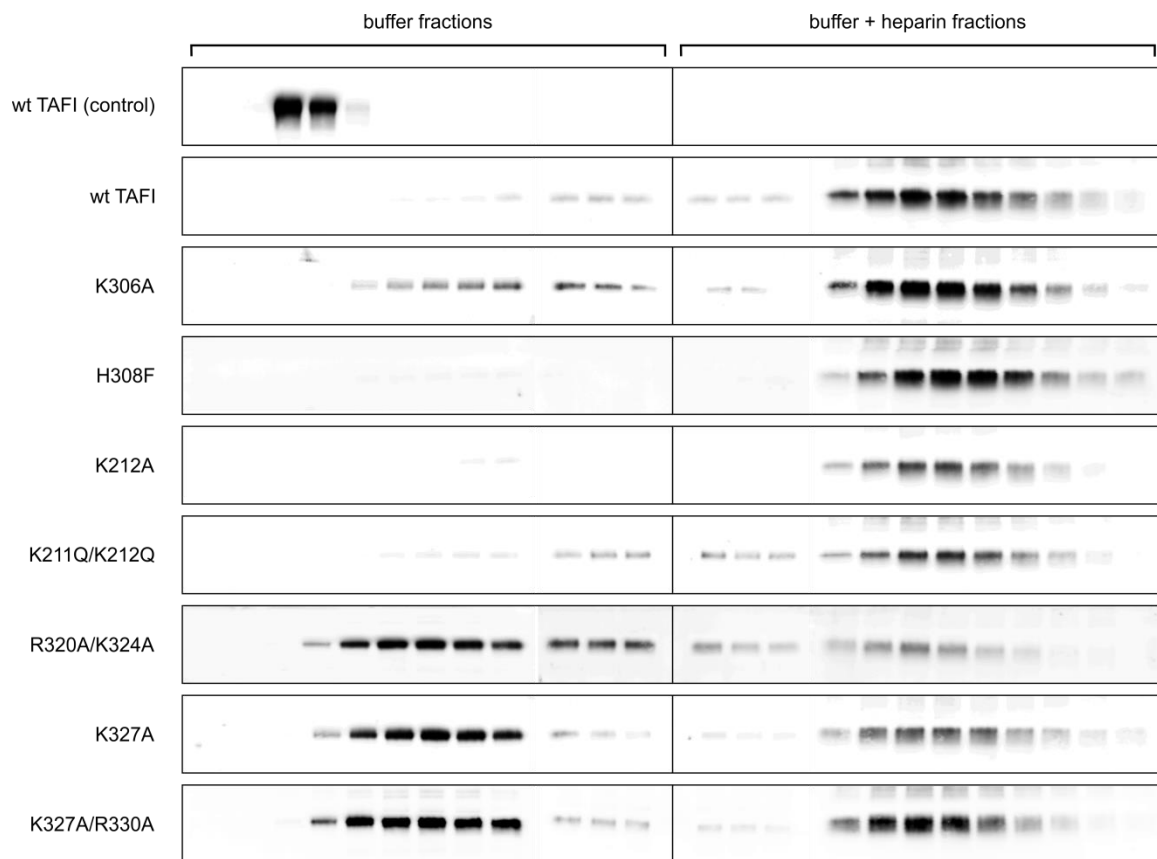


Figure 4-3. Binding of TAFI variants to heparin-agarose. Purified recombinant TAFI variants were applied to a heparin-agarose column. The column was washed with 2.4 column volumes of buffer (over 12 fractions) and eluted with 2.4 column volumes of buffer containing heparin (over 12 fractions). Each fraction was subjected to western blot analysis using a polyclonal anti-TAFI antibody. A control experiment to evaluate non-specific binding of TAFI to the column was conducted using the same agarose resin that was not derivatized with heparin (wt TAFI (control)). The data are representative of at least 3 independent experiments.

4.4.3 Kinetics of activation of TAFI variants by plasmin in the absence and presence of heparin

The kinetics of activation of various concentrations of the TAFI variants by plasmin in the absence and presence of heparin were determined (Figure 4-4). If a reduced accelerative effect of heparin was observed, this would imply that the mutated residues were important for the effect of heparin in this context. The estimated Michaelis-Menten kinetic parameters in the absence and presence of heparin are summarized in Tables 4-2 and 4-3, respectively. Our values for k_{cat} and K_M are in the same range as was reported by Mao and coworkers using a similar western blot-based method ($k_{\text{cat}} = 0.00044 \text{ s}^{-1}$; $K_M = 55 \text{ nM}$) [11]. We observe a 3.5-fold increase in k_{cat} upon addition of heparin, which compares favourably with the 5.9-fold increase observed by Mao and coworkers. However, while they observed a decrease in K_M upon addition of heparin, we observed an increase, which reduced the increment in catalytic efficiency (k_{cat}/K_M) in our data set. Nonetheless, it is clear that heparin has a notable effect on plasmin activation in our hands, most particularly at the highest concentrations of TAFI tested, which correspond to the physiological concentrations of the zymogen [23]. All the TAFI variants, to varying degrees, displayed accelerated activation in the presence of heparin, with K306A and R320A/K324A showing profiles most similar to wild-type (Figure 4-4). Several of the variants, however, displayed greatly impaired activation by plasmin that was superimposed on the effects of the mutations on acceleration by heparin. In particular, H308F, K211Q/K212Q, and K327A/R330A showed much reduced rates of activation that were little changed in the presence of heparin. K212A showed an intermediate effect, with

moderately reduced rates of activation and a moderate decrease in the effect of heparin (Figure 4-4).

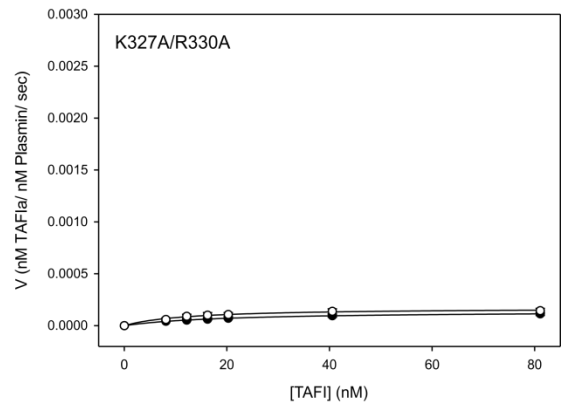
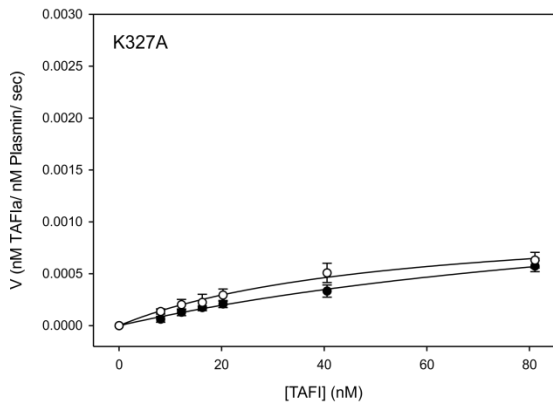
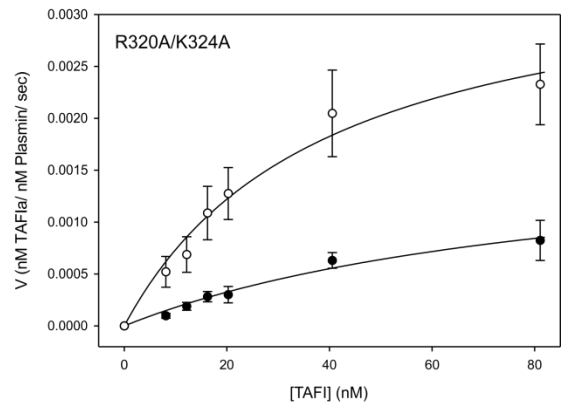
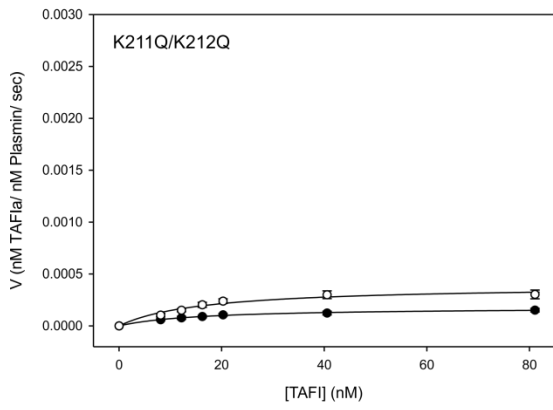
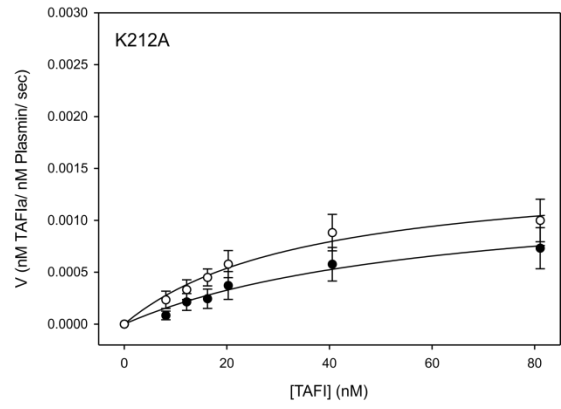
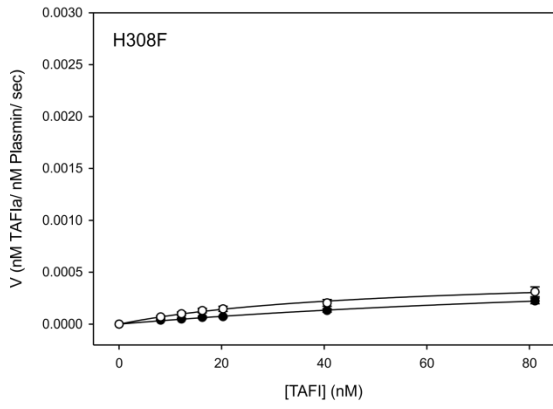
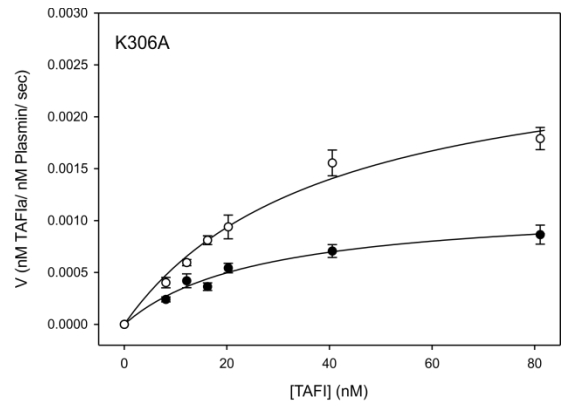
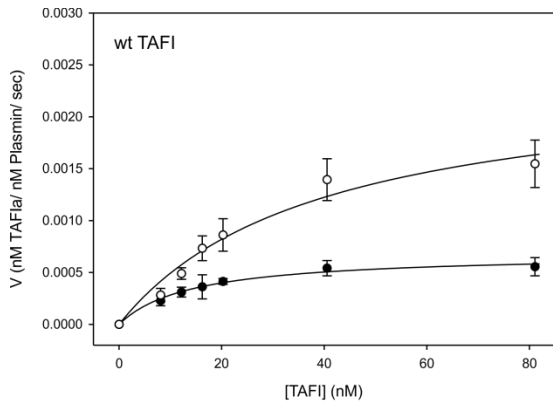


Figure 4-4. Effect of heparin on the kinetics of activation of the TAFI variants by plasmin. TAFI activation by plasmin was measured at various concentrations of TAFI and in the absence (filled circles) or presence (open circles) of heparin. The rate of TAFI activation was determined by western blot analysis. The symbols are the means \pm standard error of the mean of at least 3 independent experiments. The lines are regression lines based on the mean values of k_{cat} and K_M obtained from at least 3 independent experiments.

Table 4-2. Kinetic parameters for activation of TAFI variants by plasmin in the absence of heparin

Variant	k_{cat} s^{-1}	K_{M} μM	$k_{\text{cat}}/K_{\text{M}}$ $\mu\text{M}^{-1}\text{s}^{-1}$
TAFI wt	0.000684 ± 0.00012	0.0142 ± 0.005	0.0481 ± 0.0051
K306A	0.001157 ± 0.00010	0.0265 ± 0.002	0.0436 ± 0.0050
H308F	0.000623 ± 0.00016	0.1458 ± 0.019	0.0043 ± 0.0006
K212A	0.001323 ± 0.00010	0.0610 ± 0.018	0.0217 ± 0.00001
K211Q/K212Q	0.000179 ± 0.00003	0.0157 ± 0.002	0.0114 ± 0.0022
R320A/K324A	0.001793 ± 0.00058	0.0901 ± 0.017	0.0199 ± 0.0024
K327A	0.001502 ± 0.00016	0.1328 ± 0.038	0.0113 ± 0.0024
K327A/R330A	0.000142 ± 0.00002	0.0199 ± 0.004	0.0071 ± 0.0009

Data are the means \pm standard error of the mean of 3-9 independent experiments

Table 4-3. Kinetic parameters for activation of TAFI variants by plasmin in the presence of heparin

Variant	k_{cat} s^{-1}	K_{M} μM	$k_{\text{cat}}/K_{\text{M}}$ $\mu\text{M}^{-1}\text{s}^{-1}$
TAFI wt	0.002422 ± 0.00036	0.0391 ± 0.002	0.0619 ± 0.0095
K306A	0.002762 ± 0.00020	0.0391 ± 0.002	0.0706 ± 0.0046
H308F	0.000488 ± 0.00009	0.0484 ± 0.007	0.0101 ± 0.0022
K212A	0.001508 ± 0.00031	0.0360 ± 0.008	0.0418 ± 0.0131
K211Q/K212Q	0.000391 ± 0.00004	0.0164 ± 0.002	0.0239 ± 0.0025
R320A/K324A	0.003614 ± 0.00058	0.0392 ± 0.005	0.0922 ± 0.0018
K327A	0.001065 ± 0.00019	0.0521 ± 0.015	0.0204 ± 0.0076
K327A/R330A	0.000172 ± 0.00003	0.0121 ± 0.007	0.0142 ± 0.0052

Data are the means \pm standard error of the mean of 3-9 independent experiments

4.4.4 Thermal Stabilities of the TAFI variants in the absence and presence of heparin

Mao and coworkers [11] previously established that the half-life of TAFIa at 25°C was increased from 74 min to 170 min in the presence of heparin. The thermal stability of each variant in the presence and absence of heparin was determined to examine whether this stabilizing effect is also observed at 37°C. Half-lives of each variant were calculated from the resultant first-order decay rate constants (Table 4-4). The half-life of wild-type TAFIa was increased 1.5-fold by heparin. Many of the variants have a different stability to wild-type in the absence of heparin, and this is not unexpected given the results of previous mutagenesis studies [13, 24]. However, H308F, R320A/K324A, K327A, and (especially) K327A/R330A each showed decreases in the degree to which they were stabilized by heparin (Figure 4-5; Table 4-4).

Table 4-4. Intrinsic Stability of TAFI variants in the absence and presence of heparin

Variant	Half-life (min)		fold increase
	minus heparin	plus heparin	
TAFI wt	5.3 ± 0.5	7.9 ± 0.6	1.5
K306A	4.3 ± 0.3	8.1 ± 0.4	1.9
H308F	1.3 ± 0.1	1.7 ± 0.1	1.3
K212A	5.4 ± 0.6	9.1 ± 0.9	1.7
K211Q/K212Q	4.1 ± 0.2	6.8 ± 0.5	1.7
R320A/K324A	7.0 ± 0.9	8.9 ± 0.7	1.3
K327A	4.7 ± 0.6	5.8 ± 0.7	1.3
K327A/R330A	3.0 ± 0.04	3.1 ± 0.05	1.0

Data are the means ± standard error of the mean of 3-5 independent experiments performed in triplicate

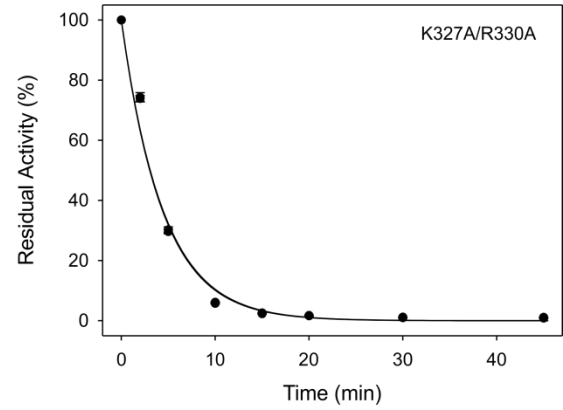
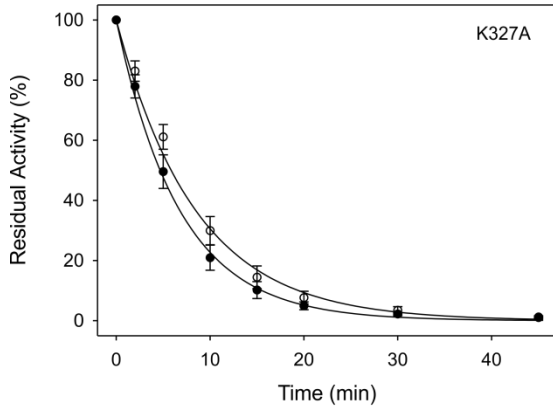
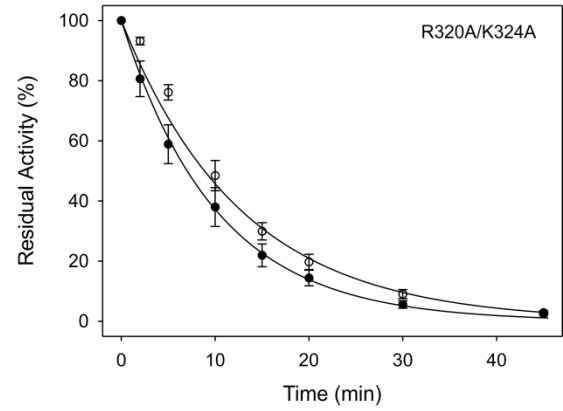
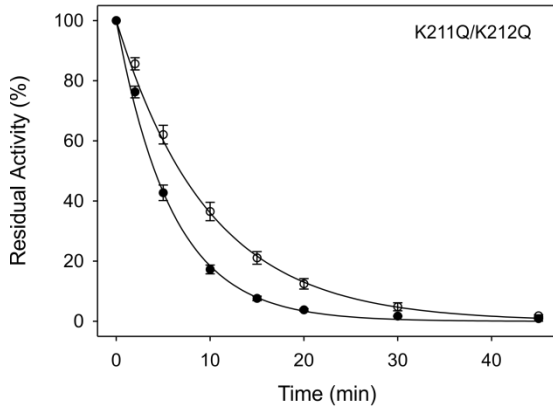
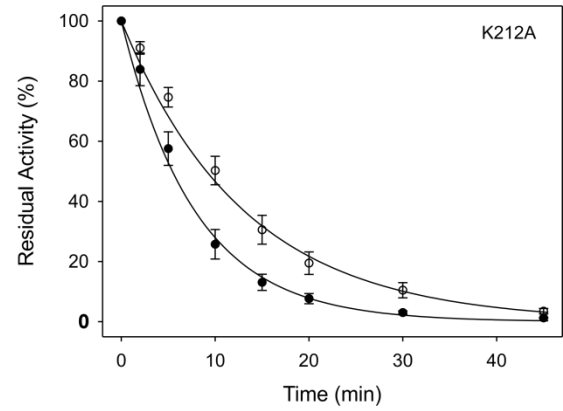
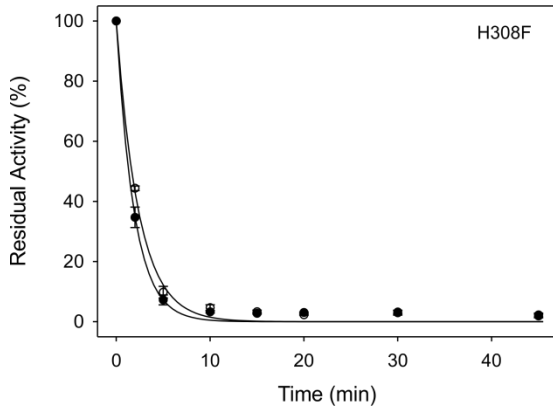
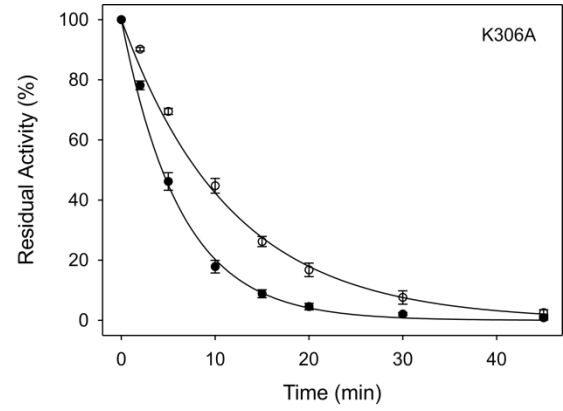
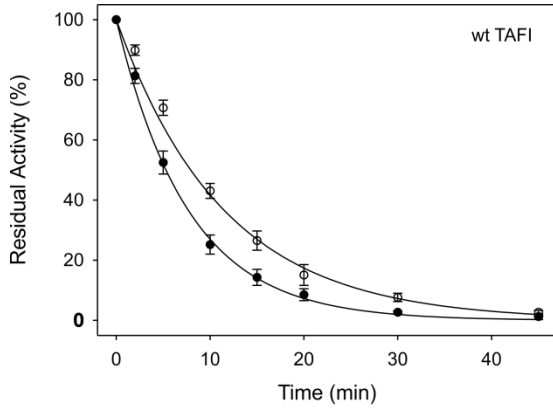


Figure 4-5. Effect of heparin on the stabilities of the TAFIa variants. Each variant was quantitatively activated by incubation with thrombin-TM. After quenching of the thrombin, the TAFIa was incubated at 37°C in the presence (filled circles) or absence (open circles) of heparin and timed aliquots removed and placed on ice. The residual TAFIa activity was measured using a TAFIa substrate and is expressed relative to the TAFIa activity present before incubation at 37°C. The symbols are the means \pm standard error of the mean of at least 3 independent experiments. The lines are regression lines based on the mean first-order rate constants for TAFIa decay obtained from at least 3 independent experiments.

4.4.5 Antifibrinolytic potential of TAFI variants

We determined the antifibrinolytic potential of the variants in the absence and presence of heparin using plasma clot lysis assays. We set up two different types of assays in order to separately evaluate the effects of heparin on plasmin-mediated activation and on stability of TAFIa.

In the first type of lysis assay, the system was manipulated such that the plasmin that was generated during lysis is the sole TAFI activator, by using TAFI-deficient, barium-adsorbed plasma and initiating clot formation using batroxobin. No thrombin would be generated during clot formation or lysis under these conditions, and it was previously shown by Mao and coworkers that addition of heparin to stimulate plasmin-mediated TAFI activation was required to observe a TAFI-dependent antifibrinolytic effect [11]. We varied the concentration of recombinant TAFI variants added to the clots and used two different concentrations of heparin (Figure 4-6). Wild-type TAFI exhibited the greatest antifibrinolytic potential with increased heparin concentration, with variants K212A and K306A also having a notable response to heparin. Heparin resulted in a small increase in lysis time at the highest concentrations of K211A/K212A, R320A/K324A, K327A, and K327A/R330A, while H308F showed very little antifibrinolytic effect that was essentially resistant to heparin (Figure 4-6).

In the second type of clot lysis assay, we quantitatively activated the TAFI variants using thrombin-TM prior to addition to the clot lysis assay. This allowed us to specifically interrogate the role of heparin-mediated TAFIa stabilization during lysis, since the maximal extent of fibrinolysis inhibition (i.e. the plateau) is a direct function of the intrinsic stability of TAFIa [13, 25]. Wild-type TAFI displays an increase in stability

in the presence of heparin and therefore an increase in the plateau lysis time, as expected (Figure 4-7). Variants K306A, K211Q/K212Q, and R320A/K324A each exhibited increases in the plateau lysis times due to heparin that were comparable to wild-type TAFIa (Figure 4-7). K212A showed a moderately decreased increase in plateau lysis time, while lysis time of H308F, K327A, and K327A/R330A were largely resistant to the effects of heparin (Figure 4-7). Notably, K327A/R330A and H308F are both much less stable than wild-type TAFIa and show correspondingly reduced antifibrinolytic potential (i.e. plateau lysis time).

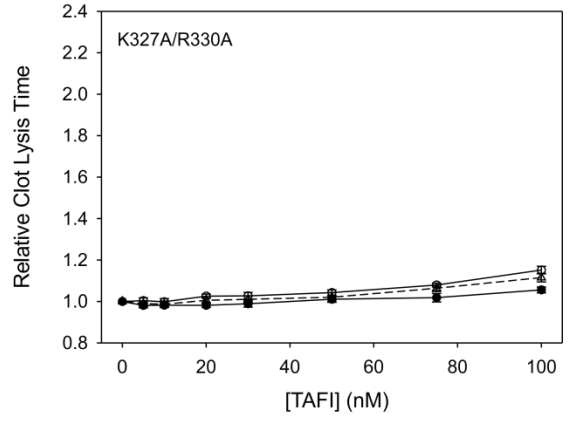
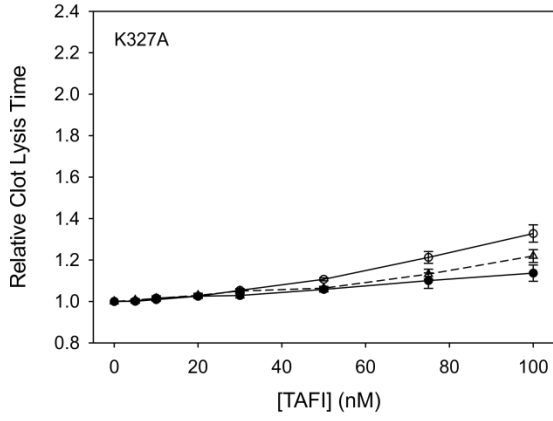
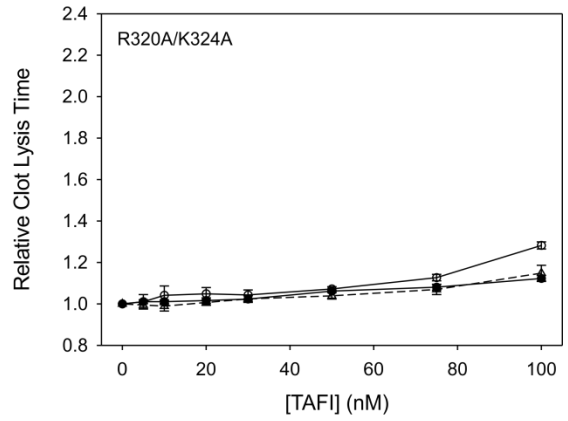
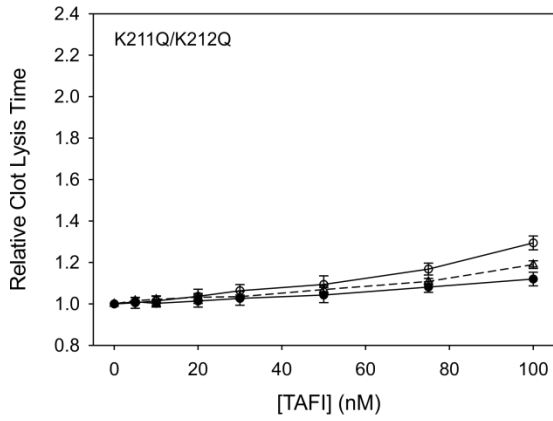
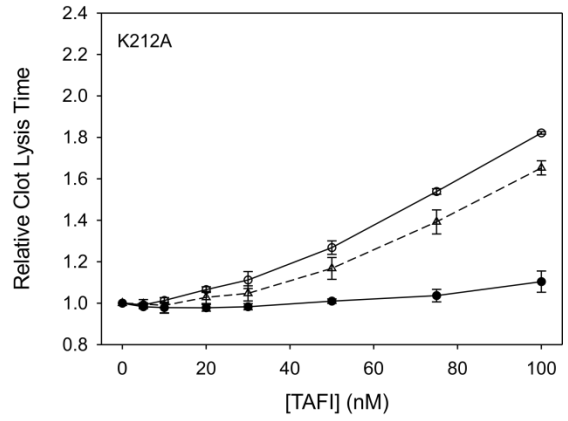
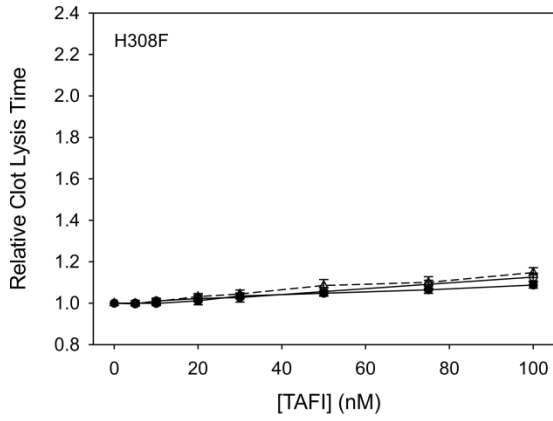
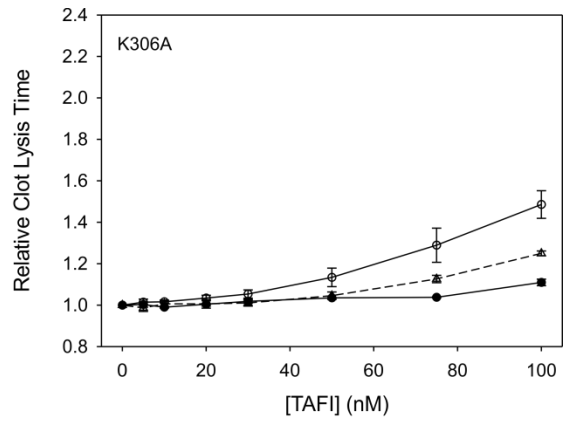
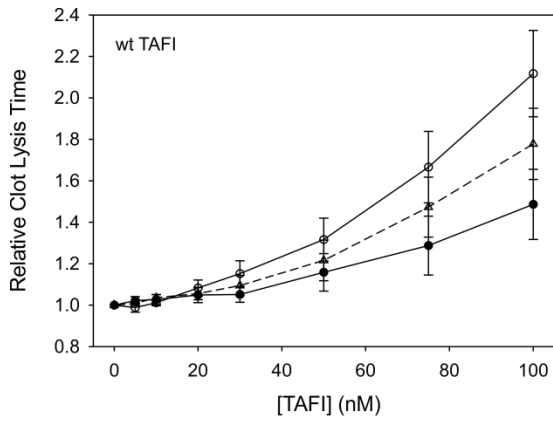


Figure 4-6. Effect of heparin on the antifibrinolytic potential of the TAFI variants.

Clots were assembled from barium-adsorbed TAFI-deficient plasma and supplemented with various concentrations of TAFI variants in the absence of heparin (filled circles) or in the presence of 25 U/mL (open triangles) or 100 U/mL (open circles) heparin. Clotting was initiated with batroxobin and lysis was initiated with tPA. Clot formation and lysis was allowed to proceed at 37°C and lysis was monitored by measurement of absorbance at 405 nm. The time to 50% clot lysis (defined as the midpoint between the maximum and minimum absorbances) was determined and plotted relative to the clot lysis times observed in the absence of TAFI. The data are the means \pm standard error of the mean of at least three independent experiments.

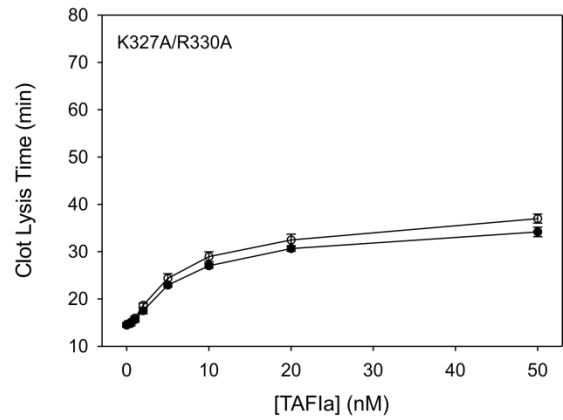
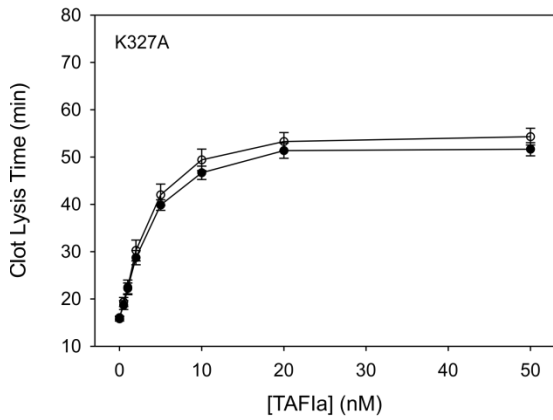
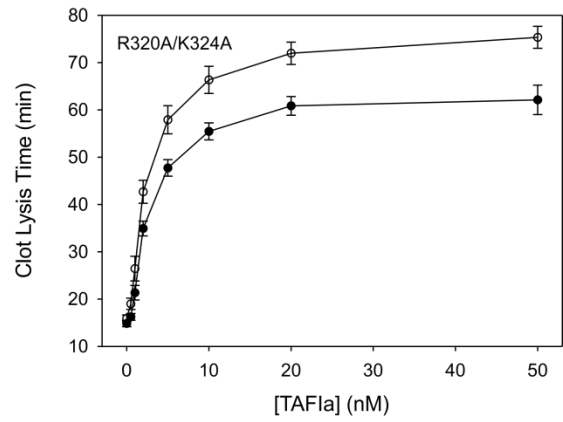
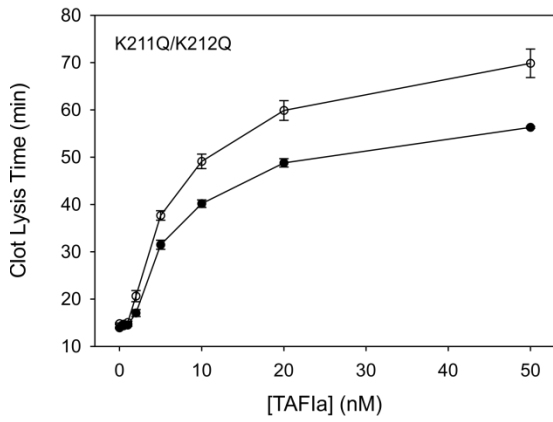
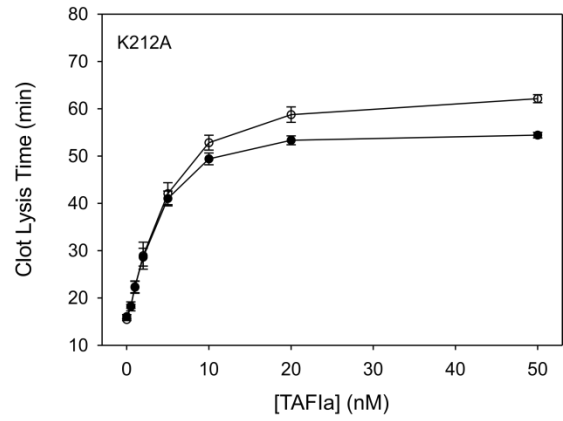
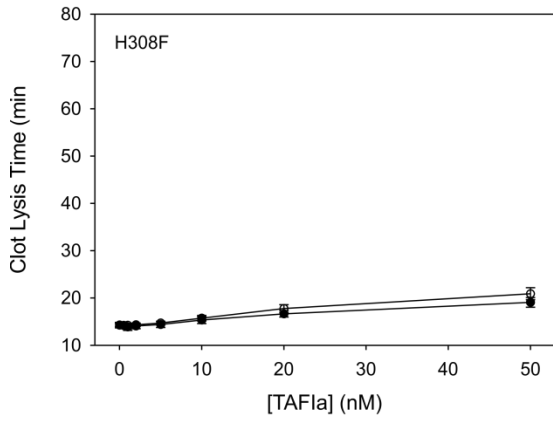
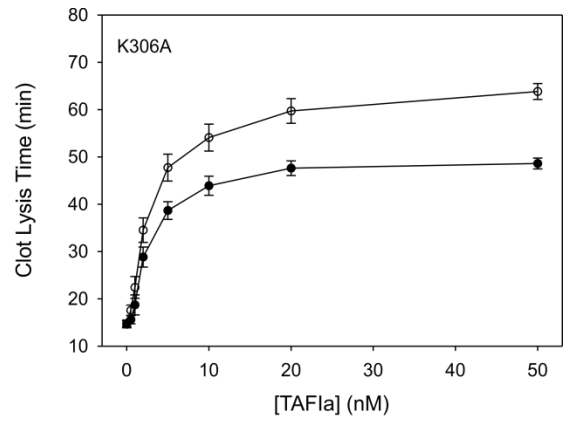
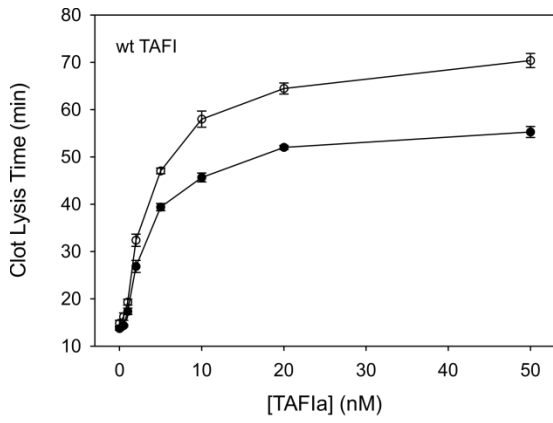


Figure 4-7. Effect of heparin on the antifibrinolytic potential of the TAFIa variants. TAFI was quantitatively activated by incubation with thrombin-TM, and the resultant TAFIa (in different concentrations) was included in clots assembled from barium-adsorbed TAFI-deficient plasma and either lacking (filled circles) or containing (open circles) 100 U/mL heparin. Clotting was initiated with batroxobin and lysis was initiated with tPA. Clot formation and lysis was allowed to proceed at 37°C and lysis was monitored by measurement of absorbance at 405 nm. The time to 50% clot lysis (defined as the midpoint between the maximum and minimum absorbances) was determined. The data are the means \pm standard error of the mean of at least three independent experiments.

4.5 Discussion

It has been reported that GAGs, such as heparin, accelerate activation of TAFI by plasmin and stabilize TAFIa [11]. However, the number and location of the heparin-binding sites on TAFI that mediate these effects are unknown. The report on the bovine crystal structure of TAFI identified several bound sulfate ions which may predict potential heparin-binding sites on TAFI. The sulfate ions bound to the instability region appeared as the most probable heparin-binding site as this would explain the increased half-life of TAFIa in the presence of heparin [16]. In addition, a potential GAG-binding site was identified by sequence homology with heparin-binding proteins [11], although only one of them is present on the surface of the protein and hence accessible for heparin-binding. It is important to consider that binding of individual sulfates may not necessarily represent *bona fide* heparin-protein interactions, since heparin itself is a polymer of varying lengths consisting of sulfated glycosidic units. Therefore, it is possible that some of the residues that are associated with a sulfate ion in the crystal structure do not represent actual heparin-binding sites. Nevertheless, the predicted sites give us clues to begin to identify the TAFI-heparin interaction.

We constructed a series of TAFI variants with mutations in surface-exposed basic residues suggested to be heparin-binding residues. We then measured heparin binding of the TAFI variants and the effect of heparin on their plasmin-mediated activation, enzyme stability, and antifibrinolytic function. Our findings show that no mutation completely eliminated heparin binding, and that Lys327 and Arg330 may represent a functional heparin binding site.

The binding of the variants to the heparin-agarose column indicated that mutations in the instability region of TAFI result in a reduced affinity for heparin since at least half of the protein eluted in the wash fractions. We do not believe that our findings represent fractions of the protein that are or are not capable of binding heparin since if we pool the TAFI in the non-binding fraction and reapply it to the column, a similar fraction does not bind to the column (data not shown).

Although the K306A variant displays slightly reduced affinity for heparin, we do not appear to observe this reflected in the functional properties of the protein, as heparin normally accelerated its activation by plasmin and stabilizes its enzyme form. It does appear to be less potent in inhibiting fibrinolysis than wild-type TAFI both in the presence and absence of heparin, however. This may be a consequence of the slightly reduced stability of the enzyme in the absence of heparin. The H308F variant binds heparin normally but is activated very poorly by plasmin and is far less stable than wild-type TAFIa. It also has almost no antifibrinolytic effect under any of the conditions we employed. In fact, it could be argued that its apparent resistance to the effects of heparin actually reflect its essential lack of functionality. Therefore, it can be concluded that Lys306 and His308 do not constitute a heparin-binding site.

Mutation of Lys211 and/or Lys212 does not significantly impact the heparin affinity of TAFI. It also does not notably alter the stability of the TAFIa variants or the effect of heparin on stability. Differences are observed in the extent of plasmin activation, however. K212A has a much increased K_M value compared to wild-type, whereas K211Q/K212Q has a much reduced k_{cat} value compared to wild-type, although the catalytic efficiency of both variants is increased by heparin to a similar extent. These

effects are reflected in the reduced potency of the respective variants in lysis assays where the antifibrinolytic effect is sensitive to plasmin-mediated TAFI activation (Figure 4-6). Overall, the data with these variants are more consistent with them influencing activation by plasmin than affecting heparin binding.

To date there are few studies characterizing the plasmin-TAFI interaction. A report by Mishra and coworkers identified a monoclonal antibody that specifically inhibits plasmin-mediated TAFI activation: MA-TCK11A9 with the major epitope binding residues Lys268, Ser272 and Arg276 [17]. The MA-TCK22G2 and MA-TCK27A4 were found to inhibit plasmin and thrombin mediated activation and plasmin, thrombin, and thrombin-TM activation, respectively. The residues important for binding MA-TCK22G2 are Thr147 and Ala148 and MA-TCK27A4 additionally binds Phe113 [17]. None of these residues are close to Lys211/212. However, Lys211/212 is on the same side of the protein as Arg92, suggesting that they may be contacted either by the protease domain of plasmin or its lysine-binding kringles.

The mutations in the instability region (dynamic flap) of TAFI had the greatest effect on TAFI binding to heparin. The weakest binding was observed with R320A/K324A. Interestingly, this variant exhibited normal activation by plasmin and acceleration of that activation by heparin. However, this result was not reflected in clot lysis assays with plasmin as the activator, where inhibition of fibrinolysis was very much impaired compared to wild-type. The reason for this observation is not clear. Although the R320A/K324A enzyme was stabilized to a lesser extent by heparin, clot lysis assays using pre-activated TAFIa showed that heparin could raise the plateau lysis time of this enzyme. Interestingly, while an R320Q variant is very unstable compared to wild-type

[13], the variant studied here is actually slightly more stable than wild-type, and has a correspondingly higher plateau lysis time in the absence of heparin. Our observations with the R320A/K324A variant raise the possibility of more than one heparin-binding site on TAFI, with one of the sites being “cryptic” and only exposed upon TAFI activation. It is possible that Arg320, Lys324, Lys327 and Arg330 are all involved in this site, as the dynamic flap is located close enough to the activation domain for its molecular motion to be constrained in the context of the zymogen.

Mutation of Lys327 and Arg330 provides the strongest evidence for the existence of a functional heparin-binding site that influences both TAFI activation and TAFIa inactivation. Both mutations abolish the increase in k_{cat} for plasmin activation seen in response to heparin, although the double mutation results in a profound impairment in plasmin-mediated activation. These effects are reflected in the clot lysis assays with plasmin as the activator; both mutants, particularly the double mutant, show a much reduced response to the addition of heparin. The K327A results in a decreased stabilization of the enzyme, while the K327A/R330A double mutant is both very unstable compared to wild-type and is completely resistant to stabilization by heparin. Both these enzymes show a similar plateau lysis time either in the presence or absence of heparin, and the plateau lysis times of the double mutant are much lower owing to the fact that this enzyme is relatively unstable.

Altogether, the results of this study shed new light on the nature of the TAFI-heparin interaction. Connections can be made between impaired heparin-binding, reduced effects of heparin on activation or stabilization, and changes in antifibrinolytic potential.

The data suggest that TAFI possesses multiple heparin-binding sites which may contribute to the different functions of heparin in regulating TAFI activity.

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Chapter 5

**A novel fluorescent assay for the measurement of activated
Thrombin-Activatable Fibrinolysis Inhibitor (TAFIa) activity**

5.1 Summary

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a procarboxypeptidase present in plasma that acts as a molecular connection between the coagulation and fibrinolysis pathways. TAFI is activated to form TAFIa through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin. Various assays are available to measure the carboxypeptidase activity of TAFIa however, the methods currently available suffer one or more disadvantages including low sensitivity, labour intensivity and complexity. Additionally, none of the available assays are able to monitor the activation of TAFI in real-time during clot formation and dissolution. *Objectives:* To develop a novel fluorescence assay to monitor the process of TAFI activation in real-time. *Methods:* We have developed a novel fluorescence based assay for the measurement of the basic carboxypeptidase activity of TAFIa. A synthetic peptide substrate was designed composed of four amino acids (Gly-Ala-Gly-Arg) modified with an amino-terminal tetramethylrhodamine (TAMRA) fluorophore. An anionic quencher interacts electrostatically with the positively charged carboxyl-terminal arginine side chain in the substrate allowing quenching of the TAMRA fluorescence. The cleavage of the carboxyl-terminal arginine by TAFIa results in the release of the substrate causing an increase in fluorescence. *Results:* The fluorescent quencher dye Evans Blue was determined as the most optimal quencher for the TAMRA-GAGR synthetic peptide substrate and the interaction between the substrate and quencher is not affected by chloride concentration. The sensitivity of the TAMRA-GAGR substrate is far superior to the sensitivity of commonly employed carboxypeptidase B substrates. Due

to its sensitive nature, the activation of TAFI *in vitro* was able to be monitored in real-time and is detectable at TAFIa concentrations as low as 100 pM. *Conclusions:* We have devised a novel fluorescence based assay to assess the function of activated TAFI. The assay is a promising means of monitoring TAFIa activity during clot formation and lysis in real-time.

5.2 Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is plasma procarboxypeptidase that functions as a molecular link between the coagulation and fibrinolysis pathways [1, 2]. The activation of TAFI through proteolytic cleavage by thrombin, thrombin in complex with the endothelial cell cofactor thrombomodulin (TM), or plasmin generate the activated form of the zymogen called activated TAFI (TAFIa) [3-5]. TAFIa possesses basic carboxypeptidase activity and functions by catalyzing the removal of carboxyl-terminal arginine and lysine residues from proteins or peptide substrates [6, 7].

TAFI is an important regulator of the fibrinolytic pathway. TAFIa attenuates fibrinolysis by interfering with the positive feedback in the fibrinolytic cascade. Specifically, TAFIa removes the newly-exposed carboxyl-terminal basic amino acid residues from partially degraded fibrin which are essential for maximally efficient plasminogen activation [6, 7]. A characteristic that differentiates TAFIa from other carboxypeptidases is its intrinsic thermal instability. TAFIa is unstable at body temperature, possessing a half-life of approximately 8-15 minutes. TAFIa undergoes a conformational change that is associated with the loss of its enzymatic activity [8-11].

Several methods have been reported for measuring the carboxypeptidase activity generated upon activation of TAFI. Hydrolysis of synthetic substrates such as hippuryl-arginine or -lysine (Hipp-Arg/Hipp-Lys) and anisylazofornyl-arginine or -lysine (AAFR/AAFK) is monitored spectrophotometrically at wavelengths 350 nm and 254 nm, respectively. While this approach can be used to quantify TAFIa levels in a purified system [10, 12-14], it is not possible to directly detect TAFIa activity in plasma. This is

primarily due to turbidity induced interference with absorbance measurements resulting from the formation of a fibrin clot and the high protein concentration present in the plasma milieu. In addition, spectrophotometric detection of cleavage of these substrates suffers limited sensitivity. Highly sensitive methods to detect Hipp-Arg cleavage that involves either detection of the products by HPLC [15] or a coupled redox fluorescence-based assay [16] have also been described, but these assays are not capable of monitoring TAFIa generation *in situ*. Likewise, a highly sensitive assay for TAFIa with a detection limit between 1-2 pM has been described that utilizes electrochemiluminescence [17]. Another sensitive assay for measurement of TAFIa takes advantage of its ability to remove basic residues from soluble fibrin degradation products and measures the release of plasminogen therefore indirectly measuring the concentration of TAFIa in plasma. However, the requirement for the preparation of numerous biomolecular components for the assay is cumbersome and can lead to variability [18].

In the present study, we have developed a novel fluorescence assay for the measurement of TAFIa activity with picomolar sensitivity. The synthetic substrate designed for the assay, termed TAMRA-GAGR, is a peptide composed of four amino acids (Gly-Ala-Gly-Arg) modified with an amino-terminal tetramethylrhodamine (TAMRA) fluorophore. An anionic quencher interacts electrostatically with the positively charged carboxyl-terminal arginine in the substrate, thereby quenching the fluorescence of the bound TAMRA fluorophore. Cleavage of the carboxyl-terminal arginine by TAFIa causes the release of the associated quencher resulting in a measurable increase in fluorescence, indicative of TAFIa activity and thus allowing the concentration of TAFI in the sample to be quantified.

5.3 Experimental Procedures

5.3.1 *Materials*

The synthetic TAFIa substrate TAMRA-GAGR was synthesized by CanPeptide Inc. (Pointe-Claire, Quebec, Canada) and was purified by HPLC. The quenching dye, Evans Blue, was purchased from Tocris Bioscience (Bristol, UK), Rose Bengal and Trypan Blue from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada), Acid Green 27 from Tokyo Chemical Industry (Kita-Ku, Toyko, Japan), and Ponceau S from BioShop Canada Inc. (Burlington, ON, Canada). The synthetic carboxypeptidase substrate anisylazofornyl-arginine (AAFR) was obtained from Bachem Americas, Inc. (Torrance, CA, USA). Hippuryl-L-arginine was obtained from Sigma-Aldrich. The thrombin inhibitor D-phenylalanylprolylarginyl chloromethylketone (PPack) was purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, penicillin/streptomycin/fungizone (PSF) were obtained from Invitrogen Canada Inc. (Burlington, ON, Canada). Newborn Calf Serum was obtained from Sigma-Aldrich. Effectene transfection reagent was purchased from QIAGEN, Inc. (Toronto, ON, Canada). Methotrexate was purchased from Mayne Pharma, Inc. (Salisbury, SA, Australia). Carboxypeptidase B (CPB), isolated from porcine pancreas, was purchased from Worthington Biochemical Company (Lakewood, NJ, USA). Thrombin, rabbit-lung thrombomodulin and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, USA). The mouse-anti-human TAFI monoclonal antibody MA-T4E3 was obtained from Dr. Ann Gils (Katholieke Universiteit Leuven, Leuven, Belgium) and coupled to CNBr Activated Sepharose 4B (GE Healthcare

Life Sciences, Mississauga, ON, Canada) according to manufacturer's instructions (2-6 mg antibody/mL resin).

5.3.2 *Recombinant Wild-type TAFI and TAFI-CIIYQ*

The expression plasmid is wild-type TAFI-pNUT vector, which contains human TAFI cDNA which contains threonine at positions 147 and 325 [10]. The stable TAFI variant, TAFI-S305C-T325I-T329I-H333Y-H335Q (TAFI-CIIYQ), contains mutations in the dynamic flap region that stabilize the region and increase the half-life of TAFIa approximately 180-fold [19].

5.3.3 *Expression and Purification of Recombinant Wild-type TAFI and TAFI-CIIYQ*

Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (1:1) (DMEM/F-12) containing 5% (v/v) newborn calf serum (NCS) and 1% (v/v) PSF in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere. An expression plasmid encoding wild-type TAFI and TAFI-CIIYQ was transfected into cells using Effectene transfection reagent (2 µg plasmid DNA and 60 µL of transfection reagent per 100 mm plate of cells). After 24 hour incubation with the transfection mixture, the cells were then cultured in DMEM/F-12 containing 5% NCS and 1% PSF supplemented with 400 µM methotrexate for approximately 2 weeks, with the medium changed every other day, for the selection of stable cell lines expressing recombinant wild-type TAFI and TAFI-CIIYQ. For recombinant wild-type TAFI and TAFI-CIIYQ production, a stably expressing line was cultured in triple flasks (500 cm², Nunc, Roskilde, Denmark) in Opti-MEM containing 1% (v/v) PSF and 40 µM ZnCl₂.

Conditioned medium was collected at 48 hour intervals and replaced with fresh medium. Harvested conditioned medium was centrifuged at $3000 \times g$ for 5 minutes, supplemented with Tris-HCl, pH 8.0 (to 5 mM), reduced glutathione (to 0.5 mM) and phenylmethanesulphonyl fluoride (PMSF) to (2 μ M) and stored at -20°C .

To purify wild-type TAFI and TAFI-CIIYQ expressed from the pNUT vector, conditioned medium was passed through a 0.22- μ m filter prior to affinity chromatography over a 1-mL MA-T4E3-Sepharose column at 4°C as previously described [14]. Protein containing fractions were pooled and then were concentrated and exchanged into HBST (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween 80) using Amicon ultra-4 centrifugal filter units, MWCO 10 kDa. Purified TAFI was quantified by measurement of absorbance at 280 nm and 320 nm ($\epsilon_{1\%, 280} = 26.4$; $M_r = 60,000$), aliquoted and stored at -70°C .

5.3.4 Spectrophotometric Measurements

The absorbance spectrum, fluorescence excitation spectrum and emission spectrum of the TAMRA-GAGR peptide substrate was obtained with a Spectramax M5e plate reader (Molecular Devices, Sunnyvale, CA, USA). The TAMRA-GAGR peptide substrate was dissolved in water to a final concentration of 1000 μ M, aliquoted and stored at -20°C . A 200 μ L sample of 1000 μ M TAMRA-GAGR sample was analyzed in a clear UV sensitive microtitre plate (Corning) or black microtitre plate (Dynatech) for absorbance or fluorescence measurements, respectively. All measurements were determined at 21°C .

5.3.5 Screening quencher dyes of TAMRA-Gly-Ala-Gly-Arg substrate

The quencher dyes Evans Blue, Ponceau S, Acid Green 27, Rose Bengal, and Trypan Blue were dissolved in water to a final concentration of 10 mM and stored in an opaque container at 21°C. The fluorescence intensity of TAMRA-GAGR was measured as a function of quencher dye concentration to determine the optimal dye concentration required to obtain maximal quenching of TAMRA-GAGR. The quencher dye was titrated (0-80 µM) with a fixed concentration of TAMRA-GAGR (5 µM) in an HBST (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween 80) buffer solution. The fluorescence intensity was measured at 550 nm excitation and 590 nm emission wavelengths, with a cut-off filter at 580 nm at 21°C. A black microtitre plate was used and a final well volume of 100 µL was maintained for each assay.

The dissociation constant between each quencher dye and TAMRA-GAGR substrate was determined based on its relative fluorescence. The relative fluorescence was calculated using the equation shown below.

$$\text{RFU}_{\text{relative}} = \frac{I_{\text{max}} - I}{I_{\text{max}} - I_{\text{min}}} \quad \text{Eq. 1}$$

Where I_{max} is the fluorescence of a solution containing 0 µM quencher, I is the fluorescence of each solution of varying quencher concentration, and I_{min} is the fluorescence of a solution containing 80 µM quencher. A plot of relative fluorescence versus quencher concentration was generated for each quencher using these calculated values. The complete data set was fit to the ligand binding equation for one-site saturation by non-linear regression analysis (Sigmaplot 10, SPSS Inc., Chicago, IL) to determine the

dissociation constant. The plots appeared sigmoidal, and so the data were fit to the Hill equation to find the Hill constant (n) and the dissociation constant K_d . The Hill equation is shown below.

$$\log\left(\frac{R}{1-R}\right) = n \cdot \log[Q] - n \cdot \log K_d \quad \text{Eq. 2}$$

Where R is the relative fluorescence quenching, n is the Hill constant, Q is the concentration of the quencher, and K_d is the dissociation constant.

5.3.6 Determination of the Effect of Chloride concentration on TAMRA-Gly-Ala-Gly-Arg substrate and quencher interaction

The NaCl concentration was varied (0-150 mM) with TAMRA-GAGR (5 μ M final concentration) and Evans Blue (40 μ M final concentration) in a HEPES buffer solution (20 mM HEPES pH 7.4, 0.01% (v/v) Tween 80). The fluorescence intensity was monitored as described above.

5.3.7 Determination of the Effect of Evans Blue quencher concentration on detection of enzymatic activity

The Evans Blue quencher dye was varied (0-80 μ M final concentration) with a fixed concentration of TAMRA-GAGR (5 μ M final concentration) in HBST buffer solution in a volume of 95 μ L. The fluorescence intensity was monitored for a short time period then 5 μ L of TAFIa or CPB was added to 1 nM final concentration in a final well

volume of 100 μL . The fluorescence intensity continued to be monitored as described above.

5.3.8 Thermal Stability and Activity of Wild-type TAFIa

Purified recombinant wild-type TAFI (1 μM) was activated by incubation with thrombin (25 nM), TM (100 nM), CaCl_2 (5 mM) in HBST at 21°C for 15 min. Thrombin activity was then quenched by the addition of PPAck (to 1 μM) before the mixture was placed on ice. The activated TAFI was divided into aliquots corresponding to various time points in the presence of TAMRA-GAGR (5 μM) or Evans Blue (40 μM) alone or in combination and incubated at 37°C. At each time point, the corresponding aliquots were removed and placed on ice. Residual TAFIa activity was determined by measurement of the rate of hydrolysis of AAFR by monitoring the absorbance at 350 nm. Non-linear regression of the data to an equation describing first-order exponential decay was used to determine decay rate constants and thus half-life of wild-type TAFIa, as previously described [14].

5.3.9 Generating a Standard Curve for Measuring TAFIa Concentration

Wild-type TAFI (1 μM) was completely activated by incubating with thrombin (25 nM), TM (100 nM) and CaCl_2 (5 mM) at 21 °C for 10 minutes. The reaction was stopped by the addition of the thrombin inhibitor PPAck (1 μM final concentration) and placed on ice. Activated TAFI was diluted to obtain known amounts of TAFIa (0 – 250 nM) and 10 μL was added into a 90 μL solution containing TAMRA-GAGR (5 μM final concentration) and Evans Blue (40 μM final concentration) in HBST and fluorescence

was monitored as described above. A standard curve was constructed by plotting the initial rates of hydrolysis of TAMRA-GAGR *versus* the concentration of activated TAFI. A standard curve was constructed for TAFIa-CIIYQ in the same manner. A standard curve was constructed for CPB as follows: a 10 μL solution of known amounts of CPB (0 – 15.6 nM) was added into a 90 μL mixture of TAMRA-GAGR (5 μM) and Evans Blue (40 μM) in HBST and fluorescence was monitored as described above.

5.3.10 *Measuring TAFI activation in real-time*

The activation of TAFI in real-time was examined utilizing the TAMRA-GAGR substrate and Evans Blue quencher. An 87.5 μL solution consisting of TAMRA-GAGR (5 μM final), Evans Blue (40 μM final), CaCl_2 (5 mM final) and varying TAFI concentrations (0-200 nM final) in HBST was added to the wells of a black microtitre plate. Then a 12.5 μL mixture of thrombin (25 nM final) and thrombomodulin (100 nM final) was added to the wells and the fluorescence was immediately monitored. From the linear portion of the initial rate of substrate hydrolysis the RFU/min^2 was calculated and plotted *versus* the concentration of TAFI zymogen.

5.3.11 *Activity of TAFIa toward synthetic substrates*

Various concentrations of TAFIa (0 – 22 nM), generated as described above, were added into a solution containing the synthetic carboxypeptidase substrate hippuryl-L-arginine (HR) (1.2 mM final) or AAFR (120 μM final) to achieve a final volume of 100 μL . All reactions were performed in the wells of a microtiter plate which had been pre-treated (overnight) with HBS/1% Tween 80 and thoroughly rinsed with deionized

distilled water. Absorbance was monitored at 254 nm (HR) or 350 nm (AAFR) using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA). The initial rates of hydrolysis of HR or AAFR were determined from the slope of the absorbance *versus* time relationship. At the same concentrations of TAFIa, the activity towards TAMRA-GAGR was monitored under the same conditions described above. The initial rate of TAMRA-GAGR hydrolysis was determined from the slope of the RFU *versus* time relationship.

5.4 Results

5.4.1 TAMRA-Gly-Ala-Gly-Arg substrate Assay Concept

A schematic representation of the assay is presented in Figure 5-1. The future application of this assay is to be able to detect TAFIa activity in real-time as it becomes activated during lysis assays since currently there is no simple method for measuring TAFIa activity in this manner.

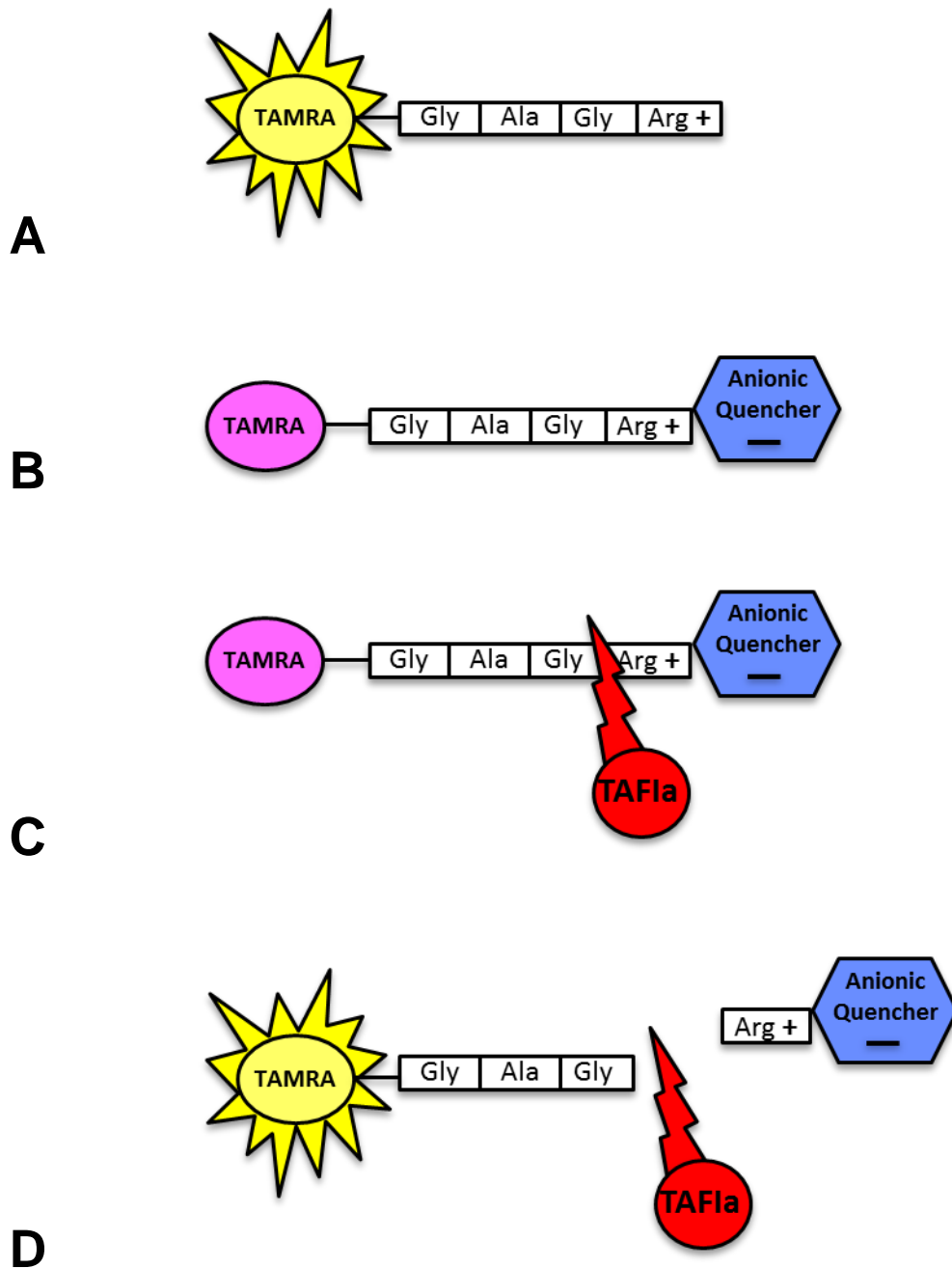


Figure 5-1. Schematic representation of the fluorescent carboxypeptidase assay. A) The synthetic peptide substrate is composed of four amino acids conjugated to the fluorophore TAMRA (TAMRA-Gly-Ala-Gly-Arg). B) An anionic quencher in solution will interact with the positively charged carboxyl-terminal arginine in the substrate. C) Addition of TAFIa, a basic carboxypeptidase, will result in cleavage of the carboxyl-terminal arginine. D) Dissociation of arginine and the associated quencher from the substrate will result in a measurable increase in TAMRA's fluorescence.

5.4.2 *Spectral Properties of TAMRA-Gly-Ala-Gly-Arg substrate*

To determine the maximum excitation and emission wavelength prior to performing fluorescence assays, the excitation spectrum and emission spectrum of the TAMRA-GAGR substrate was obtained. The excitation spectrum of the intact TAMRA-GAGR substrate is bell-shaped with a maximum at 550 nm. The emission spectrum of the substrate possesses a similar shape with a maximum at 590 nm. The absorbance spectrum possesses a broad peak between 450 and 650 nm. The fluorescence and absorbance spectra of the intact TAMRA-Gly-Ala-Gly-Arg substrate is presented in Figure 5-2.

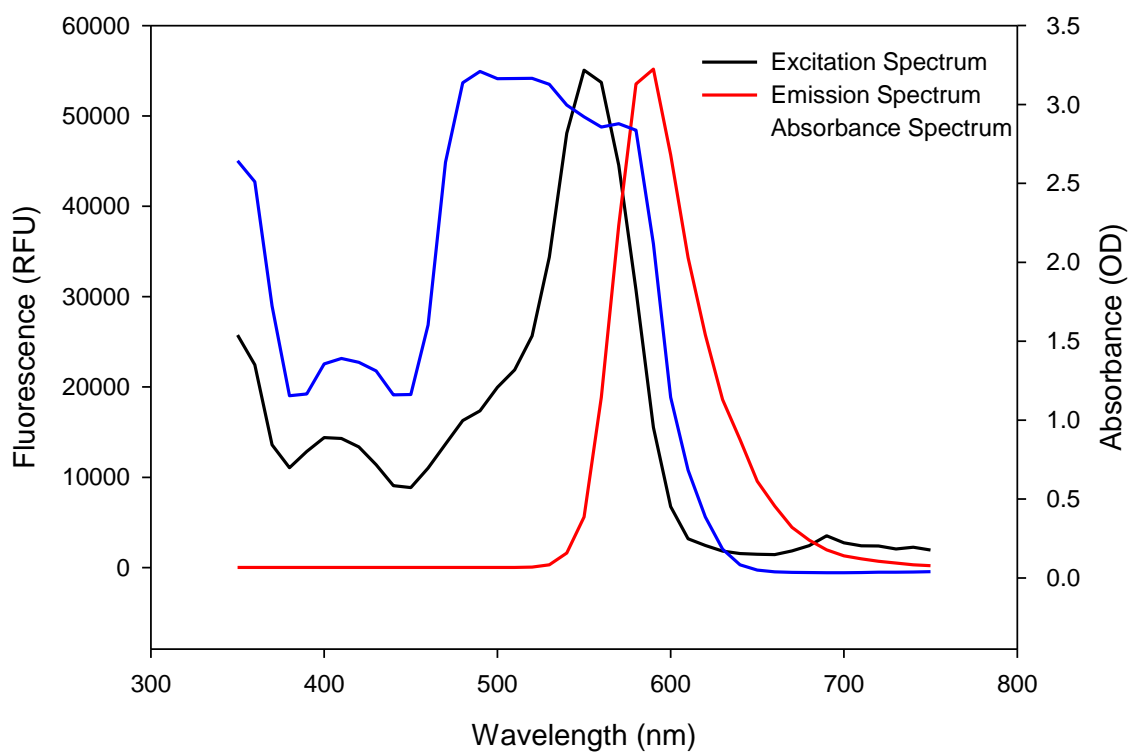


Figure 5-2. Spectral properties of TAMRA-Gly-Ala-Gly-Arg substrate. The absorbance spectrum (blue), excitation (black) and emission (red) spectrum of the TAMRA-GAGR peptide substrate. The substrate (1000 μ M) was dissolved in water.

5.4.3 Fluorescence *quenching of TAMRA-Gly-Ala-Gly-Arg substrate by binding of quencher*

The extent of fluorescence quenching of the TAMRA-GAGR substrate by different quencher dye molecules was examined in order to identify the best quencher of the TAMRA fluorescent moiety and to determine the optimal ratio of quencher to 5 μM TAMRA-GAGR substrate to use for the fluorescence assays. The following quencher dyes were screened against TAMRA-GAGR to identify the optimal quencher: Evans Blue, Ponceau S, Acid Green 27, Rose Bengal, and Trypan Blue (Figure 5-3). Quenching of the fluorescence of TAMRA-GAGR by Evans Blue is readily observable under ultraviolet light (Figure 5-4). The fluorescence quenching of the substrate by the quencher dyes was evaluated by titrating the TAMRA-GAGR substrate with different concentrations of quencher and monitoring the fluorescence. The fluorescence quenching of TAMRA-GAGR by the quencher dye molecules from most to least pronounced is as follows: Evans Blue, Ponceau S, Trypan Blue, Rose Bengal and Acid Green 27 (Figure 5-5). As the binding curves appeared to be sigmoidal in shape, the quenching data were fit to the Hill equation (Figure 5-6), which showed positive cooperativity was occurring (Hill constants > 1 ; Table 5-1). The extent of positive cooperativity was consistent at all concentrations of quencher in all cases except for Evans Blue, where the Hill constant was observed to increase at the higher concentrations of quencher (Figure 5-6). The concentration of Evans Blue to use in the fluorescence assays was chosen to be the concentration at the beginning of the plateau where the substrate becomes significantly quenched (40 μM), but before the large increase in positive cooperativity.

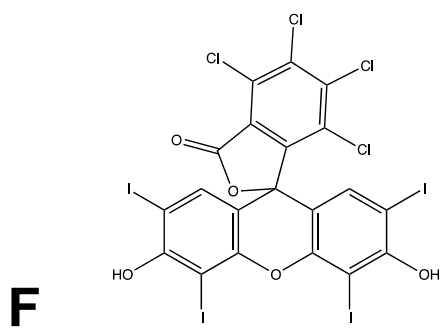
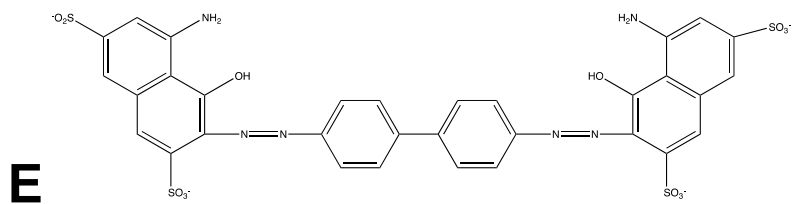
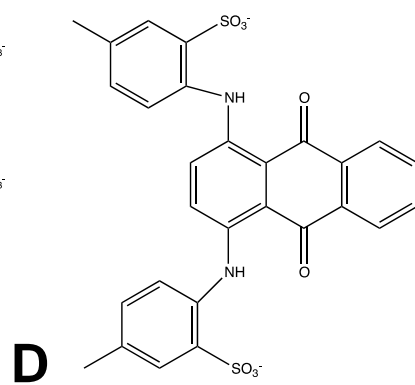
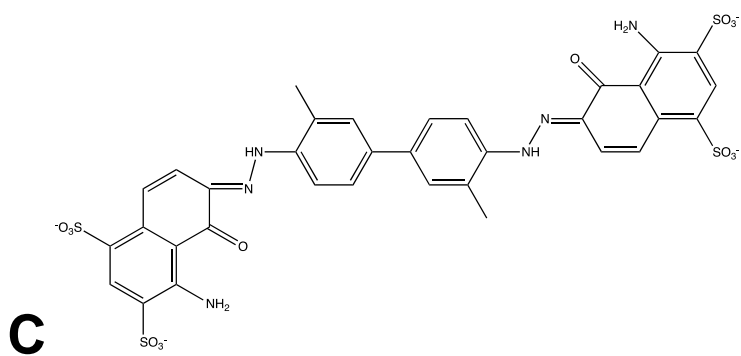
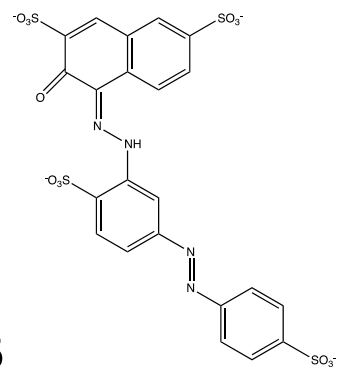
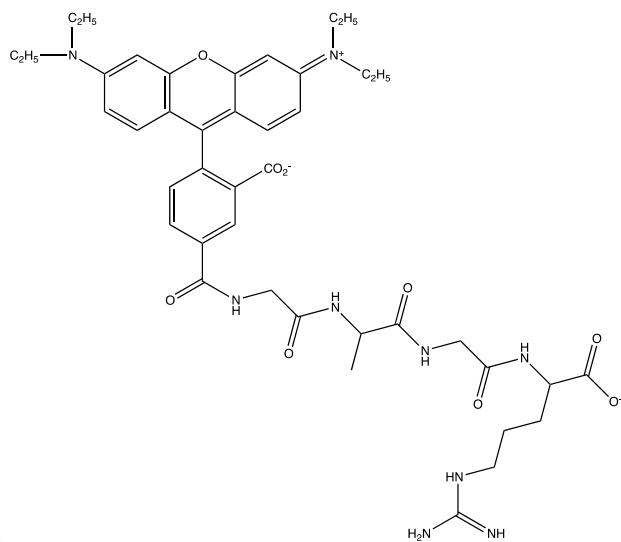


Figure 5-3. Structure of components of the fluorescent assay. This assay involves A) a synthetic peptide, Gly-Ala-Gly-Arg with an amino-terminal fluorophore, TAMRA. Various quencher dyes were analyzed for their ability to quench TAMRA's fluorescence including: B) Ponceau S, C) Evans Blue, D) Acid Green 27, E) Trypan Blue, F) Rose Bengal.

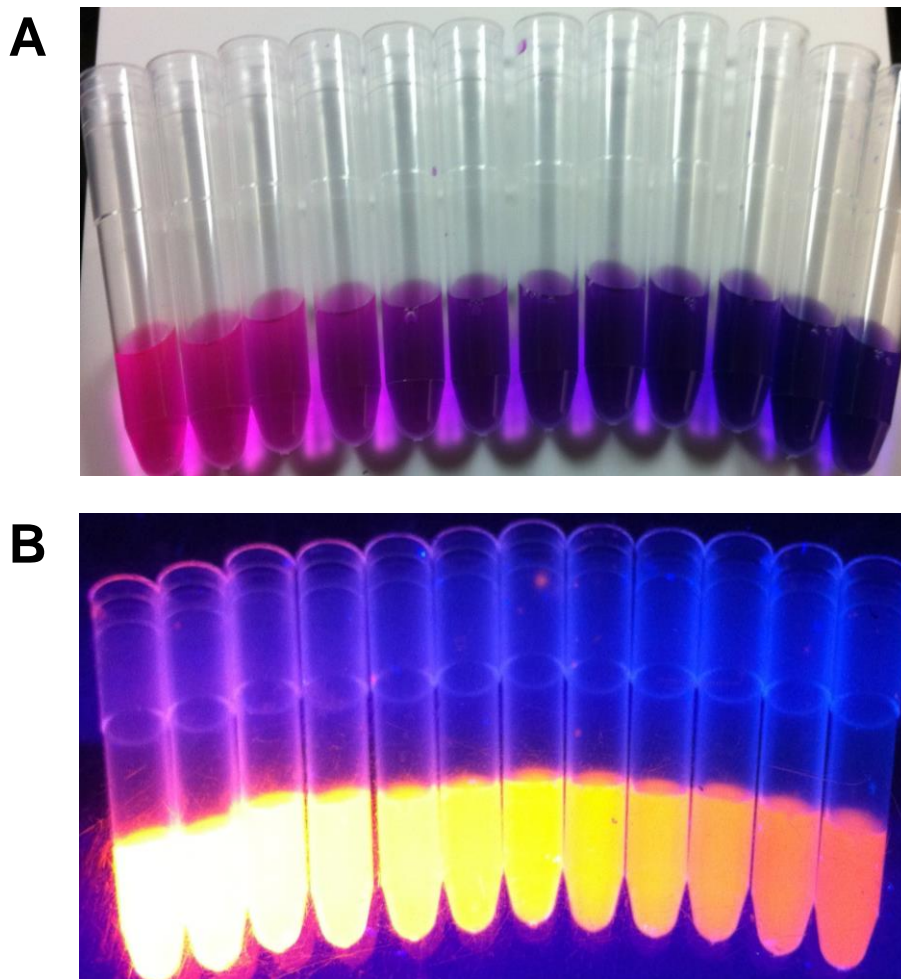


Figure 5-4. Visualization of Evans Blue titration with TAMRA-Gly-Ala-Gly-Arg substrate. Titertubes containing varying concentrations of Evans Blue (titrated from 0-80 μM , left to right) into 5 μM TAMRA-GAGR and HBST buffer were imaged in A) visible light and B) ultraviolet light.

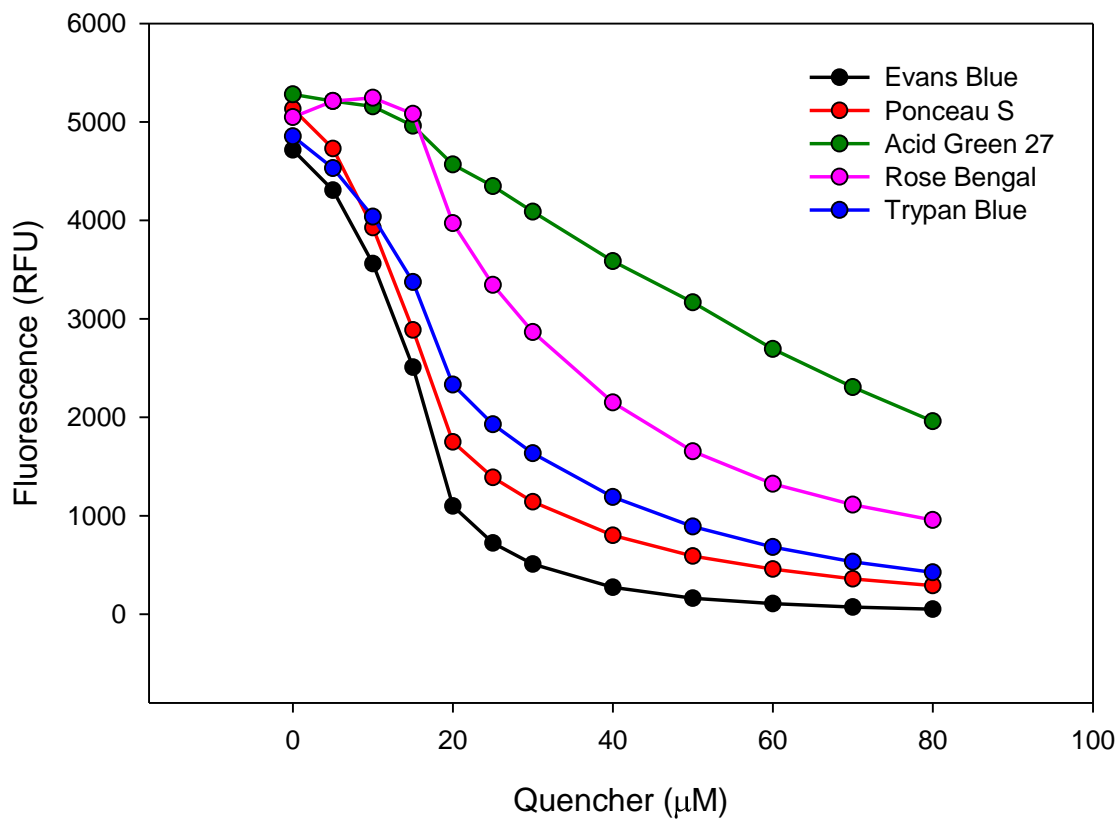


Figure 5-5. Quencher titration of TAMRA-Gly-Ala-Gly-Arg substrate. The concentration of the TAMRA-GAGR peptide substrate was fixed at 5 μM in HBST buffer and varying concentrations of quencher were titrated from 0-80 uM and the fluorescence was monitored. The quenchers are indicated: Evans Blue (black), Ponceau S (red), Acid Green 27 (green), Rose Bengal (magenta), and Trypan Blue (blue).

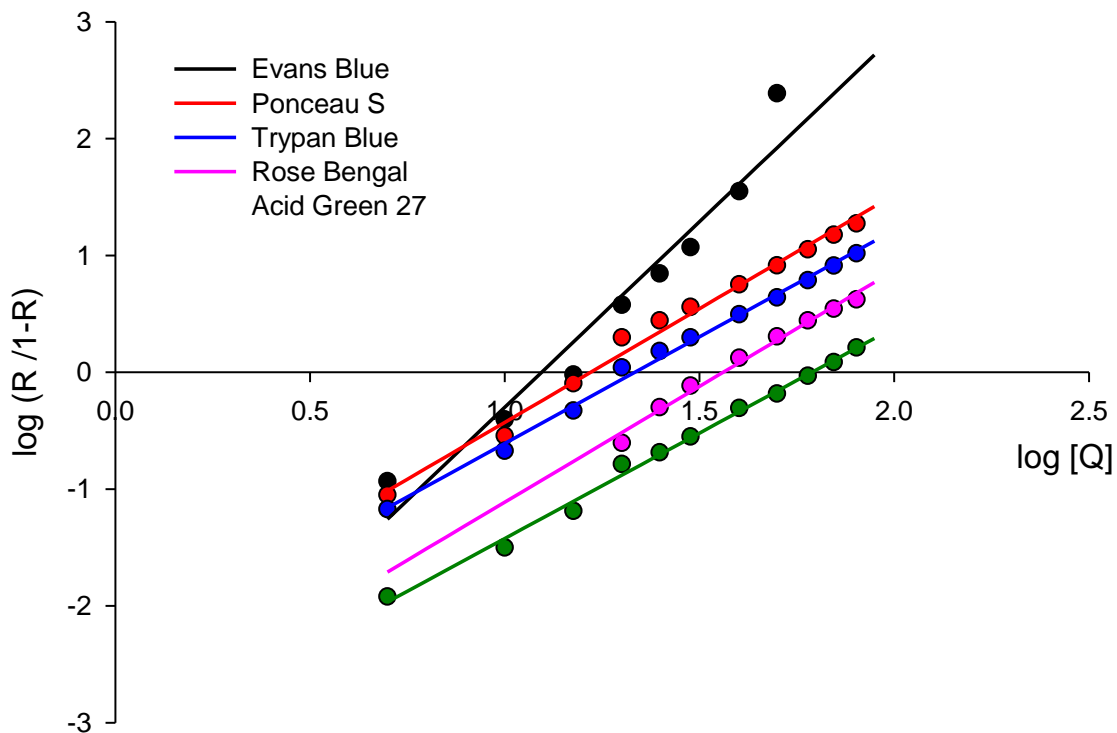


Figure 5-6. Hill plot of TAMRA-Gly-Ala-Gly-Arg substrate with quenchers. The quencher titration data was fit using the Hill equation $\log(R/(1-R)) = n\log([Q]) - n\log(K_d)$, where R is the relative fluorescence quenching, n is the Hill constant, K_d is the dissociation constant, and $[Q]$ is the quencher concentration. The slope of the curve represents the Hill constant and the intercept with the x-axis provides $n\log(K_d)$. The quenchers are indicated: Evans Blue (black), Ponceau S (red), Acid Green 27 (green), Rose Bengal (magenta), and Trypan Blue (blue).

Table 5-1. Dissociation constant and Hill constant between quencher and substrate^a

Quencher	K_d μM	Hill constant n
Evans Blue	2.2 ± 0.1	3.18
Ponceau S	4.2 ± 0.1	1.94
Acid Green 27	9.8	1.80
Rose Bengal	6.1	1.98
Trypan Blue	5.4	1.83

^aData are the means \pm standard errors of the mean of 1-3 independent experiments performed in triplicate

5.4.4 Influence of Chloride on TAMRA-Gly-Ala-Gly-Arg substrate and quencher interaction

The influence of sodium chloride concentration on the interaction between the TAMRA-GAGR substrate and Evans Blue quencher was evaluated by varying the sodium chloride concentration and evaluating the fluorescence intensity. Although there was a slight increase in the fluorescence intensity as a function of chloride concentrations, the fluorescence intensity remained quenched relative to the absence of quencher at 150 mM NaCl (Figure 5-7).

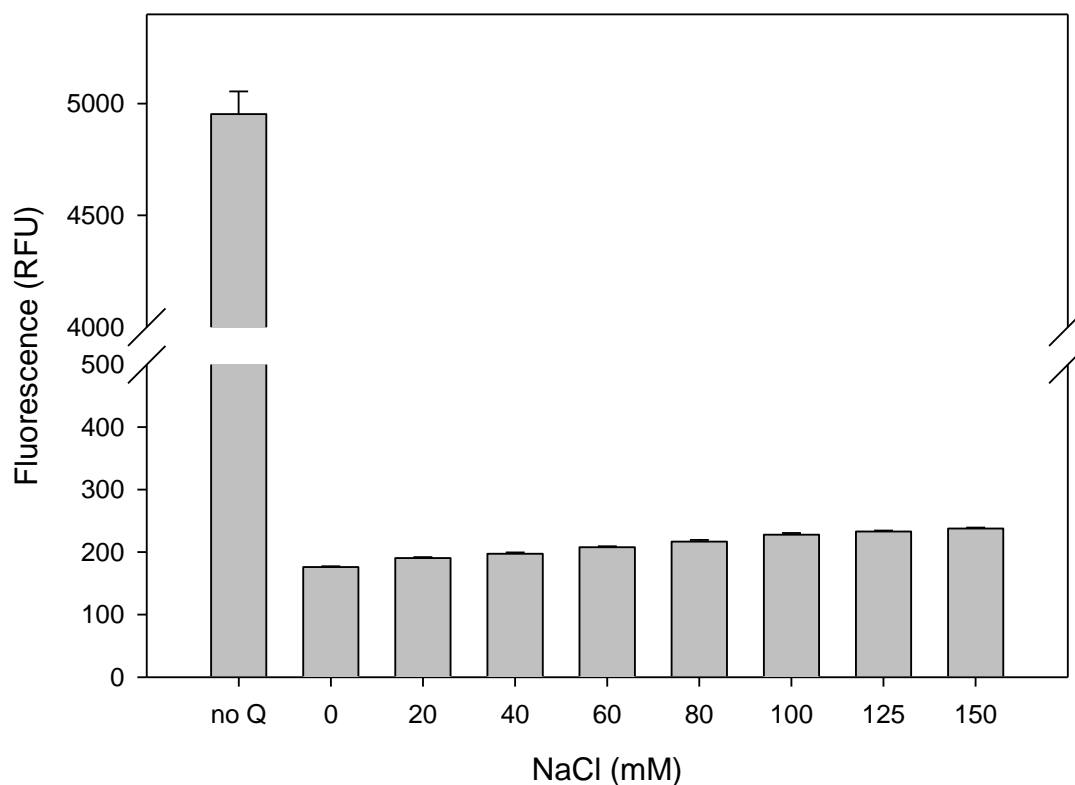


Figure 5-7. Effect of NaCl on TAMRA-Gly-Ala-Gly-Arg substrate and quencher interaction. The NaCl concentration was varied and the initial fluorescence intensity was evaluated to assess the interaction between the TAMRA-GAGR substrate and Evans Blue quencher. The first bar on the left represents the initial intensity of the TAMRA-GAGR substrate in the absence of quencher (Q) but in buffer containing 150 mM NaCl. The subsequent bars indicate the initial intensity of TAMRA-GAGR and Evans Blue in buffer containing varying NaCl concentrations. The data are the means \pm standard errors of the mean of three independent experiments.

5.4.5 *Influence of Evans Blue on detection of enzymatic activity*

The effect of the concentration of the Evans Blue quencher on the detection of wild-type TAF1a or CPB enzyme activity towards the TAMRA-GAGR substrate was examined by varying the concentration of the quencher in an HBST buffer solution with a constant TAMRA-GAGR concentration while monitoring the fluorescence intensity over time. Upon addition of the enzyme, the rate of TAMRA-GAGR hydrolysis increased with increased Evans Blue concentration and reached a maximum at 40 μM and 25 μM Evans Blue for wild-type TAF1a (Figure 5-8A) and CPB (Figure 5-8B), respectively, and then the rate of hydrolysis gradually decreased.

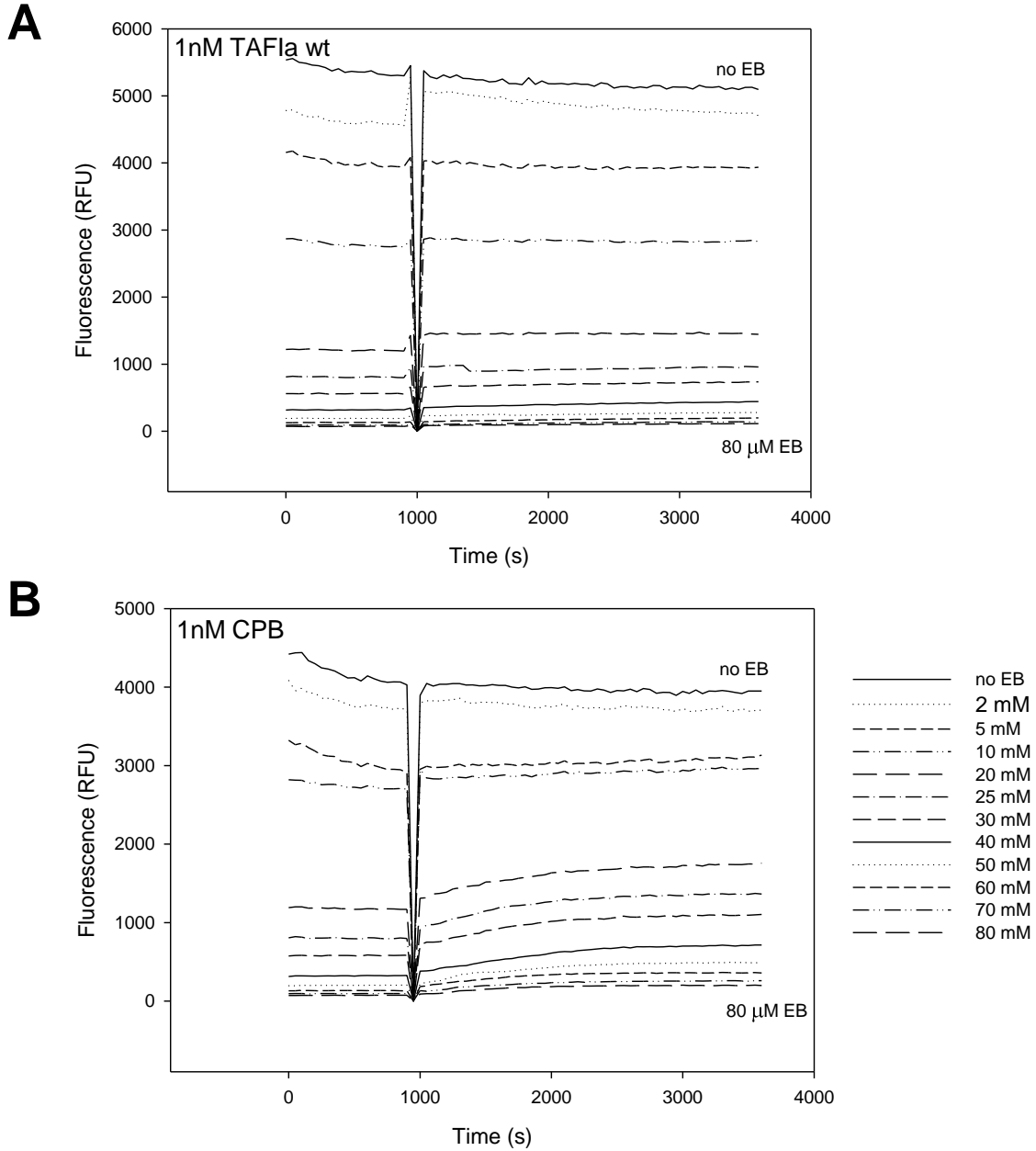
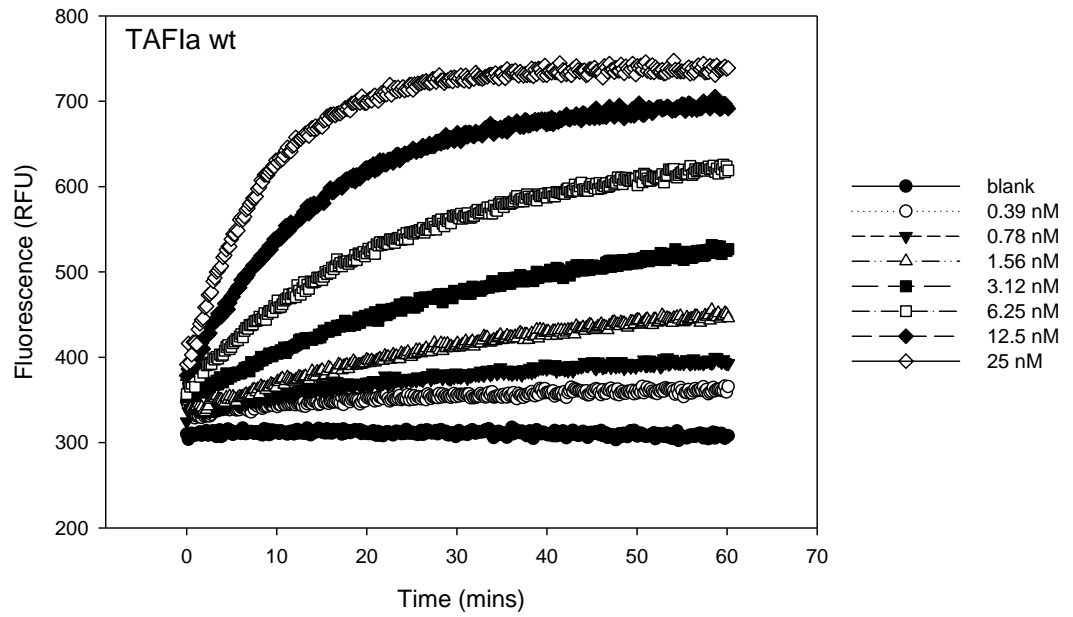
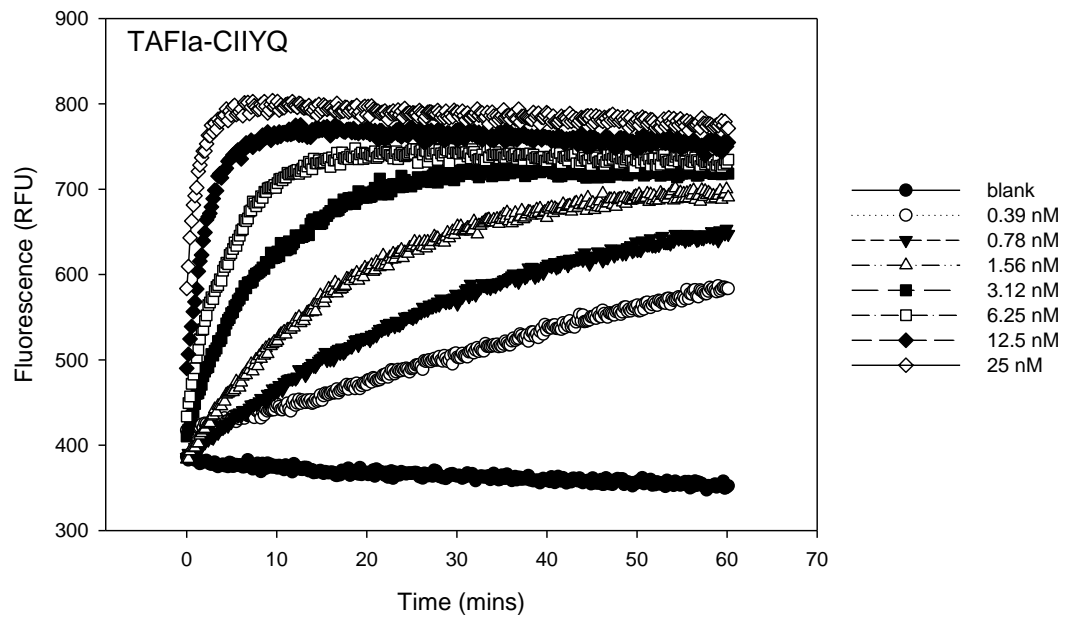


Figure 5-8. Effect of Evans Blue on detection of enzymatic activity. The Evans Blue quencher concentration was varied (0-80 μ M) at a fixed concentration of TAMRA-GAGR (5 μ M) in HBST buffer and fluorescence intensity was monitored. Following addition of 1 nM enzyme, A) wild-type TAFIa and B) CPB to buffer containing varying Evans Blue concentrations the fluorescence intensity continued to be monitored.

5.4.6 *Standard Curves*

Standard curves were created for wild-type TAFIa, TAFIa-CIIYQ and CPB to determine whether a relationship exists between the concentration of the enzyme and the rate of hydrolysis of the TAMRA-GAGR substrate to enable quantitative analysis. The enzymes were serially diluted to various known concentrations then added to the TAMRA-GAGR and Evans Blue substrate mixture and fluorescence intensity was monitored over time. An example of the fluorescence profiles of wild-type TAFIa, TAFIa-CIIYQ and CPB that demonstrate increase in fluorescence due to the cleavage of TAMRA-GAGR by the respective enzymes is shown in Figure 5-9. Standard curves were constructed by plotting the initial rates of hydrolysis of TAMRA-GAGR *versus* the concentration of enzyme (Figure 5-10). The limit of detection for wild-type TAFIa and TAFIa-CIIYQ is 0.0977 nM and for CPB is 0.0061 nM (Figure 5-10). Additionally, to determine whether the TAFI zymogen would have activity toward the TAMRA-GAGR substrate a standard curve was generated using the same concentrations of zymogen. No activity was observed when the TAFI zymogen was used (data not shown).

A**B**

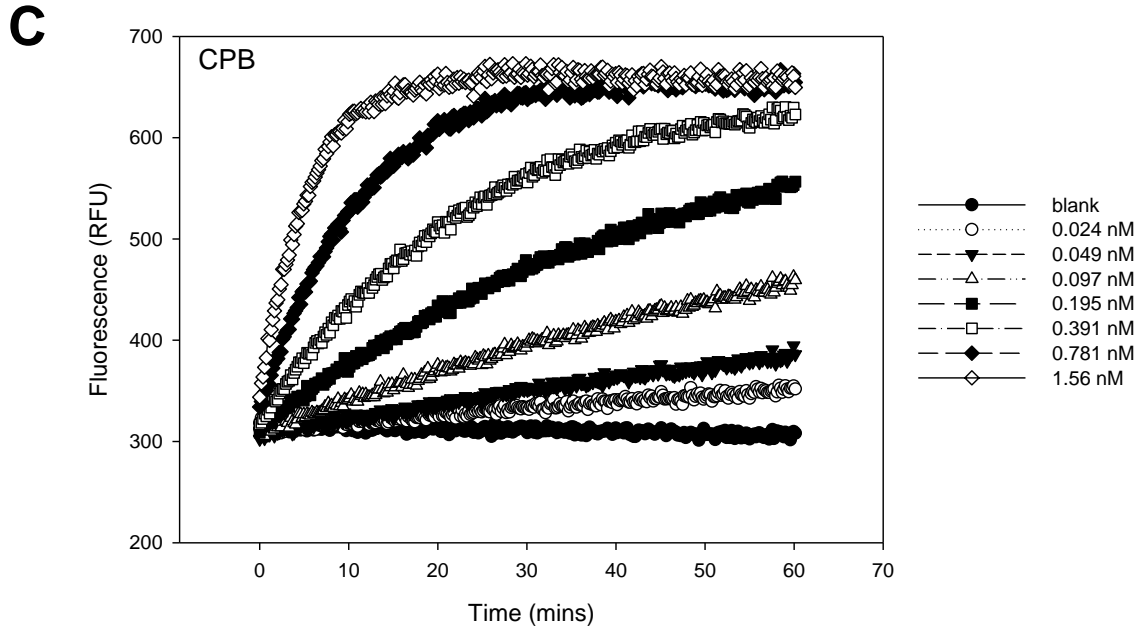


Figure 5-9. Standard fluorescence progress curves. Known concentrations of enzyme were added to a TAMRA-GAGR and Evans Blue substrate mixture and the hydrolysis of the substrate was monitored over time. Representative standard progress curves are shown for A) wild-type TAFIa, B) TAFIa-CIIYQ, and C) CPB.

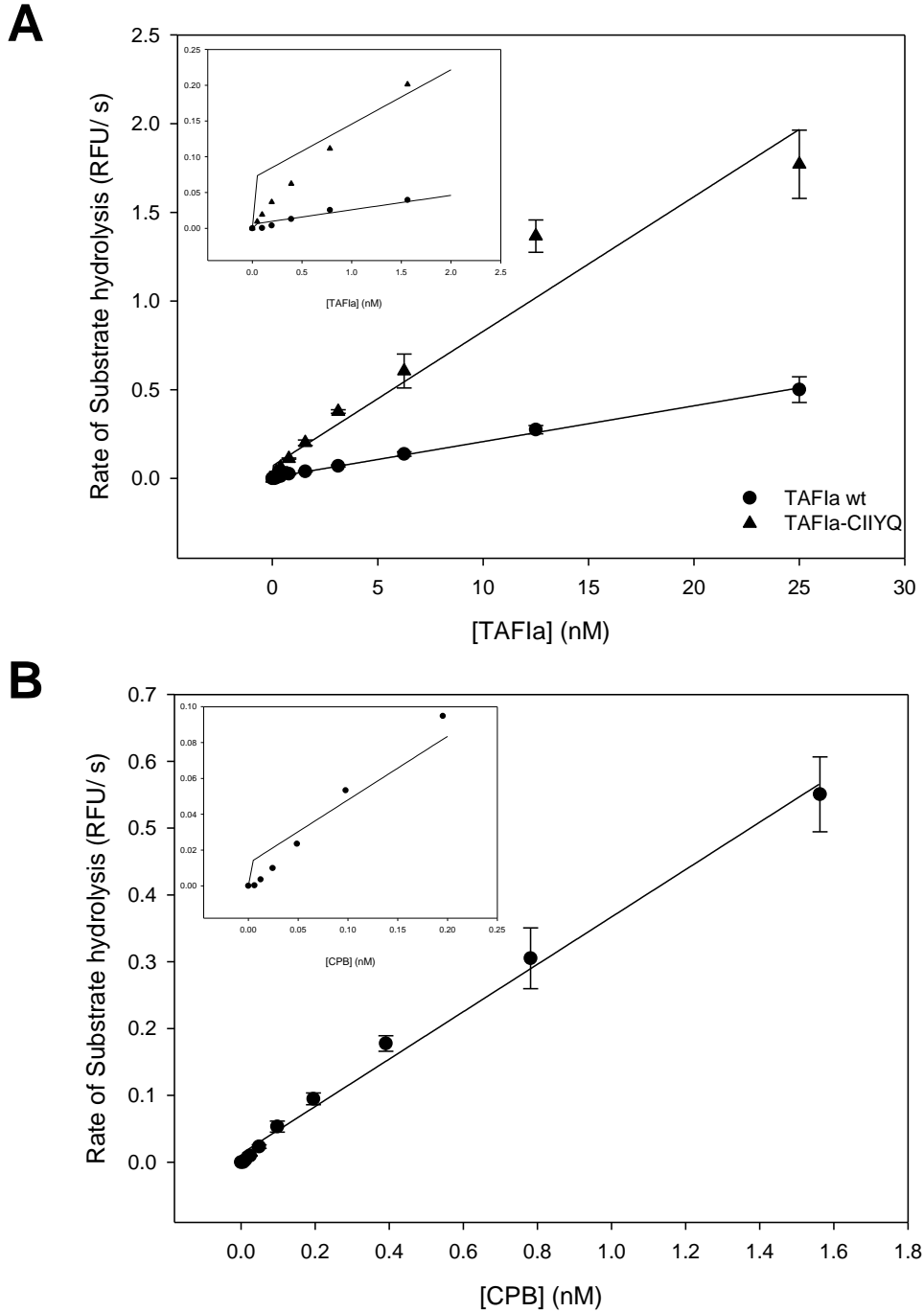


Figure 5-10. Standard curve plots. Standard curves were constructed by plotting the initial rate of hydrolysis of TAMRA-GAGR *versus* the concentration of enzyme. Standard curve plots are shown for A) wild-type TAFIa and TAFIa-CIIYQ. The inset is the magnification of the plot at the 0 - 2 nM concentration. B) A standard curve plot for CPB with the inset magnifying the plot at the 0 – 0.2 nM concentration. The data are the means \pm standard errors of the mean of three independent experiments.

5.4.7 Thermal Stability and Enzymatic Activity of Wild-type TAFI in the absence or presence of assay components

The thermal stability and enzymatic activity of wild-type TAFI in the presence and absence of TAMRA-GAGR or Evans Blue alone or in combination was determined to examine whether any of the assay components affect the stability or activity of TAFIa, respectively. The half-life was calculated from the resultant first-order decay rate constants (Table 5-2). None of the assay components either alone or in combination had an observable effect on the stability or activity of TAFIa.

Table 5-2. Intrinsic Stability and activity of Wild-type TAFIa in the absence and presence of assay components^a

Variant	Half-life (min)	Activity (mOD/min) ^b
TAFIa wt	7.0 ± 0.2	1.97 ± 0.0483
TAFIa wt plus TAMRA-GAGR	7.0 ± 0.2	1.79 ± 0.0536
TAFI wt plus Evans Blue	7.0 ± 0.3	2.02 ± 0.0214
TAFI wt plus TAMRA-GAGR & Evans Blue	7.1 ± 0.2	1.50 ± 0.0926

^aData are the means ± standard errors of the mean of 2-3 independent experiments performed in triplicate

^bData are the initial rates of substrate hydrolysis following activation of wild-type TAFI

5.4.8 *Monitoring Activation of TAFI in real-time*

The activation of wild-type TAFI by the thrombin-TM complex was detected upon incubation with the TAMRA-GAGR and Evans Blue substrate mixture. The conversion of TAFI to the activated TAFIa enzyme results in hydrolysis of the TAMRA-GAGR substrate and an increase in fluorescence intensity (Figure 5-11A). An apparent linear relationship exists between the RFU/min² *versus* the concentration of TAFI zymogen (Figure 5-11B).

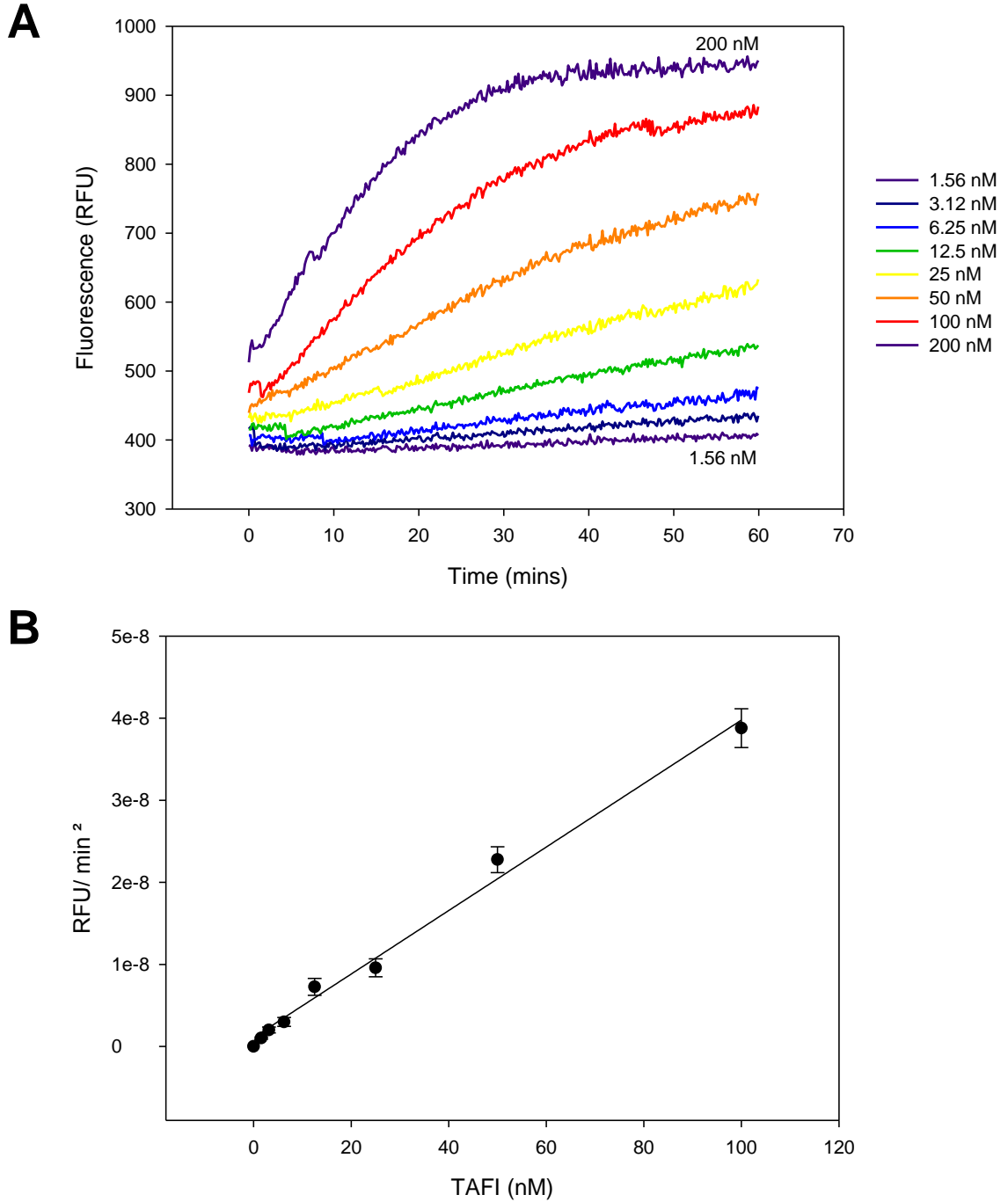
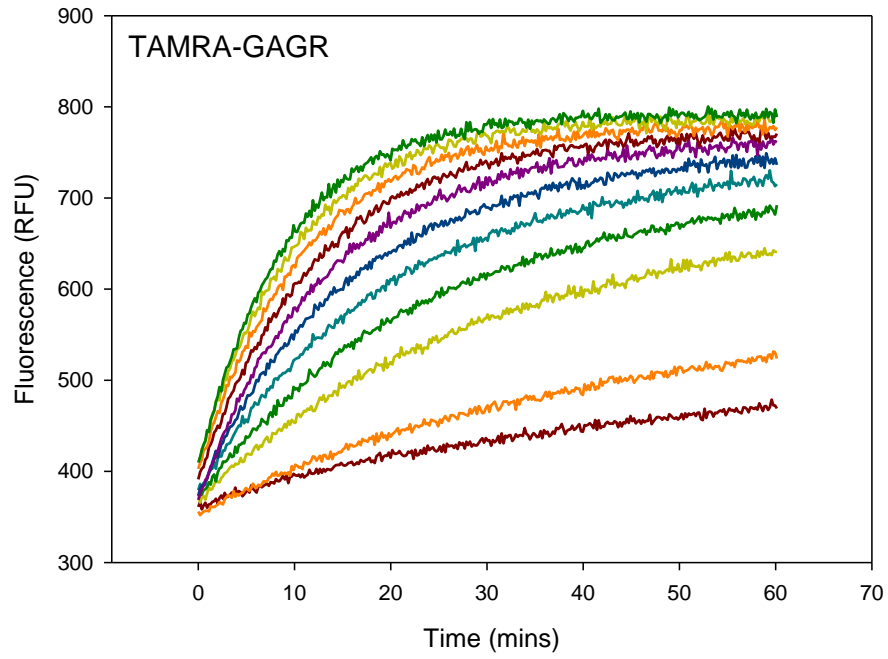
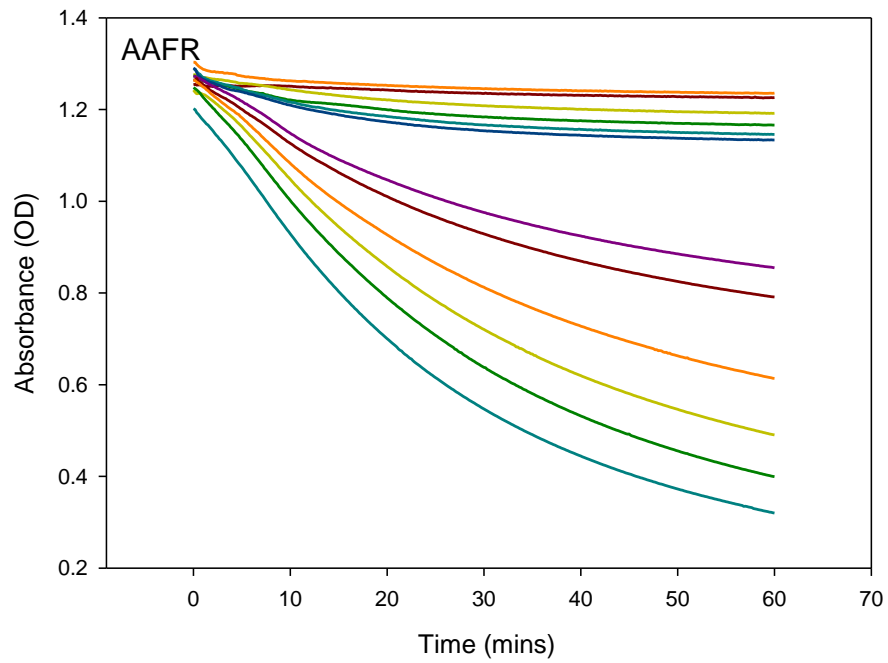


Figure 5-11. Activation of TAFI progress curve. A) Wild-type TAFI of varying concentrations was incubated with an activation mixture (25 nM thrombin, 100 nM TM and 5 mM CaCl₂) and the TAMRA-GAGR and Evans Blue substrate mixture at 21°C. The rate of TAFIa formation was assessed by monitoring the hydrolysis of the substrate over time. B) A plot of RFU/min² versus zymogen TAFI concentration was constructed. The data are the means \pm standard errors of the mean of three independent experiments.

5.4.9 *TAFIa activity toward synthetic substrates*

The enzymatic activity of wild-type TAFIa toward the available carboxypeptidase B substrates AAFR and HR was compared to the fluorescent TAMRA-GAGR substrate. The same concentrations of TAFI (1-22 nM) were incubated with each of the substrates and the absorbance or fluorescence was monitored over time (Figure 5-12). A plot was constructed of the rate of substrate hydrolysis *versus* the concentration of TAFIa and the slope represented the efficiency of detection of substrate hydrolysis by TAFIa per nanomolar of TAFIa (Figure 5-12D). The TAMRA-GAGR substrate is almost 1000 times more sensitive than AAFR and almost 14,000 times more sensitive than HR (Table 5-3).

A**B**

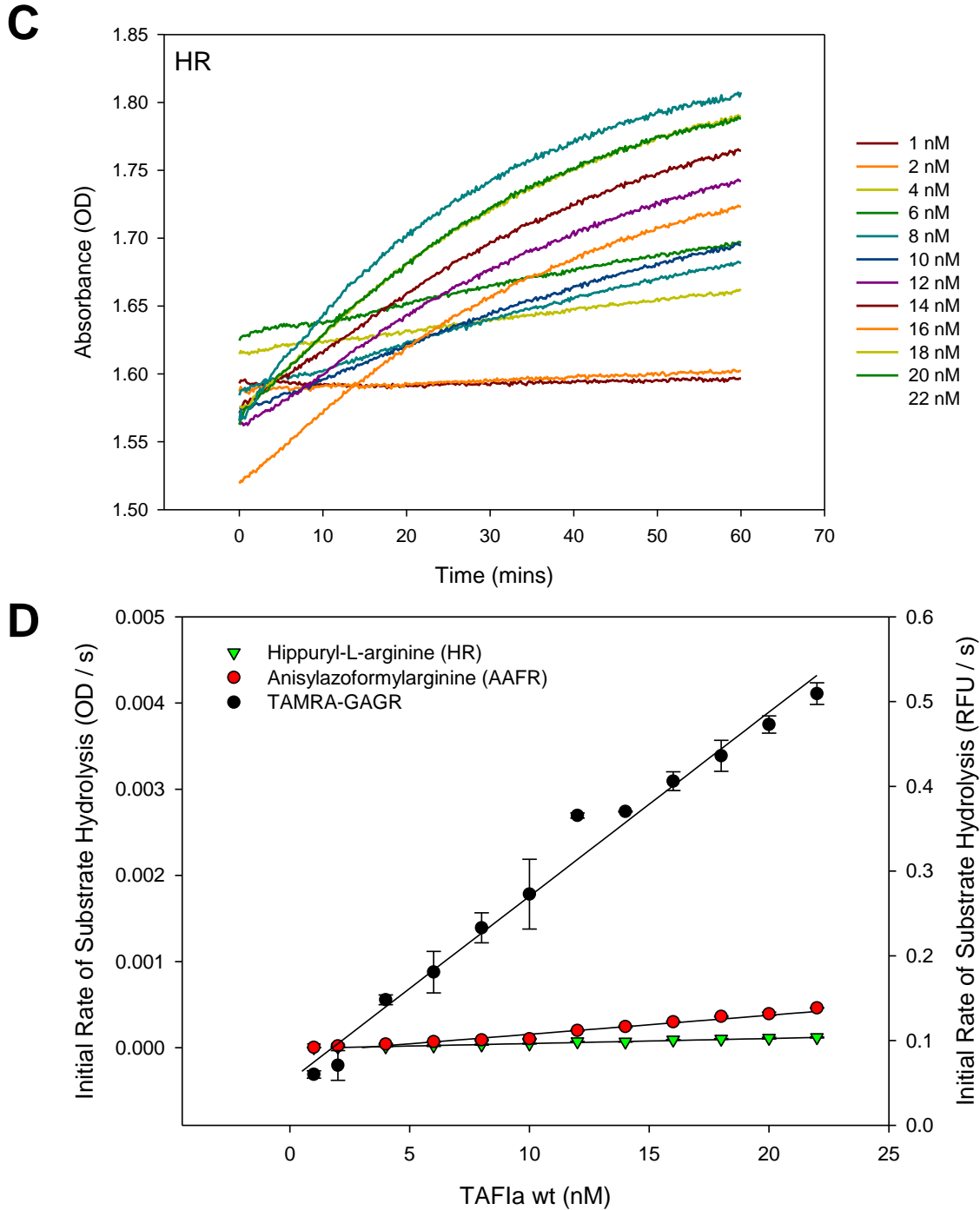


Table 5-3. TAFIa Activity toward substrates

Substrate	Equation of a Line	Efficiency of Detection
TAMRA-	$y = 0.02174 + 0.05284$	-
GAGR	$R^2 = 0.98$	
AAFR	$y = 0.00002213 - 0.00005463$ $R^2 = 0.97$	983
HR	$y = 0.00000156 + 0.00007766$ $R^2 = 0.97$	13,924

5.5 Discussion

In the present study, we have designed a novel fluorescence based assay for measuring the basic carboxypeptidase activity of TAFIa. This is the first fluorogenic substrate of any kind to be reported for the detection of TAFIa activity. The assay consists of a synthetic peptide substrate composed of four amino acids (Gly-Ala-Gly-Arg) modified with an amino-terminal TAMRA fluorophore. The basis of this assay method is the ability of the TAMRA moiety to be quenched through electrostatic interaction between an anionic quencher and the positively charged carboxyl-terminal arginine in the substrate. The ability of activated TAFI to catalyze the cleavage of the carboxyl-terminal arginine in the substrate causes the release of the quencher from the proximity of the fluorophore and results in an increase in fluorescence which can be monitored using a fluorescence plate reader. The assay was inspired by an assay system for arginine deaminases developed by Wang and coworkers [20], where conversion of arginine to citrulline results in elimination of the positive charge of the guanidinium functional group of arginine and loss of interaction with the anionic quencher.

The ability of several anionic quencher dyes to quench the fluorescence of the TAMRA-GAGR substrate was assessed. The study by Wang and coworkers [20] demonstrated the fluorescence quenching of TAMRA-containing substrates using numerous quencher dyes. The dyes that were reported to have the greatest quenching ability were Acid Green 27, Evans Blue, Ponceau S, Rose Bengal and Trypan Blue. Therefore, these were candidate anionic quenchers used to test against the substrate. Evans Blue (followed closely by Ponceau S) was determined as the most optimal

quenchers for the TAMRA-GAGR synthetic peptide substrate. Evans Blue had a slightly lower K_d compared to Ponceau S and therefore was chosen as the optimal quencher with a ratio of 1:8 for the fluorescent assays. Moreover, preliminary investigations with TAFIa showed that Evans Blue resulted in the greatest increase in fluorescence following substrate hydrolysis (data now shown).

Interestingly, all of the quenchers showed positive cooperativity in their interaction with the TAMRA-GAGR substrate. The basis for this behaviour is not immediately evident from the chemical structures of the substrate and quenchers. It is possible that there is some degree of self-association between molecules of TAMRA-GAGR that is disrupted by the quencher, thus facilitating binding of additional TAMRA-GAGR molecules by quencher. In any event, we see no evidence that this cooperative binding has a serious impact on the assay. For example, standard curves constructed with varying concentrations of TAFIa or CPB are consistently linear (Figure 5-9).

On the other hand, the assay was shown to be quite sensitive to the substrate:quencher ratio. Ratios higher or lower than the optimal (approximately 1:8 substrate:quencher) result in underestimation of the TAFIa or CPB activity present (Figure 5-8). Therefore, it is reasonable to conclude that the assay performs the best when a relatively small proportion of the substrate is hydrolyzed by TAFIa/CPB. As such, the rates of hydrolysis should be measured early in the time course and dilution of the enzyme may be required to ensure it falls within the linear range.

Indeed, some unusual behavior of the assay was observed in the case, most notably, of wild-type TAFIa. We often observed that the plots of fluorescence versus time deviated from linearity at extended times, apparently reaching a premature plateau in

some cases. This is evident, for example, by comparing panels A and C of Figure 5-9. Since this behavior seemed somewhat restricted to wild-type TAFIa, we suspected that some component of the assay was destabilizing the enzyme, especially since the plateau effect was occurring far earlier than might be explained by the half-life of wild-type TAFIa (approximately 2 hours at room temperature). However, direct assessment of this possibility using a different TAFIa substrate appeared to rule out an effect on stability (Table 5-2). Thus, the reason for the plateau effect with TAFIa remains to be determined, although the measures described in the previous paragraph should be sufficient to mitigate these effects.

Since the quencher and substrate interact electrostatically to form a fluorescently quenched complex it was assessed whether this interaction is disrupted by the chloride concentration in the buffer. At all chloride concentrations tested, however, the interaction was minimally perturbed (Figure 5-7). This indicates the chloride concentration in the buffer used for the assays is not sufficient to dissociate the quencher from the substrate and therefore, the quencher is able to quench the substrate despite the presence of only a single arginine residue. Therefore, any increase in fluorescence is attributable to TAFIa cleavage of substrate, not random dissociation of the quencher from the substrate.

One of the major advantages of the TAMRA-GAGR substrate is its high sensitivity compared to other commonly used TAFIa substrates such as AAFR and HR. This fluorescence assay is sensitive for detecting concentrations of TAFIa as low as 100 pM. The future application of this assay is to be able to detect TAFIa activity in real-time as it becomes activated during clot formation and dissolution assays, in a manner analogous to how the endogenous thrombin potential is determined during plasma

clotting in the presence of a fluorogenic thrombin substrate [21, 22]. Currently, there is no method for measuring TAFIa activity in this manner. We are able to monitor the activation of TAFI in buffer in real-time however further optimization is required to begin to examine the activation of TAFI in the context of plasma/clot. Indeed, preliminary experiments in the presence of plasma (data not shown) showed that sensitivity of the assay is much decreased, perhaps because of competition for binding to the anionic quencher by other elements in plasma.

This study provides a platform to begin to optimization of this fluorescence based assay. Before we begin to fully quantitate the concentration of TAFIa with high accuracy and reliability the limitations of the substrate and quencher need to be addressed. First, the substrate only contains a single arginine residue that is susceptible to quenching. Designing a substrate with more than one positively charged residue that can be removed by TAFIa will allow improved ability to quench the substrate and will generate an enhancement in the fluorescence generated upon exposure to an enzyme thereby increasing the signal to noise ratio. Moreover, as with many fluorescence based assays the fluorescent substrate suffers from inner-filter effects as determined from the absorbance spectrum showing the absorbance at the excitation and emission wavelength exceeding 0.1. This causes the measured fluorescence intensity and the substrate concentration to deviate from linearity resulting in an underestimation of the fluorescence signal. Therefore, a correction factor for the inner-filter effect needs to be taken into account when using different substrate concentrations [23]. As well, the intra- and interassay variability must be determined to assess the reproducibility of the assay.

The full potential of this assay method has yet to be completely explored and appreciated. One research application of the assay will be to study the time course of TAFIa activation during clot formation and lysis. The assay can also be used as a sensitive method to routinely measure the activity of TAFIa and other basic carboxypeptidases such as CPB. In addition, due to the simplicity and sensitivity of the assay it could ultimately be used as a tool in clinical settings to identify individuals with hemostatic diseases based on the variation in TAFIa generation or altered TAFIa activation and function.

5.6 References

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Chapter 6

General Discussion

The balance of the coagulation and fibrinolytic systems is in part mediated by the activation of the thrombin-activatable fibrinolysis inhibitor (TAFI) pathway [1, 2]. TAFI is activated by thrombin alone, thrombin in complex with thrombomodulin (TM), or plasmin. The thrombin-TM complex greatly accelerates the activation of TAFI to activated TAFI (TAFIa) in comparison to the weak activators thrombin and plasmin [3-5]. However, the structural basis for the acceleration of TAFI activation by thrombin in the presence of TM has been relatively elusive. In Chapter 2, we mutated surface-exposed charged residues on TAFI to alanine to determine the sites on TAFI that allow the acceleration of activation by TM. We determined that Arg12 is essential and Arg377 is very important for the activation of TAFI by the thrombin-TM complex. We speculate that residue Arg12 directly binds to TM whereas residue Arg377 could affect the interaction between thrombin and TAFI, particularly in the presence of TM. Additionally, during our study we identified that the activation domain of TAFI is an important region influencing its accelerated activation by the thrombin-TM complex, as evidence by the effect of additional mutations beyond Arg12. In Chapter 3, the potential role of cleavage of TAFI after Arg12 on activation by thrombin-TM was tested by constructing amino-terminal truncation mutants in the activation domain of TAFI. At this time, however, the results of the study are inconclusive since the proteins were not secreted. Plasmin is a poor activator of TAFI but the presence of glycosaminoglycans (GAGs) is able to accelerate its activation as well as stabilize the thermally unstable TAFIa enzyme. However, the structural basis of these effects is unknown. In Chapter 4, we identified residues Lys327 and Arg330 as part of a heparin-binding site on TAFI that allows GAGs to mediate their effect on TAFI function. Finally, in Chapter 5, we designed a novel

fluorescence assay for the measurement of TAFIa activity which has significantly increased sensitivity compared to the commonly used carboxypeptidase B (CPB) substrates hippuryl-arginine or -lysine (Hipp-Arg/Hipp-Lys) and anisylazofornyl-arginine or -lysine (AAFR/AAFK).

6.1 The interaction between TAFI and thrombomodulin

Based on several studies to date it is becoming increasingly evident that the mechanisms by which TM mediates the enhancement of TAFI and protein C (PC) activation by thrombin feature both similarities and differences. Previous studies have focused on comparing TAFI and PC because they have similar kinetics of activation. They are both weakly activated by thrombin and in the presence of TM their rate of activation is greatly accelerated by over 1000-fold [5]. In both cases it is thought that EGF-5 and EGF-6 bind to thrombin with high affinity while upstream EGF-like domains interact with the substrate (either TAFI or PC) to present it optimally to thrombin for cleavage [6, 7]. Although crystallographic analysis of the thrombin-TM structure appeared to rule out previously-hypothesized allosteric effects of TM acidic residues in the thrombin active site [6], subsequent reports have suggested some allosteric effects of TM including relieving a Ca^{2+} -dependent clash between basic residues in thrombin and PC [8], altering the conformation of the PC activation domain [9], and altering the conformation of the thrombin active site [10]. Whether these or analogous allosteric effects play a role in TAFI activation by thrombin-TM has not been explored.

Most relevant to the work in this dissertation, however, is the fact that TAFI and PC require different structural elements of TM to allow efficient activation. The smallest primary structure of TM that allows efficient activation of PC requires the residues of EGF-4 through EGF-6 plus the six residues connecting EGF-4 to EGF-3 whereas TAFI requires residues of the c-loop of EGF-3 through EGF-6 [11, 12]. In particular, the residues within the c-loop of EGF-3 are essential for TAFI activation. A report by Wang and coworkers identified residues in the c-loop of EGF-3 that are required for TM cofactor function for TAFI activation. They used alanine scanning mutagenesis of TM and demonstrated that residues within the c-loop of TM-EGF-3 including Val340, Asp341, Glu343 and Asp349 are essential for the cofactor function of TM in TAFI activation but not PC activation. As well, mutation of residues Tyr337, Asp338, Leu339, Val345 within the c-loop of TM-EGF-3 and residues Glu346 and Val348 within the interdomain region between EGF-3 and EGF-4 resulted in significantly reduced activation of TAFI [12]. This study indicated that the residues within the c-loop of TM-EGF-3 that are essential for TAFI activation are primarily negatively charged. In Chapter 2, we elected to focus our study on mutating surface-exposed charged residues on TAFI to alanine in order to identify the regions on TAFI that influence TM-dependent activation. We did find that the greatest effects on thrombin-TM-dependent activation of TAFI were observed when basic residues were mutated, consistent with the role for acidic residues in TM. It is important to note that the interaction between TAFI and TM is not exclusively mediated through ionic interactions. There are likely also some hydrophobic and polar uncharged residues on TM that mediate its interaction on the surface of TAFI through hydrophobic interactions or hydrogen-bonding interactions, respectively. Future

studies should be aimed at validating the TM interaction surface on TAFI with reciprocal mutations in the putative region in TM.

In Chapter 2, we identified residue Arg12 as critical for the interaction between TAFI and TM. Indeed, this residue's involvement in the interaction confirmed an earlier report by Plug and coworkers [13] however, our R12A mutant was essentially resistant to the accelerative effects of TM which is the first mutation of TAFI to be reported with such an effect. The mutation reported by Plug and coworkers, R12Q, did not yield such a dramatic effect on TM likely due to the fact that the mutant of TAFI is most likely still able to make some productive contact with TM [13]. Our mutant, R12A, is novel in that it essentially disrupted the interaction between TAFI and the cofactor ability of TM. The location of residue Arg12 on the crystal structure of TAFI is on a surface exposed loop directly below and in close proximity to the Lys42/Lys43/Lys44 residues within the activation domain that was reported by Wu and coworkers which had only an 8-fold reduced catalytic efficiency (k_{cat}/K_M) of TAFI activation by the thrombin-TM complex [14]. It would be interesting to mutate other surface exposed residues in the vicinity of Arg12 in order to examine this TM binding region on the surface of TAFI.

In addition, TAFI and PC each require different structural elements of thrombin for activation. A study by Hall and coworkers, using a collection of thrombin mutants where polar or charged surface residues were mutated to an alanine, found that three mutants were able to specifically decrease the activation of TAFI without affecting activation of PC [15]. The thrombin mutations that affected TAFI activation mapped to a region on thrombin above the active site cleft. Also, thrombin mutations were found that specifically affected PC activation but had no effect on TAFI activation, and these were

located below the active site cleft. This study therefore showed that thrombin interaction with its substrates, TAFI and PC, is through distinct domains [16]. In Chapter 2, we also identified residue Arg377 as important for the activation of TAFI by the thrombin-TM complex. Mutant R377A showed a 17-fold reduced catalytic efficiency of TAFI activation by the thrombin-TM complex but a less than two-fold decreased activation by thrombin alone. Examining the model of TAFI docked to the thrombin-TM structure [14], this residue appears in close proximity to where thrombin binds to TAFI. We therefore speculated that Arg377 of TAFI interacts with thrombin most consequentially in the ternary complex containing TM, and hence this finding suggests that TAFI interacts with thrombin in a subtly different manner in the presence and absence of TM. It would be interesting to determine through mutagenesis and biochemical studies whether Arg377 of TAFI contacts the region in thrombin above the active site cleft; the binding of TM to thrombin may position the enzyme active site and the region of TAFI Arg377 in a manner that causes a tighter interaction between the thrombin-TM complex and TAFI.

Moreover, in Chapter 2, we identified residues within or in close proximity to the activation domain of TAFI (Arg15, Glu28, Lys59, Glu99 and Glu106) that exhibited a decreased catalytic efficiency of activation by the thrombin-TM complex and little difference in catalytic efficiency of activation by thrombin alone. These results indicate that the variants that showed the strongest TM dependence were focused around the activation domain which would be near where the c-loop of TM-EGF-3 would interact with TAFI [14]. The crystal structure of human α -thrombin bound to TM-EGF-4-6, which is the smallest structure of TM that is capable of activating PC, has been determined and has been a useful tool in evaluating the structural basis for the acceleration of PC

activation by TM through the modeling of PC with the complex [6]. Wu and coworkers docked the crystal structure of TAFI onto this thrombin-TM complex structure, however, the charged residues of the c-loop of TM-EGF-3 required for TAFI activation are absent from this structure [14]. This model as well as the crystal structure of TAFI aided in the selection of residues on TAFI to study in Chapter 2 to assess its interaction with TM however, the location of TM-EGF-3 relative to the TAFI structure is relatively unknown. To date, however, a crystal structure of the complex between thrombin and EGF-3-6 of TM has not been reported. Such a structure would allow additional insights to be gained regarding the interaction between the TAFI activation domain and thrombin-TM. One reason for the absence of a crystal structure might be that the extended structure of EGF-3-6, compared to EGF-4-6, inhibits crystallization. It would be worthwhile to try and overcome this effect by attempting to crystallize thrombin-TM in the presence of TAFI, because TAFI would bind to and perhaps conformationally stabilize the extended EGF-3-4 region of TM. Such a structural model would be a valuable tool in elucidating the mechanism by which TM enhances the acceleration of TAFI activation by thrombin.

As the results of Chapter 2 were being prepared for submission, a paper was published that identified Arg12 as important for the acceleration of TAFI activation by thrombin-TM [13]. Intriguingly, Arg12 was also reported to be a cleavage site (albeit an inefficient one) for thrombin and plasmin. The authors speculated that the reason why their Arg12 to Gln12 mutation was impaired as a substrate for thrombin-TM was because thrombin cleavage at Arg12 yields a form of TAFI that is a better substrate for thrombin-TM. However, the slow kinetics of Arg12 cleavage did not support such a scenario, and the authors acknowledged that they could not rule out that Arg12 formed a TM-binding

exosite. The results in Chapter 2 firmly put to rest the notion that cleavage of TAFI at Arg12 accelerated its activation: cleavage should be equally affected by mutation of Arg12 to Gln and Ala, yet the mutation to Ala has a much more profound effect on thrombin-TM activation. Nonetheless, to attempt to more directly address the role of Arg12 cleavage, we undertook the studies in Chapter 3, with the construction of amino-terminal truncation mutants of the activation domain of TAFI. We attempted to express the respective recombinant TAFI variants to examine their ability to be activated by the thrombin-TM complex. Unfortunately, it is difficult to conclude with certainty whether removal of residues 1-12 has functional consequences since the mutant proteins were not secreted. Analysis of the crystal structure of TAFI indicates that these residues are likely crucial for folding of the activation domain, which is likewise probably required to stabilize the enzyme domain during folding of the protein in the ER lumen. We speculated that the carboxyl-terminal 6×-His tag may have prevented folding and secretion of the truncated variants, since studies of a perhaps analogous variant lacking residues 1-73 showed that this variant could be secreted in the context of an amino-terminal 6×-His tag (or no His tag) and the CIIYQ mutations [17]. We did not observe secretion of the Δ 1-73-CIIYQ variant with a carboxyl-terminal 6×-His tag, although expression of the mRNA for this variant was greatly diminished compared to the other variants. Future studies will involve construction of amino-terminally His-tagged versions of these truncation variants of TAFI.

Although we utilized soluble forms of TM in all our studies, it should be noted that TM is a transmembrane protein that is expressed on the luminal surface of endothelial cells [18-20]. Therefore, TAFI activation occurs on the surface of endothelial

cells and indeed its activation has been demonstrated on human umbilical vein endothelial cells [7, 21]. However, TM expression has been detected in a variety of other cell types including human blood monocytes, neutrophils, and smooth muscle cells [22-24]. Studies have shown that the level of cellular TM antigen increases upon differentiation of the monocytic cell line THP-1 [25]. TM has also been found in human platelets present at approximately 60 molecules per platelet whereas approximately 30,000 to 100,000 molecules are found on the endothelial cell surface [20]. These cell surfaces are all likely to play a role in the activation of TAFI *in vivo* and should be evaluated in further studies. As well, soluble TM is found in plasma due to proteolysis of the protein or damage to endothelial cells due to infections, sepsis, and inflammation [26-31]. Therefore, the contribution of soluble TM to promoting TAFI activation should be assessed. Soluble TM has been shown to promote activation of TAFI in the context of *in vitro* clot lysis assays [21].

6.2 The interaction between TAFI and Heparin

Although plasmin is a relatively weak activator of TAFI, its activity against TAFI has been shown to be markedly enhanced in the presence of heparin and other GAGs [4]. Therefore, plasmin may be an important activator at vascular injury sites which expose an abundance of GAGs present in the extracellular matrix. Additionally, these injury sites will be comparatively deficient in TM, therefore in the presence of GAGs, plasmin mediated TAFI activation may dominate thereby contributing to stabilizing the fibrin rich clot. A study by Mao and coworkers also demonstrated that the presence of GAGs is able

to stabilize the active enzyme TAFIa causing a 2.3-fold increase in its half-life at 25°C. A putative GAG-binding site in TAFI was identified by sequence homology with heparin-binding proteins spanning Trp210 through Ser221 [4]. Anand and coworkers later identified several bound sulfate molecules in the crystal structure of bovine TAFI and postulated that the sulfates evidenced the existence of a heparin-binding site. The bound sulfates were found in three locations: at the activation domain, within the active site cleft, and near the instability/dynamic flap region where it interfaces with the activation domain [32]. These studies suggested the existence of an interaction between TAFI and heparin; however, the elements of TAFI structure that interact with heparin to impart these characteristics remained to be definitively identified.

In Chapter 4, to identify the GAG-binding site(s) on TAFI and assess their function in regulating TAFI activity, we have expressed recombinant TAFI variants containing mutations in basic (i.e. positively charged) residues in the predicted regions that might participate in ionic interactions with the highly anionic GAGs. We chose to focus on the instability region, since it was the most relevant region to explain the increased stabilization of TAFIa, as well as the heparin-binding protein-homologous region spanning Trp210-Ser221 which are predicted to interact with heparin. Interestingly, many of the residues implicated on bovine TAFI are not conserved in human TAFI, so we had to either not mutate some residues or mutate other nearby positively-charged residues. We identified the instability region of TAFI as an important heparin interaction site on the structure since mutations within this area resulted in the weakest binding interaction to heparin. In particular, residues Lys327 and Arg330 within the instability region were identified as part of a functionally-relevant heparin-binding

site and nearby residues Arg320 and Lys324 may also be involved. The results of TAFI mutant R320A/K324A hinted at the existence of more than one heparin binding site present on the TAFI structure due to the weak binding of this variant to heparin, yet its normal sensitivity to the effects of heparin during plasmin activation and TAFIa stabilization indicates that these residues are not functionally relevant.

Our study was based on the individual bound sulfates in the crystal structure of bovine TAFI and therefore each bound sulfate does not necessarily represent a heparin-TAFI interaction since GAGs are long, unbranched polysaccharide polymers of varying lengths containing variable amounts of sulfate or carboxyl groups on their sugars [33, 34]. In addition, GAG-binding consensus sequences do not exist in all heparin-protein interactions indicating that several factors determine the interaction between GAGs and proteins including length, anionic character and stereochemistry of the polysaccharide, and conformation of the protein structure [35, 36]. Yet, our study provided clues into the interaction between TAFI and heparin. Future studies should be aimed at identifying other heparin-binding regions on TAFI through mutagenesis studies. The TAFI structure contains several exposed positively charged surface patches which may provide interaction sites with heparin since its interaction with proteins is primarily mediated through ionic interactions. In addition, we used only a semi-quantitative method for assessment of heparin binding to TAFI through the use of an affinity chromatography approach. A previous graduate student in our group had attempted to measure the binding of TAFI to heparin by measurement of the intrinsic fluorescence of TAFI or by using fluorescently-labeled heparin. No binding was revealed by these methods, which indicates that if binding was occurring, perhaps it did not influence the quantum yield of the

intrinsic or extrinsic fluorophores. Another possible method to measure binding would be surface plasmon resonance. Importantly, this technique would readily be able to detect the presence of multiple binding sites for heparin on TAFI as well as their respective affinities.

To date, the structural basis for the interaction of plasmin with TAFI is relatively uncharacterized. However studies by Mishra and coworkers utilizing monoclonal antibodies have identified some important residues on TAFI for interaction with plasmin including residues Lys268, Ser272, Arg276, Thr147, Ala148 and Phe113 [37]. In Chapter 4 we identified residues Lys212 and Lys211 as potentially participating in plasmin binding to TAFI. The position of residues Lys212 and Lys211 on the three-dimensional structure of TAFI is located on the surface near the scissile bond (Arg92-Ala93). Plasmin's protease domain or lysine-binding kringles may interact with residues near Arg92 which would explain why these variants showed an increase in K_M as well as reduced plasmin activation in the absence of heparin compared to wild-type.

Additionally, whether the exposed positively charged surface patches on TAFI are sites for interacting with the kringle domains of plasmin is unknown since residues near the scissile bond may not be the only determinants of substrate specificity. Activation of plasminogen to plasmin, gives the protease a more open conformation which may allow it to stretch out to these regions on the surface of the TAFI protein allowing it cleave the scissile bond while being bound to lysine-containing exosites elsewhere on the substrate. As a compounding effect, the impaired interaction with plasmin could possibly impair the ability of heparin to elicit its effects. For example, in the case of thrombin inhibition by antithrombin, heparin catalyzes the reaction by binding to both thrombin and

antithrombin [38, 39]. Therefore, it is possible that heparin binds to both plasmin and TAFI, thus allowing heparin's accelerating effect on TAFI activation by plasmin. In that case, impaired binding of TAFI to plasmin would also affect heparin binding to TAFI. To investigate this possibility, a truncated form of plasmin can be used. Microplasmin lacks all 5 kringles, but has an active catalytic domain [40], thus it could still activate TAFI. This approach may give insight on whether heparin can still elicit its catalyzing effect on plasmin-mediated activation of TAFI in a kringle-independent manner. To fully appreciate the accelerating effects of heparin on TAFI activation by plasmin we need to understand how plasminogen/plasmin interacts with TAFI. Until then it will be difficult to differentiate heparin interaction sites on TAFI that promote activation and heparin interaction sites that promote stability. On a more fundamental level, the mechanism underlying the effect of heparin on plasmin-mediated activation of TAFI remains unclear. Importantly, heparin has been shown to bind to kringle 5 of plasminogen [41]. This suggests that heparin could act as a bridge to promote an encounter complex between plasmin and TAFI. Further kinetic and mutagenesis studies will be required to address this possibility.

As expected, mutations in the dynamic flap region of TAFI had the greatest effect on stabilization of TAFIa by heparin. The dynamic flap is held in place in the zymogen (whose potential activity is stable) through interactions with the activation domain. Upon removal of the activation domain, the dynamic flap is proposed to undergo a structural transition that alters the position of the catalytically critical Glu363 residue present where the dynamic flap re-enters the core of the enzyme [42]. The spontaneous loss of TAFIa activity is associated with a substantial (almost 50%) decrease in the intrinsic

fluorescence of the enzyme [43, 44] as well as a markedly decreased solubility [45, 46]. It is difficult to imagine that these effects could be accounted for by a change in the conformation of the dynamic flap alone. Presumably, movement of the dynamic flap triggers additional structural changes, evidence for which has been found in fluorescence [43] and antibody-binding studies [47]. The structure of “inactive” TAFIa is unknown. In any event, movement of the dynamic flap certainly is the key step in TAFIa inactivation. We speculate that the ability of heparin to bind in this region reduces the conformational freedom of the dynamic flap thus stabilizing the enzyme. Crystallization of TAFI/TAFIa in the presence of heparin (or, more likely, a homogenous GAG analogue such as fondaparinux) should shed light on this issue.

Mao and coworkers also demonstrated that other GAGs/proteoglycans such as chondroitin sulfate, dermatan sulfate, and high concentrations of heparan sulfate and keratan sulfate are capable of stimulating plasmin-mediated TAFI activation [4]. However, the study only showed semi-quantitatively the efficacy of the different GAGs in plasmin activation and it is unknown how these GAGs affect stabilization of TAFIa. Further studies should determine the effects of the different GAGs on TAFI activation by plasmin through kinetic analysis as well as on TAFIa stabilization. In addition, it has been shown that GAGs can inhibit the activation of TAFI by the thrombin-TM complex however the structural basis of these effects is not known [48]. The K327A and K327A/R330A variants constructed in Chapter 4 could be used as tools to assess the activation of TAFI by the thrombin-TM complex in the presence of heparin since they bind very poorly to heparin. It is important to determine the molecular basis for these effects since TM and GAGs do not exist in isolation physiologically. Moreover, the role

of cell surface and extracellular matrix GAGs on TAFI activation by thrombin-TM and plasmin should be investigated. The loss of function mutants that have been generated and characterized in this dissertation would be very useful for this task. It essential to try to understand the how the activation of TAFI is regulated by these factors as it will give us clues into how the TAFI pathway is regulated *in vivo*.

6.3 Monitoring TAFIa activity with a Fluorescence-based assay

Many methods have been described for measuring the carboxypeptidase activity of the TAFIa enzyme. Synthetic substrates such as Hipp-Arg/Hipp-Lys and AAFR/AAFK are able to detect TAFIa activity in a purified system through spectrophotometric means [44, 49-51]. However, these substrates cannot be used to detect TAFIa activity in the context of plasma since they suffer from low sensitivity and absorbance detection will not be possible during clot lysis assays. Other, indirect, assays have been reported to detect TAFIa activity; however, they either require many components that are not readily available or are time-consuming due to the complexity of the assay method [52-54]. No matter how sensitive, no indirect method can measure the accumulation (and disappearance) of TAFIa in real-time in the context of a clot.

In Chapter 5, we describe the development of a novel fluorescence assay to detect TAFIa activity utilizing a fluorescent substrate. This is the first direct fluorogenic substrate system ever described for TAFIa or any other carboxypeptidase. The basis of the assay is the ability of TAFIa to catalyze the removal of carboxyl-terminal arginine residues from a synthetic peptide substrate modified with an amino-terminal

tetramethylrhodamine (TAMRA) fluorophore. The carboxyl-terminal arginine in the synthetic substrate electrostatically interacts with the anionic quencher, Evans Blue, to allow fluorescence quenching of the TAMRA fluorophore. The cleavage of the carboxyl-terminal arginine by TAFIa results in the release of the synthetic substrate bound to the quencher thus producing an increase in fluorescence. This assay was shown to be detectable at TAFIa concentrations as low as 100 pM. The fluorescence based assay has advantages over the existing substrates used to measure TAFIa activity including its greatly increased sensitivity compared to the currently available carboxypeptidase B substrates Hipp-Arg/Hipp-Lys and AAFR/AAFK and the simplicity of the assay method.

We undertook various strategies to optimize the assay, most notably by comparing and titrating various anionic quenchers. Such optimization is crucial in the context of this assay for the maximization of the signal-to-noise ratio of the assay and for ensuring that it faithfully reports the concentration of TAFIa at all time points. One reason why careful optimization is necessary is the dynamic equilibrium that exists between the anionic quencher and the arginine side chain in the substrate. If the quencher is too abundant, then insufficient “free” carboxyl-terminal arginine is available at any time to be recognized by TAFIa. If the quencher is too scarce, an insufficient degree of quenching of the TAMRA moiety would result and the sensitivity of the assay would suffer. In addition, the equilibrium would be perturbed as the assay proceeds and the substrate is consumed, which might affect the rate of substrate cleavage and/or residual quenching. In our first attempts at running the assay, we used TAFIa, and observed that the fluorescence plateaued at a different level depending on the concentration of TAFI present (see Figure 5-9A and C, for example). We speculated that either the equilibrium perturbation was

having an effect, or that TAFIa activity was being prematurely quenched in the context of the assay. When we used stable TAFIa-CIIYQ and, particularly, CPB, however, these plateau effects were either greatly diminished or absent. This would tend to rule out an effect arising from perturbation of quencher binding equilibrium. On the other hand, we could see no effect of the assay components on the specific activity or the stability of TAFIa. Another possibility is some sort of product inhibition that specifically affects TAFIa. In any event, these effects underscore the need to carefully optimize the assay and to measure rates of hydrolysis when substrate consumption is linear.

At this point in time there is no assay available that is able to continuously measure the enzymatic activity of TAFI in the context of plasma as it becomes activated during lysis assays. The preliminary experiments conducted thus far provide a starting point for beginning the optimization of this assay in a plasma containing system. We are able to detect the activation of TAFI *in vitro* in real-time in a purified system however, further studies require the optimization of the assay to be able to measure the activation of TAFI in plasma. Future studies require the determination of correction factors for the inner-filter effects in the context of a purified and plasma system. As well, the intra- and interassay variability needs to be assessed to determine the reproducibility of the assay. Following future optimization, this assay may provide a means to examine TAFIa generation in real-time within clots which will provide insight into the regulation of TAFI activity.

6.4 Concluding Remarks

It is essential that a balance exists between the activities of the coagulation and fibrinolytic pathways. Imbalances lead to excessive blood loss as well as inappropriate thrombus formation. Excessive activation of plasma TAFI poses a risk factor for the development of thrombotic diseases whereas inefficient activation of plasma TAFI is detrimental to the formation of a stable fibrin clot. This dissertation was generally focused on analysis of the control of the elaboration of TAFIa activity. The identity of the activators of TAFI in particular physiological and pathophysiological contexts remains largely unexplored. Other groups have utilized antibodies that specifically prevent TAFI activation by one or more activators to probe this question. Our approach is distinct but complementary: to generate specific loss-of-function variants that are resistant to activation by specific activators or modulation of activation. The findings in this dissertation as well as prior work from our group [51] has resulted in a “tool-kit” of well-characterized variants that can be brought to bear on these questions. Future studies should therefore be aimed at assessing the relative contribution of each of the activators in activating TAFI as well as determine the activator of TAFI in various physiological and pathophysiological contexts. This could be done both *in vitro* with recombinant protein or even *in vivo* with mutations knocked into mice or rats using genome editing approaches. The R12A variant, in particular, with a specific and profound defect in thrombin-TM mediated activation, would be a particularly attractive candidate in this context.

The studies described provide insight into the elements of structure required for the activation of TAFI by the thrombin-TM complex and well as the structural basis for

the acceleration of plasmin-mediated TAFI activation by GAGs and the stabilization of the TAFIa enzyme. These studies will aid in further propelling the determination of the mechanism of TAFI activation by the respective activators. They may also provide the rationale for the development of therapeutics aimed at manipulating the activity of the TAFI pathway. For instance, activation of TAFI has been shown to decrease the efficacy of thrombolytic therapy [55]. Promotion of TAFI activation, however, would be a potential strategy to ameliorate the propensity for bleeding that is observed in hemophilia and other bleeding diatheses.

Finally, the fluorescence-based TAFIa assay described could provide a useful tool for examining the regulation of the TAFI pathway in different settings in the vasculature and for the continuous measurement of TAFIa generation during clot formation and lysis assays, respectively. Previous studies have shown a donor-dependent difference in the functional elaboration of TAFIa activity that was associated with risk for ischemic stroke [56]. Our assay could be applied clinically to identify subjects with impaired or accelerated TAFI activation thus reflecting dysfunction of the fibrinolytic system.

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