MAMMALIAN CELL SURFACE DISPLAY OF FUNCTIONAL ALPHA 1-PROTEASE INHIBITOR: BUILDING "DESIGNER" SERPINS

By

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the

Requirements for the Degree

Doctor of Philosophy

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DOCTOR OF PHILOSOPHY (2015)		McMaster University	
(Medical Sciences Program, Blood and Vasculature)		Hamilton, Ontario	
TITLE:	Mammalian Cell Surface Displa 1- Protease Inhibitor: Building	y of Functional Alpha 'Designer'' Serpins	
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NUMBER OF PAGES	ix, 135		

ABSTRACT

Human serpins belong to a superfamily of serine protease inhibitors involved in the regulation of essential physiological processes, including coagulation via thrombin inhibition by AT. Inhibitory serpins undergo a remarkable folding mechanism to a thermodynamically unstable (i.e. metastable) conformation, highlighted by a long and flexible RCL, prior to secretion as soluble proteins into circulation. The serpin alpha-1-proteinase inhibitor (API) normally protects tissues from proteases released from inflammatory cells (e.g., neutrophil elastase). Importantly, a variant of API (i.e. API-Pittsburgh or API-M358R) was reported to be the cause of a fatal bleeding disorder in a young patient in the late 1970's; the point mutation M358R at P1 of the RCL resulted in a dramatic shift in function toward thrombin inhibition.

This dissertation summarizes the results from experiments performed with serpins expressed as membrane proteins tethered to the surface of mammalian cells. Serpins API-M358R, AT, HCII, and the non-inhibitory double mutant API-M358R/T345R were anchored to 293 and COS cell plasma membranes with N-terminal non-cleavable protein sequences derived from either the human asialoglycoprotein (AR) or transferrin (TF) receptors. Sub-cellular fractionation (with or without monolayer exposure to thrombin) immunoblots confirmed serpin localization to the integral membrane fraction using either anchoring approach or cell type. The blots also revealed that tethered API-Pittsburgh in particular, and AT to a lesser extent formed serpin-enzyme complex (SEC) with thrombin, while HCII and API-M358R/T345R (as expected) did not. While tethered API-M358R maintained inhibitory function, kinetic studies revealed that the rate of SEC formation was less rapid compared to its soluble counterpart. Additional testing by immunofluorescence microscopy, and flow cytometry confirmed the status of tethered API-M358R as a robust inhibitor of thrombin.

That tethered serpins maintained the ability to inhibit thrombin provided the underlying rationale for the thesis hypotheses: surface displayed serpins can be used in gene therapy to temper thrombogenicity associated with certain diseased tissues (i.e. cancer), and tethered serpins can be used as a platform for screening RCL mutant libraries to identify "better" protease (i.e. thrombin) inhibitors.

The potential gene therapy scheme was tested by expressing tethered API-Pittsburgh on the surface of T24/83 cancer cells constitutively co-expressing tissue factor (TF), and then measuring endogenous thrombin generation in the presence of re-calcified, and defibrinated human plasma by either discontinuous or continuous fluorescence-based thrombin generation assays (TGA). Unexpectedly, the displayed API-Pittsburgh did not appear to reduce discontinuous TGA thrombin suggesting that the difference may have been too low for accurate measurement by this method. Moreover, the results were identical when the same reaction was continuously monitored by fluorescencebased TGA, indicating that the levels of API-Pittsburgh expression were simply insufficient to effectively counter thrombin generation. The high levels were confirmed when up to 1 μ M of hirudin variant 3, or soluble API-M358R, were required to completely abolish the thrombin profile. With this in mind, a measureable reduction in TGA was achieved when 293 cells were co-transfected with DNA ratios of API-M358R: TF adjusted to 9:1.

Mammalian cell display, in combination with FACS/flow cytometry, has previously been employed to successfully develop improved monoclonal antibodies. However, there was never any certainty that the technique was applicable to the screening of displayed serpin mutant RCL libraries. The method was tested with a modest library degenerate only at P1; the rationale was that successful sorting would generate the expected wild-type (WT) P1 Arg (i.e. API-Pittsburgh). Unfortunately, repeated attempts did not result in enrichment, and flow cytometry was abandoned.

An alternate protocol based on bacterial expression, and previously developed in our lab, was implemented in order to perform the library screens. This technique involved incubating lysates, containing soluble serpin RCL mutant candidates, with immobilized thrombin. Encouragingly, the P1 library screen identified the WT candidate at the expected frequency (5 in 150 lysates) as well as the more rare P1 mutant Lys (1 in 150 lysates). A kinetic comparison between mutant proteins containing the three basic residues revealed that P1 Arg ($k_2 \sim 10^5 \text{ M}^{-1} \text{sec}^{-1}$) was approximately two orders of magnitude more efficient than either Lys or His (both with $k_2 \sim 10^3 \text{ M}^{-1} \text{sec}^{-1}$) at inhibiting thrombin.

The bacterial expression technique was then enhanced through kinetic optimization in order to facilitate the screening of more complex libraries. Analysis of the P7 to P1 (always Arg) library returned a selection of anticipated non-polar residues (5 in 100 lysates; 2 x Pro, 2 x Leu and Met) at P2.

Extensive screening (~ 1300 colonies) of a second expanded library with the repeated VNN nucleotide sequence (i.e. no stop codons to eliminate truncated proteins) at P7 to P2 and P1 Arg, identified 7 x Pro mutants at P2 further confirming the results from the original library screen. Importantly, the assay also identified the novel mutant TLSATPR which registered the largest kinetic response ($k_2 \sim 5 \times 10^5 \text{ M}^{-1} \text{sec}^{-1}$), and even exceeding API- M358R by a factor of ~ 3 ($k_2 \sim 1.5 \times 10^5 \text{ M}^{-1} \text{sec}^{-1}$).

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor, Dr. William Sheffield, for his generous support, patience, and guidance throughout the course of my PhD studies. I am especially grateful for the opportunity to truly learn what is meant by "leave no stone unturned" and for all the help I received developing my skills in scientific writing. These were vital for me to become a better scientist. I would also like to thank the members of my PhD supervisory committee, Dr. Patricia Liaw and Dr. Murray Junop, for the many helpful discussions.

Thank you also to the Sheffield lab staff Varsha Bhakta, Louise Smith and Sharon Gataiance for all their help. Varsha was particularly helpful with the tricky aspects of some of the cloning.

Finally, I would like to thank my patient, understanding and remarkable wife, MaryAnn, and my two wonderful kids Jenna and Nicholas. This could never have happened without the love and support from my entire family. This degree belongs to them as much as it does to me.

TABLE OF CONTENTS

Title page	i
Descriptive notei	ii
Abstractii	ii
Acknowledgements	V
Table of Contents	/i
List of abbreviationsvi	i
1 INTRODUCTION	1
1.1 Serpins In Health and Disease	1
1.2 Overview of Blood Coagulation	1
1.2.1 Cellular Coagulation Components	5
1.2.1.1 Tissue Factor	5
1.2.1.2 Thrombomodulin	6
1.2.1.3 Platelets	6
1.2.2 Soluble Coagulation Components	7
1.2.2.1 von Willebrand Factor (vWF)	7
1.2.2.2 Coagulation Cofactors	7
1.2.2.3 Coagulation Factors	8
1.2.2.4 Fibrinogen/Fibrin1	0
1.2.2.5 FXIII/FXIIIa 1	0
1.2.2.6 Proteins C and S 1	1
1.2.2.7 Tissue Factor Pathway Inhibitor (TFPI)1	1
1.2.2.8 Plasminogen/Plasmin1	2
1.2.2.9 Thrombin Activated Fibrinolysis Inhibitor (TAFI) 1	2
1.2.3 The Contact Activation Pathway 1	3
1.2.3.1 Factor XII1	3
1.2.3.2 Factor XI and PreKallikrein1	3
1.2.3.3 High Molecular Weight Kininogen 1	3
1.2.4 Coagulation Testing 1	4
1.2.4.1 Hemostasis Screening Assays1	4
1.2.4.2 Thrombin Generation Assays	4
1.3 Thrombin 1	5
1.3.1 Structure and Function	5
1.3.2 Exosites I and II 1	6
1.3.3 The 60s and γ -loop	6
1.4 Serpins and their Roles in Coagulation 1	6
1.4.1 Serpin Structure 1	6
1.4.2 Serpin Mechanism of Protease Inhibition 1	7
1.4.3 The Serpin Branched Pathway Mechanism 1	7
1.4.4 X-Ray Crystallography – Serpin Structure and Function 1	8
1.4.5 GAG Cofactors Accelerate Serpin Inhibitory Activity 1	9
1.4.6 Variant Thrombin Inhibitory Serpins	0

1.4.6.1	Alpha-1 Protease Inhibitor and the Pittsburgh Variant	
1.4.7 Na	atural Thrombin Inhibitory Serpins	
1.4.7.1	Antithrombin	
1.4.7.2	Heparin Cofactor II	22
1.4.7.3	Protein C Inhibitor	
1.4.7.4	Protease Nexin-1	
1.5 Engine	ered Protein Display and Mutant Library Screening	
1.6 Purpos	e of Investigation	25
1.6.1 Ot	bjectives and Hypotheses	25

5	SU	MMARY & PERSPECTIVES	. 94
	5.1	Rationale for Investigation	. 94
	5.2	Achieving Serpin Display on Mammalian Cells	. 95
	5.3	Serpin Targeting to the Plasma Membrane	. 95
	5.4	Measuring Inhibition Efficiency: Tethered vs. Soluble Serpins	. 96
	5.5	Inhibition of endogenous thrombin generation by AR-API M358R	. 98
	5.6	Mammalian Display Screening of Mutant RCL Libraries	100
	5.7	Thrombin Capture Screening of Degenerate RCL Libraries	102
	5.8	Future directions	104

List of Figures

Figure 1.1 - Tertiary structure of human serpin API	2
Figure 1.2 - The coagulation cascade.	4
Figure 1.3 - The serpin branched pathway mechansim.	. 18
Figure 1.4 - The stoichiometry of inhibition (SI).	. 18
Figure 2.1 - Soluble and membrane-targeted serpin constructs	34
Figure 2.2 - Subcellular fractionation of transfected mammalian cells	39
Figure 2.3 - Reaction of tethered or soluble recombinant API with thrombin	41
Figure 2.4 - Enzymatic deglycosylation of AR-API M358R and AR-	API
T345R/M358R	42

Figure 2.5 - Microscopic and flow cytometric detection of cell-surface bound
thrombin
Figure 2.6 - Comparison of initial rates of thrombin-API complex formation of
tethered or free serpins
Figure 2.7 - Reaction of tethered or soluble recombinant AT 47
Figure 2.8 - Reaction of tethered or soluble recombinant HCII 48
Figure 3.1 – TF and AR-API M358R expression by cultured T24/83 cells 62
Figure 3.2 - Analysis of thrombin generation assay (TGA) of T24/83 cells 64
Figure 3.3 - TF and API expression by transfected or co-transfected HEK 293
cells
Figure 3.4 - Immunoblot analysis of singly and doubly transfected HEK 293 cells.
Figure 3.5 - Immunoblot and thrombin generation of singly and doubly
transfected HEK 293 cells
Figure 4.1 - Subcellular fractionation of transfected mammalian cells
Figure 4.2 - Flow cytometric detection of bound thrombin
Figure 4.3 - Screening of a bacterial expression library of API randomized at
residue 358 (P1)
Figure 4.4 - Immunoblot and kinetic characterization of thrombin reactions with
recombinant API variants
Figure 4.5 - Thrombin capture assay time course
Figure 4.6 - Chracterization of API variants identified by screening of a bacterial
expression library of API partially randomized at residues P7-P2 (residues 352 -
357, no termination codons, all amino acids except F, C, W, or Y)

List of Tables

Table 1.1 - Disaccharide composition of coagulation GAGs	20
Table 2.1 - Sequence and purpose of oligonucleotides used in this study	33
Table 4.1 - Sequence of deoxyribonucleotide primers employed in this study	79

LIST OF ABBREVIATIONS

APC	Activated protein C
API	α_1 -proteinase inhibitor
APTT	Activated partial thromboplastin time
AR	Asialoglycoprotein receptor
AT	Antithrombin
DS	Dermatan sulphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
ETP	Endogenous thrombin potential
FII	Prothrombin
Fg	Fibrinogen
Fn	Fibrin
GlcNAc	N-Acetyl-D-glucosamine
GAG	Glycosaminoglycan
Gla	γ-Carboxyglutamic acid
GU	D-Glucuronic acid
GalNAc	N-Acetyl-D-galactosamine
Н	Heparin
HCII	Heparin cofactor II
HK	High molecular weight kininogen
HS	Heparan sulphate
PAR	Protease activatable receptor
PCI	Protein C inhibitor
Pg	Plasminogen
РК	Prekallikrein
Pn	Plasmin
PN-1	Protease nexin 1
PT	Prothrombin time
RCL	Reactive centre loop
SHBG	Sex hormone binding globulin
TAFI	Thrombin activatable fibrinolysis inhibitor
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TR	Transferrin receptor
uPA	Urokinase plasminogen activator
vWF	von Willebrand factor

1 INTRODUCTION

Blood coagulation is a critical physiological process mediated by enzymatic coagulation factors and co-factors that involves both soluble and cellular components. The enzymes of blood coagulation are proteases regulated by protease inhibitors. Most of these inhibitors belong to the serpin superfamily. None of the over thirty human serpins are membrane proteins. In this thesis, the effects of converting several human serpins into membrane proteins were studied, with an emphasis on human α_1 -proteinase inhibitor (API). The consequences of API mutations on the inhibition of the key coagulation protease thrombin were explored in genetically engineered mammalian and bacterial expression systems. In the following sections, detailed background information provides the scientific context and rationale for the hypotheses that were tested in this work.

1.1 Serpins In Health and Disease

Serpins have evolved with a very complex structure and mechanism of inhibition (see Figure 1.1): an exposed reactive center loop (RCL) irreversibly traps the target protease, and undergoes a dramatic conformational change that renders both inhibitor and enzyme permanently non-functional (1). In human health, inhibitory serpins regulate a number of biological processes essential for maintaining life, including inflammation and complement activation (API (2), and kallistatin (3)), angiogenesis (maspin (4)), fibrinolysis (α_2 -antiplasmin (5)), and blood coagulation (antithrombin (AT)) (6)). The main physiological inhibitor of thrombin, the key protease of coagulation, is AT (7), and to a lesser extent, heparin cofactor II (HCII) (8), protein C inhibitor (PCI) (9) and protease nexin 1 (PN-1) (10).

Certain serpin mutations facilitate misfolding and polymerization of the complex serpin structure resulting in a number of pathologies (i.e. serpinopathies), including the early onset of dementia (neuroserpin (H338R)(G392E) (11)), emphysema and cirrhosis of the liver (API (E342K) or the Z variant (11)), and thrombosis (AT (P80S)) (12). These mutations interfere with the correct folding of β -sheet A, resulting in the exchange ("swapping") of domains, comprised of two β -strands (i.e. s5A and s4A/RCL), between similarly misfolded serpin monomers, and irreversibly expanding the polymer (13,14).

1.2 Overview of Blood Coagulation

Hemostasis (i.e. maintaining normal blood flow) is a delicate balance between bleeding and clotting, and involves a series of amplifying biochemical activation reactions (i.e. coagulation cascade) (15) that culminate in the formation of the serine protease thrombin from its precursor protein prothrombin (factor II, or FII) (16). Thrombin is pivotal to the formation of a stable clot (i.e. thrombus) through its activation of platelets, coagulation enzymes and cofactors, and its conversion of fibrinogen (Fg) to fibrin (Fn) (17).

The coagulation cascade (see Figure 1.2) is triggered by injury to the vascular endothelium and exposure of circulating blood to tissue factor (TF): a membrane protein cofactor tightly associated with phospholipids (18), and localized to the adventitia (19). Circulating factor VII (FVII), once bound to TF (1:1), is activated by activated factor X



Krishnan & Gierasch Nature Structural & Molecular Biology 18, 222-225 (2011)

Figure 1.1 - Tertiary structure of human serpin API.

Human serpins share a conserved tertiary structure comprised of ca. 400 amino acids, 3 β -sheets (e.g., A, B and C), 8 or 9 α -helices, and a flexible reactive center loop (RCL) of ca. 20 residues. The intact RCL, with P1-P1' scissile peptide bond (blue, left image), acts as bait for protease (red, not to scale). Serpins in this native conformation maintain a unique tertiary structure that confers high-energy "meta-stability". While API does not require activation to inhibit protease, the coagulation serpins AT, and HCII require GAG (H, HS, and DS) binding to the helix D region for optimal reactivity. The inhibition reaction involves: cleavage of the P1-P1' scissile bond, and concomitant covalent bond formation between P1 and active site S1, RCL loop insertion into β -sheet A (right image), translocation of the protease to the opposite pole of the serpin rendering both non-functional, release of free energy, and a more "relaxed" and stable serpin conformation. The yellow balls are sites of mutation and are unrelated to the present discussion. With permission from Nature Publishing Group.



Figure 1.2 - The coagulation cascade.

Coagulation refers to a series of plasma amplifying biochemical reactions that culminate in thrombin formation. At the time of vascular injury, thrombin plays a pivotal role in reestablishing haemostasis (i.e. normal blood flow) through stable clot formation via direct and indirect activation reactions. Early plasma thrombin generation, initiated by the extrinsic tenase complex (TF - FVIIa), is sufficient to "prime" the cascade by directly activating coagulation cofactors (FVa, FVIIIa), factors (FXIa, FXIIIa), platelets (PAR 1), and Fn (from Fg), culminating in a dramatic "burst" of thrombin produced by the intrinsic tenase complex (FVIIIa - FIXa; see thick "U" shaped arrow), and a secure thrombus. As the clot matures, and thrombin generation reaches a maximum, the protease acts to inhibit its own formation (i.e. thrombin behaves like an anticoagulant) by binding endothelial cell TM (a transmembrane glycoprotein). The thrombin-TM complex activates PC (a serine protease with Gla domain) bound to EPCR, to form APC. Upon release, APC binds cofactor PS (non-enzymatic with Gla domain) on the surface of activated platelets blocking FVa, and FVIIIa formation, shutting down the prothrombinase (FXa - FVa), and intrinsic tenase (FVIIIa - FIXa) complexes. The thrombin - TM complex also activates TAFI (a carboxypeptidase that removes C-terminal basic residues from Fn) limiting tPA and Pg binding to Fn (i.e. thrombin behaves like a pro-coagulant). As thrombin generation diminishes, the negative feedback on fibrinolysis lifts, and the clot dissolves as Direct thrombin activation is represented by dotted lines. tissue repair proceeds. Cofactors are in green font while key inhibitory pathways are in red. Small black dots identify coagulation factors with platelet-binding Gla domains. Normally, cofactor FVIII circulates bound to vWF for transport to the surface of activated platelets, where thrombin activates, and then releases the cofactor. The bottom left corner summarizes the contact activation pathway where FXII auto-activates in the presence of negatively charged surfaces, and contributes to FXIa formation. While this pathway forms the basis for the in vitro APTT test, the physiological relevance to haemostasis remains uncertain.

(FXa) (20), and forms the extrinsic tenase complex (TF-FVIIa) that catalyses the conversion of FX to FXa (21), and factor IX (FIX) to FIXa (although FIXa plays only a minor role during the early stages of coagulation (22)). FXa, the only coagulation protease that can activate prothrombin (23), binds cofactor activated factor V (FVa) (1:1) to form the prothrombinase complex (FXa-FVa), and in the presence of Ca^{+2} and activated platelets catalyzes the conversion of FII to thrombin (24).

The relatively small ("priming") amounts of thrombin generated are sufficient to activate platelets by binding PAR1 (25), and release cofactor FV from platelet α -granules (26). Some thrombin binds non-PAR platelet surface receptors (i.e. GP-Ib/IX-V complex) and remains active (27). Circulating complexes of von Willebrand factor (vWF) and factor VIII (FVIII) also bind GP-Ib/IX (28); upon cleavage by thrombin, FVIIIa is released from vWF (29). The newly generated cofactors FVa and FVIIIa are now available for binding the activated platelet surface (30). FXI, also bound to the platelet surface, is activated by thrombin to FXIa (31), and is now capable of converting even more FIX to FIXa (32) which binds activated FVIIIa to form the intrinsic tenase complex (FVIIIa-FIXa) on the platelet surface (33). This complex now generates large amounts of FXa leading to the accelerated thrombin production required for stable clot formation (28).

As coagulation progresses, thrombin continues to stimulate the conversion of Fg to Fn (34), and activate the transglutaminase, factor XIII, to FXIIIa (35). FXIIIa strengthens the clot by introducing covalent cross-links into the polymerized fibrin monomers (36). Thrombin also helps to maintain the clot by activating thrombin-activatable inhibitor of fibrinolysis (TAFI) (37). TAFIa is a carboxypeptidase that delays fibrinolysis by inhibiting the activation of plasminogen (Pg) to plasmin (Pn) (37).

The thrombin that diffuses away from the injury site will be either inhibited by soluble factors like the serpin antithrombin (AT) (7) or be bound by the vascular endothelial cell receptor, thrombomodulin (TM) (38). The thrombin-TM complex activates endothelial receptor (i.e., EPCR) bound (39) protein C to APC (40), and APC then binds with protein S to the surface of activated platelets and inactivates FVa and FVIIIa (41). The non-serpin tissue factor pathway inhibitor (TFPI) inhibits early thrombin activation by binding the extrinsic tenase complex and forming quaternary complex with TF, FVII/FVIIa and FX/FXa (42).

1.2.1 <u>Cellular Coagulation Components</u>

Two membrane proteins and the entire platelet form the primary cellular components that contribute to the process of blood coagulation.

1.2.1.1 Tissue Factor

TF is a single-chain, 263-amino acid, 46 kDa integral membrane glycoprotein (43) predominantly localized to smooth muscle cells in the tunica adventitia of veins and arteries (44), and away from the lumen of blood vessels. It is comprised of three domains: a C-terminal 21-residue intracellular domain, a 23-residue trans-membrane

domain, and an N-terminal 219-residue extracellular domain (45). TF is a known cellular receptor for FVII and FVIIa (46). Site-directed mutagenesis studies have identified three separate regions on the extracellular domain for contact with FVIIa: Ile(22), and Lys(20), Asp(58) and Arg(135), Phe(140) (47). Formation of the extrinsic tenase (TF-VIIa) complex initiates the coagulation cascade, thrombin generation, and the formation of stable thrombi (48). The constitutive expression of TF on the cell surface is also a hallmark of several different types of cancers (49).

Under normal physiological conditions (i.e., hemostasis) there is no interaction between TF constitutively expressed on the surface of vascular sub-endothelium cells, and coagulation factors (i.e., FVIIa) in circulation. In this way, TF remains encrypted (i.e., inactive) with minimal procoagulant activity (PCA) (50); only upon injury is the TF activated to the prothrombotic state. While a definitive mechanism for decryption is not currently well known, *in vitro* studies have shown that activation can occur by various stimuli (e.g., cycles of freeze/thaw, oxidizing agents, calcium ionophores) resulting in sequestration of anionic phospholipids (i.e., phosphatidylserine) to the cell surface, without *de novo* protein synthesis (51). Importantly, recent studies suggest that the redox status of the protein disulfide isomerise (PDI)-dependent Cys186-Cys209 disulphide bond influences TF extracellular domain conformation, and ultimately PCA(52).

1.2.1.2 Thrombomodulin

Thrombomodulin (TM) is a 557-residue (53), 74 kDa (54) transmembrane glycoprotein expressed on the surface of endothelial cells and has no protease activity (55). Its structure is highlighted by: a 36-residue C-terminal cytoplasmic domain, a 35-residue Ser/Thr-rich domain, a 35-residue transmembrane domain, the 240-residue six epidermal growth factor (EGF)-like domains, a 68-residue hydrophobic domain and an 154-residue N-terminal lectin-like domain (56,57). Thrombin binds TM via exosite I at EGF domains 5 and 6 (53) while substrate protein C (PC), bound to endothelial protein C receptor (EPCR), interacts with TM at EGF domain 4 and the Ser/Thr-rich domain (58). In this way TM functions as an anticoagulant, by restricting the ability of thrombin to interact its numerous substrates, including fibrinogen, PAR-1, FV, FVIII, FXI, and FXIII and also as a procoagulant by promoting TAFI generation (57,59).

1.2.1.3 Platelets

Thrombin activates platelets by binding the G-protein coupled, protease-activated receptor, PAR1 (60). Thrombin binds, and then cleaves the N-terminal extracellular domain of PAR1 (25,61) exposing a new N-terminal tethered ligand domain that binds the cleaved receptor to complete the activation (62). Activation depends on thrombin interaction with cofactor platelet receptor GP-Ib/IX (63) and involves both exosites I and II (17,27). The constitutively expressed platelet glycoprotein (GP) Ib-IX-V complex is comprised of four transmembrane GPs: the GP Ib α chain, the GP Ib β chain, the GP IX and GP Ib β -chain (64).

Upon activation, platelets release the contents of their α -granules (i.e. fibrinogen, fibronectin, vWF, plasminogen, protein S, FV, FVIII) (65) and electron dense particles (Ca⁺², serotonin, ATP/ADP, pyrophosphate) (66) into the circulation, while phospholipid scramblase facilitates the transfer of phosphatidylserine (PS) to the platelet surface (67), providing a surface on which activated coagulation factors (thrombin, FIXa, FXIa) and cofactors (FVa, FVIIIa) can assemble (28).

Although the GP-Ib/IX complex is an important receptor for thrombin, it can also bind vWF, and because the binding domains occupy separate locations they can coexist simultaneously (68). In this way, vWF promotes its function as an important adhesion molecule (69).

Platelet activation also results in conformational changes in other key receptors, such as the major platelet integrin glycoprotein IIb/IIIa, also known as integrin $\alpha_{IIb}\beta_3$, that render it competent to bind vWF, fibrinogen, fibrin, or fibronectin. These adhesive proteins can simultaneously bind to two different platelets at once, and the resulting aggregation of enmeshed platelets explains why both aggregated platelets and polymerized, cross-linked fibrin contribute importantly to the physical integrity of physiological blood clots (70).

1.2.2 Soluble Coagulation Components

Soluble components important in blood coagulation include the coagulation factors and cofactors, the fibrin precursor fibrinogen, adhesive/carrier proteins such as von Willebrand factor, anticoagulant protein C, and proteins that act primarily in the fibrinolytic pathway.

1.2.2.1 von Willebrand Factor (vWF)

The precursor (pre-pro-vWF) is a 2813-amino acid glycoprotein that includes a 12-amino acid signal peptide, a 751-residue pro-peptide and a 2050-residue mature subunit (71). Intracellular processing leads to the polymerization of the mature subunits by multiple disulphide bonds. After synthesis in endothelial cells and megakaryocytes, the vWF multimers are stored in Weibel-Palade bodies (endothelial cells), or α -granules (megakaryocytes/platelets) and released upon activation (72). The vWF multimers are also constitutively secreted by endothelial cells into circulation (72).

The vWF functions as a transporter of cofactor FVIII in circulation and to facilitate platelet adhesion at the wound site (69). The vWF binds to the platelet receptor GPIb-IX-V complex (73), and to $\alpha_{IIb}\beta_3$ (70), and to components of the vascular sub-endothelium (74). The importance of vWF as an anchoring protein is underscored in vWF disease patients whose platelets lack the ability to adhere to the wound site (75).

1.2.2.2 Coagulation Cofactors

Factor V is a large, 2196-residue, single-chain non-enzymatic glycoprotein with Mr = 330 kDa (76) consisting of six domains: A1-A2-A3-B-C1-C2 (77). It is present in plasma at a concentration of 7 µg/mL (78). Thrombin activation yields FVa with a structure

comprised of an N-terminal-derived, 105 kDa heavy-chain (domains A1, A2), and a Cterminal-derived 74 kDa light-chain (domains A3, C1, C2) linked by hydrophobic interactions, and stabilized by a single Ca⁺² ion (79,80). Upon activation, cofactor FVa combines with the serine protease FXa to form the prothrombinase (FVa/FXa) complex on the phosphatidylserine-rich surface of activated platelets, in the presence of Ca⁺² (81). FVa interaction with the platelet surface occurs via the two C-domains (77,82). Historically, reports on FV in disease primarily involved bleeding disorders (83). However, in 1994 the Arg506Gln mutation (FV_{Leiden}), which causes resistance to APCmediated degradation of FVa, was reported and has since been shown to represent the most common defect in patients presenting with thromboembolism (84).

FVIII is a 2332 residue non-enzymatic polypeptide with Mr = 265 kDa (85), is synthesized primarily in the liver (86), and has the domain structure: A1-A2-B-A3-C1-C2 (85). FVIII undergoes intracellular proteolysis (87) resulting in the secretion of a 200 kDa heavy chain (A1-A2-B) non-covalently linked to a 80 kDa light chain (A3-C1-C2) in the presence of metal ion (Cu/Ca⁺²) (88). In plasma FVIII is stabilized by forming a complex with vWF (89) in a ratio of 1:100 (90), circulating in plasma at 0.2 µg/mL (91). Thrombin-activated FVIIIa is comprised of a 90 kDa N-terminal derived heavy chain non-covalently linked with a 69 kDa C-terminal derived light chain (92). After cleavage by thrombin, FVIIIa associates as a cofactor with serine protease FIXa on the surface of activated platelets to form the intrinsic tenase complex required for the conversion of FX to FXa (93). The two C domains of the light-chain are essential for binding vWF and the surface of activated platelets (94). The importance of this cofactor in coagulation is highlighted in haemophilia A, an X-linked bleeding disorder resulting from a deficiency in FVIII (95).

1.2.2.3 Coagulation Factors

The serine protease zymogens FII, FVII, FIX, and FX are vitamin K-dependent coagulation proteins and possess a conserved N-terminal γ -carboxyglutamate (Gla) domain (96). The Gla domain is essential for protein anchoring, in a Ca^{+2} -dependent interaction, to the phospholipid (PL)-rich surface of cell membranes (i.e. FVII) or the phosphatidylserine (PS)-rich surface of activated platelets (i.e. FII, FIX, FX) (67). The domain has up to twelve Gla residues depending on the coagulation factor (FII 10 Gla residues (97); FVII 10 Gla residues (98); FIX 12 Gla residues (99); FX 11 Gla residues (100)). The mechanism involves Ca^{+2} binding to the Gla side-chain carboxyl groups, triggering a conformational change (101), stabilization of the tertiary structure (102) and exposure of a hydrophobic patch (ω loop) within the domain that facilitates membrane binding (103). A more recent study has reported that the mechanism for bovine FII binding involves PS interaction with Gla-domain bound Ca⁺² ions and Gla residues 17 and 21 (104). Although activated factor XI (FXIa) is also a serine protease, it lacks the Gla domain and is dependent on the non-covalent association with high Mr kininogen to facilitate binding to platelets and activation (105). Once bound, the proteases interact with their respective cofactors to propagate the cascade of reactions leading to thrombin generation and a stable clot. Although coagulation factors FVII, FIX and FX have widely

different functions in coagulation they share a high degree of structural similarity suggesting that they evolved from a common ancestral gene (106).

FVII is a 406-residue, single chain glycoprotein (98) with Mr = 50 kDa (107), is synthesized in the liver, and circulates at 0.5 µg/mL (96). The domain structure includes a N-terminal, membrane-binding ten residue Gla domain, two epidermal growth factor (EGF)-like domains and a C-terminal serine protease domain (21). Upon activation by FXa, FVIIa is converted to a heterodimer consisting of a 152-residue light chain with the Gla and EGF-like domains covalently linked by a single disulphide bond to a 254-residue heavy chain with the catalytic domain (108). FVIIa alone possesses very little activity (109), and only when bound to cofactor TF to form the extrinsic tenase (FVIIa/TF) complex, does FVIIa achieve its full activity (110).

FIX is a 415-amino acid, single chain glycoprotein (99) with Mr = 56 kDa (111) synthesized in the liver, and circulates at 5 µg/mL (96). Structurally, FIX consists of an N-terminal Gla domain (12 Gla residues), two EGF-like domains and a C-terminal catalytic domain (112). Upon activation by FXIa, FIXa forms a heterodimer with a light chain (145 amino acids) containing the Gla domain and two EGF-like domains linked by a single disulphide bond to a heavy chain (235 amino acids) containing the catalytic domain (113). FIXa, once bound to cofactor FVIIIa to form the intrinsic tenase (FVIIIa/FIXa) complex on an activated platelet surface, becomes a potent activator of FX (114). Haemophilia B is an X-linked genetic bleeding disorder caused by a deficiency in FIX (115).

FX is a 488-amino acid, single-chain glycoprotein (100,116) with Mr = 56 kDa synthesized in the liver, and circulates at 10 µg/mL (96). The structure consists of an N-terminal Gla domain (11 Gla residues), two EGF-like domains and a C-terminal catalytic domain (106). Upon activation, by either the extrinsic tenase (FVIIa/TF) or intrinsic tenase (FVIIa/FIXa) complex, FXa forms a heterodimer with a 139-residue light chain (comprised of a single Gla domain and two EGF-like domains), linked by a disulphide bridge to a 303-residue heavy chain with the catalytic domain (117). FXa bound to cofactor FVa forms the prothrombinase complex (FVa/FXa) and converts FII to thrombin (106). FX deficiency is a rare autosomal recessive trait and is among the most severe of the bleeding disorders (118).

FXIa is also a serine protease, but lacks the membrane-binding Gla domain, and instead relies on complex formation with high molecular weight (HMW) kininogen in order to bind the surface of activated platelets or other surfaces (119). It is an unusual protease because it circulates as a homodimer of two identical polypeptide chains linked together by a single disulphide bond (120). Each chain has 607-amino acids (Mr = 70 kDa) (121), and is comprised of four Apple domains, a catalytic domain and a heparin-binding domain (120,122). Upon activation, one or both chains may be is cleaved, yielding two 238-amino acid, C-terminal derived light chains and two 369-amino acid, N-terminal derived heavy chains (123). FXIa circulates at a concentration of 5 µg/mL (96) and is

important to the intrinsic pathway by activating FIXa (124). A deficiency in FXIa is a rare autosomal bleeding disorder in which some patients lack symptoms while others suffer from variable bleeding tendencies depending on the site of the injury and the associated fibrinolytic potential (i.e. increased TAFI production) (125).

1.2.2.4 Fibrinogen/Fibrin

Fibrinogen (Fg) and fibrin (Fn) are essential structural components of a stable clot (126). Fibrinogen is a soluble (2964 amino acid), 340 kDa glycoprotein (127), synthesized in the liver (128), and consisting of three pairs of identical polypeptides chains ($A\alpha_2B\beta_2\gamma_2$) (129): two A α chains (610 residues each); two B β chains (461 residues each); and two γ_A or γ' (411 residues or 427 residues) linked by 29 disulphide bond (129). It circulates at 3 mg/mL (128). The N-termini of the six polypeptides converge at the central E-domain, and extend outward in either direction to the C-termini, forming α -helical coiled coils that lead to the globular D domain (130). The two A α chains extend beyond the D-domain, forming two flexible α C regions that normally associate with the E-domain (131).

Thrombin cleaves the N-termini of the two A α chains and two B β chains, releasing their respective fibrinopeptides A (16 amino acids) and B (14 amino acids) to form fibrin (Fn) (132). Fibrinopeptide A forms more quickly, exposing the E domain N-terminal sequence GPRVVE (the "A" binding site), specific to a γ -chain D domain binding pocket "a" on two other fibrin molecules (133). Fibrinopeptide B forms more slowly, exposing the E domain N-terminal GHRP sequence (the "B" binding site) that is specific to the B β -chain D domain binding pocket "b" from other fibrin molecules (133). These non-covalent "A:a" and "B:b" interactions between fibrin molecules promote polymerization through protofibril formation (134).

The C-terminal of the major γ_A chain binds the platelet fibrinogen receptor, $\alpha_{IIb}\beta_3$, and facilitates platelet aggregation (135). The minor γ' chain represents ca. 8% of the entire γ chain population (136), and arises from alternate mRNA splicing (137), is unable to bind platelets (138), but does bind plasma FXIII subunit B and serves as a carrier for the catalytic subunit A (139). In addition to facilitating fibrin assembly, the "B" binding region also interacts with heparin (140) and is involved with the release of vWF (140).

1.2.2.5 FXIII/FXIIIa

FXIIIa is a transglutaminase important in cross-linking Fn monomers (141). The plasma zymogen FXIII, is a 320 kDa heterologous tetramer (142) comprised of two catalytic A-subunits, synthesized in the liver and bone marrow (megakaryocytes), non-covalently linked to two carrier B-subunits, also produced in the liver (143). Importantly, about half of all the A-subunit dimers are localized to the platelet cytoplasm (142) without their carrier B-subunits, making them readily available for clot stabilization (144).

Activation by thrombin to FXIIIa occurs in the presence of fibrin polymer cofactor (145), and involves cleavage of the plasma and platelet A-subunits (146). The C-terminal region of γ -chains contains a cross-linking site where FXIIIa catalyzes the formation of γ dimers

(147) consisting of intermolecular covalent bonds between the γ donor Lys(406) and the γ acceptor Glu(398)/(399) (148). The formation of γ trimers, γ tetramers and the intermolecular cross-linking between A α or α -chains also occurs but these reactions evolve more slowly (149).

1.2.2.6 Proteins C and S

Activated protein C (APC) is a serine protease that inactivates FVa and FVIIIa (150). The zymogen protein C (PC) is a 460-residue (Mr = 52 kDa), vitamin K-dependent, single chain glycoprotein (151) synthesized in the liver and circulates at 2.3 ng/mL (152). PC shares considerable structural homology with FVII, FIX, FX, and more generally with FII, and is comprised of a Gla domain (nine Gla-residues), two EGF-like domains and a catalytic domain (153). PC circulates as a heterodimer with an N-terminal derived light-chain comprised of the Gla and EGF-like domains, linked by a disulphide bond, to a C-terminal heavy chain containing the protease domain (154).

As coagulation proceeds, the concentration of total thrombin increases, making more available for protein C activation. This reaction requires the binding of thrombin and PC to their respective endothelial cell-displayed receptors, thrombomodulin (TM) and protein C receptor (endothelial PCR, or EPCR) (155). The binding of thrombin to TM via exosite I, results in the sheltering of this fibrinogen-binding site, and promotes catalyzing the activation of protein C to activated protein C (APC) (156). APC then binds its non-proteolytic cofactor protein S to facilitate activated platelet binding, and in the presence of Ca⁺², catalyzes the inactivation of cofactors FVa and FVIIIa (157). The importance of PC is underscored by the severe and often fatal thrombotic complications in newborns with homozygous PC deficiency (158).

The APC cofactor Protein S (PS) is a single-chain 635-residue, non-enzymatic vitamin Kdependent glycoprotein with Mr = 80 kDa (159-161) synthesized primarily in the liver and by endothelial cells (162), which circulates in plasma at a concentration of 0.35 μ M (163). The PS structure includes an N-terminal Gla domain, a thrombin sensitive region (TSR), four epidermal growth factor (EGF)-like domains, and a C-terminal sex hormone binding globulin (SHBG)-like domain (164). About 60% of total PS in circulation binds C4b-binding protein (C4BP) via the SHBG-like domain while 40% is available as APC cofactor (165). PS is a cofactor in the activated protein C (APC) catalyzed inactivation of FVa (166) and FVIIIa (167). The PS Gla domain, TSR and EGF1 domain facilitate binding to APC while separate regions of the Gla domain are responsible for binding the entire complex, in the presence of Ca⁺², to the surface of activated platelets (164). The importance of PS is demonstrated in children born with a homozygous PS deficiency who present with serious thrombotic complications (168).

1.2.2.7 Tissue Factor Pathway Inhibitor (TFPI)

Tissue factor pathway inhibitor (TAFI) is the main inhibitor at the early stages of blood coagulation and functions by forming strong complexes with TF-FVIIa and FXa (169). Two different isoforms of human TFPI have been identified as TFPI α (170) and TFPI β

(171). Both isoforms are comprised of two Kunitz-type inhibitory domains, K1 and K2, that bind and inhibit FVIIa and FXa, respectively (172). The mechanism of inhibition is FXa-dependent and involves TAFI binding the ternary complex of TF-FVIIa-FXa with K1 binding the FVIIa active site and K2 binding the active site of FXa (42).

1.2.2.8 Plasminogen/Plasmin

Plasmin (Pn) is serine protease important in fibrinolysis (173). The zymogen plasminogen (Pg) is a glycoprotein that is synthesized in the liver comprised of 791amino acids (Mr = 92 kDa) and 24 disulphide bonds which circulates at a concentration of approximately 2 μ M (174,175). Structurally, Pg is comprised of an N-terminal Pan-apple (Pap) domain, five kringle domains (KR1 to KR5) and C-terminal serine protease domain (176). The Lys-binding properties of KR1 and KR4 (177) are important for fibrinolysis by modulating the interactions of Pg with Fn (178), cellular receptors(179), extracellular matrix (180) and Pn inhibitor α_2 -plasmin inhibitor (α_2 -PI) (181). Pg activation to the two-chain protease Pn, by either tissue plasminogen activator (tPA) (182), or urokinase plasminogen activator (uPA) (183), occurs by cleavage of a single Arg (560)-Val (561) bond (184). Plasminogen may be activated by either tissue-type or urinary plasminogen activators (tPA or uPA).

Human tPA is a 527-amino acid serine protease that is secreted by vascular endothelial cells (185), and is comprised of an N-terminal fibronectin type-1 domain, an EGF-like domain, two Kringle domains (KR1, KR2) and the catalytic domain (182). It has been proposed that the initial tPA binding to Fn is mediated by the fibronectin domain, while enhanced binding is promoted by KR2 domain binding to the C-terminal Lys residues exposed due to Pn degradation (186). Kinetic data shows that optimal tPA performance requires Fn binding and supports a mechanism involving ternary complex formation between tPA, Fn and Pg (187).

Human uPA is a single-chain (scu-PA) 411-amino acid and is comprised of an N-terminal EGF-like domain, that contains a region important for binding its cell-surface receptor (uPAR), a kringle domain, and a C-terminal catalytic domain (183). Two-chain uPA (tcu-PA) occurs by the Pn cleavage at Lys(158)-Ile(159) and Lys(135)-Lys(136) (188). Although tcu-PA shows a much higher activity toward chromogenic substrates than scu-PA, it is scu-PA bound to partially digested Fn (189,190) and uPAR (191) that is the more efficient activator of Pg.

Inhibition of fibrinolysis occurs by blocking the Pg activators tPA and tcu-PA, but not scu-PA with serpin PAI-1(192,193), or by blocking Pn with α_2 -antiplasmin (194,195).

1.2.2.9 Thrombin Activated Fibrinolysis Inhibitor (TAFI)

Thrombin activated fibrinolysis inhibitor (TAFI) is a 432-amino acid (58kDa) glycoprotein synthesized in the liver and circulates at a concentration of ca. 100nM (196). The activation of TAFI is enhanced by thrombin binding to TM (197). The resulting carboxypeptidase, catalyzes the removal of C-terminal basic amino acids (Lys, Arg) that

become exposed on fibrin strands degraded by plasmin, and inhibits plasminogen activation (198).

1.2.3 <u>The Contact Activation Pathway</u>

Contact activation forms the basis for the routinely employed in vitro Activated Partial Thromboplastin Time (APTT), and provides an alternate path to fibrin formation (199). It includes the plasma serine protease zymogens FXII, FXI, prekallikrein (PK), and cofactor high molecular weight kininogen (HK) (200). FXII interaction with negatively charged surfaces yields FXIIa via autoactivation (201-204), and the assembly of FXIIa with FXI, PK, and HK results in the formation of the activation complex (203). In this way, FXIIa converts FXI and PK to their respective active serine proteases FXIa (205) Kallikrein generates even more FXII via reciprocal and kallikrein (K) (206). amplification (202,207), and converts HK to bradykinin (B) (208) while FXIa leads to fibrin generation via the intrinsic tenase complex (32). Although a deficiency in the contact factors results in a severely prolonged APTT (209), individuals with a genetic deficiency in FXII (210) or FXI (i.e. hemophilia C (211)) are either asymptomatic, or experience only mild bleeding. Based largely on these observations, the contact pathway for decades has been described as unimportant in hemostasis (212), while more recent reports suggest a possible role in thrombogenesis (213).

1.2.3.1 Factor XII

Plasma FXII (209) is a 596-residue (214,215) (*M*r 76 kDa) (216) single-chain glycoprotein serine protease zymogen (217) synthesized in the liver (218), and circulates at 30 µg/mL (0.375 µM) (219). Autoactivation (220) leads to the formation of FXIIa (or α XIIa) consisting of two polypeptide chains linked by a single disulphide bond (221). The N-terminal heavy chain (350-residues, *M*r 50kDa) is comprised of two fibronectin-type domains (types I, II), two EGF-like domains, and a kringle domain, and is responsible for surface binding (222), while the C-terminal light chain (243 residues, *M*r 28 kDa) contains the catalytic domain (221). Activated FXII can also exist as XIIf (or β XIIa): a 30 kDa fragment derived from α XIIa (223) that does not bind negatively charged surfaces, and activates PK but not FXI (224,225). The plasma inhibition of α XIIa, and β XIIa occurs mainly by the serpin C1 inhibitor (226,227).

1.2.3.2 Factor XI and PreKallikrein

Current coagulation schemes include FXIa as part of a FXII-independent thrombinmediated feedback loop that generates large amounts of thrombin via FIXa, and the intrinsic tenase complex (FVIIa/FIXa) (228-230). Although FXI differs from the vitamin K-dependent coagulation proteases described above, it is structurally homologous to PK (120), and like PK (231) circulates bound to HK (232). Interestingly, recent evidence suggests that FXIa and PK likely shared a common ancestral gene (233).

1.2.3.3 High Molecular Weight Kininogen

In order for HK to be an efficient cofactor in the activation of PK, and the FXIIadependent activation of FXIa (234), it must be cleaved by K to the two-chain form (HKa), while also releasing the vasodilator bradykinin (235). Cleavage by FXIa leads to an inactive HK cofactor (236).

1.2.4 <u>Coagulation Testing</u>

The complexity of the coagulation system and associated pathways, as well as the low concentration of some components in plasma provides challenges to the in vitro testing of coagulation activities. Simplified assays based either directly on fibrin clot formation in the test tube or on thrombin generation are therefore widely applied.

1.2.4.1 Hemostasis Screening Assays

The coagulation system is routinely screened for bleeding disorders (237) by the *in vitro* tests prothrombin time (PT) (238,239), and activated partial thromboplastin time (APTT) (240,241). Although both tests measure the time to clot, and use citrate-chelated, platelet-poor plasma (i.e. no Ca^{+2} or phospholipid (PL)), they differ in the way that clotting is initiated (239,241).

The PT test is initiated by adding high concentrations of commercial tissue factor (TF), supplemented with PL and Ca^{+2} , and is a measure for the presence of functional FII, FVII, FV, FX and fibrinogen (i.e. "extrinsic" coagulation) (242). A normal PT time range is between 11 to 15 sec. (239), while a prolonged time may indicate a deficiency in one of FII, FVII, FV, FX, a deficiency in vitamin-K, or liver disease (242). PT is a standard test performed to monitor the effect of Warfarin, a vitamin K antagonist, for targeting dosage adjustments to give a PT between 150 and 200% of the normal mean value; in standardized form it is called the International Normalized Ratio (INR).

The APTT test is initiated with the addition of PL and Ca^{+2} , in the presence of an activating surface/compound (kaolin; hydrated aluminum silicate), and measures for the presence of functional FVIII, FIX, FXI and FXII (i.e. "intrinsic" coagulation) (243). The APTT is routinely used to assess a patient's response to heparin treatment relative to a normal APTT time of 35 sec (241).

1.2.4.2 Thrombin Generation Assays

Although the PT and APTT tests are routinely performed in the clinic to assess the coagulation system, the results can be affected by a number of variables (243), and are subject to challenges in interpretation (242). Over the years numerous groups have developed tests attempting to more closely mimic coagulation *in vivo*, and provide a continuous display of thrombin levels, an essential coagulation factor also known to activate non-hemostatic pathways (i.e. cell proliferation) (244). The kinetics derived from thrombin generation assay (TGA) traces can provide a more powerful means of assessing general hemostasis compared to PT or PTT (245,246).

One of the original TGA, developed over 60 years ago by Hartert (247), was thromboelastography (TEG), a mechanical technique that measures the elastic properties of whole blood as it clots, and is still in clinical use today (248). Macfarlane and Biggs

(1953) reported on a technique where thrombin generation was measured in whole blood (249). In this labour-intensive method, a blood subsample was transferred every minute to a tube containing a fibrinogen solution, and the time to clot is recorded. Thrombin generation, as a function of time, was determined by comparing the time data to a standard curve prepared using known amounts of thrombin added to a similar fibrinogen solution.

The Hemker group was first to move away from the "time to clot" methods and developed a more precise discontinuous spectrophotometric-based TGA employing thrombin-specific chromogenic substrates (S2238 (250) and SQ68 (251)), and adopted a computer program for detailed analysis. It was now possible to characterize the explosive nature of thrombin generation with quantifiable parameters, including lag time, peak height, thrombin potential (TP) (i.e. area under the peak) (251), and endogenous thrombin potential (ETP) (i.e. TP minus area under the curve for α 2-macroglobulin-bound thrombin) (245). By 2000, the same group reported on the fluorogenic substrate-based TGA that continuously displayed thrombin generation (i.e. thrombogram), and was several orders of magnitude more sensitive than chromogenic substrates (252). Currently, fluorescence-based TGA has developed into a useful research tool with potential for diagnostic use in the clinic (246).

1.3 <u>Thrombin</u>

1.3.1 <u>Structure and Function</u>

Thrombin (α -thrombin), a serine protease, is central to blood coagulation and hemostasis (17). The zymogen prothrombin (FII), is a 579 residue 72-kDa (97,253), glycoprotein (254) synthesized in the liver (255) which circulates at a concentration of 100 µg/mL (96). Key structural domains include an N-terminal Gla domain (10 of 40 residues are Gla (256)), two homologous kringle domains (257), and the C-terminal catalytic domain (258). The Gla domain is an important vitamin K-dependent post-translational modification where glutamate (Glu) is modified to γ -carboxyglutamate (Gla) by γ -glutamyl carboxylase (259). The binding of Ca⁺² to the Gla domain results in a conformational change that promotes FII anchoring to phospholipid surfaces (260,261). The Gla domains of FII, and FXa (262), and the C-domains of FVa (77) mediate the assembly of the prothrombinase complex (263) on the phosphatidylserine-rich surface of activated platelets resulting in thrombin activation (264). Once formed, thrombin escapes its anchoring domains, and diffuses into circulation to interact with its many substrates/inhibitors (17,262,265).

Thrombin consists of a light A chain (36 residues) covalently linked by a disulphide bridge to a heavy B chain (259 residues) containing the catalytic domain (16,266). Some studies have reported that the A-chain has no physiological role, and is simply a residual appendage from FII processing (267). However, others have described the severe bleeding in patients with naturally occurring mutations in the A-chain underscoring its importance (268-270).

Thrombin has unique structural features that confer specificity toward coagulation-related molecules; anion-binding regions; an active site located in a cleft in the middle of the molecule; and protective insertion loops (258).

1.3.2 Exosites I and II

The tertiary structure of thrombin reveals a molecule with an uneven charge distribution embodied in positively charged patches called exosites I and II, an unusually large proportion of polar amino acids, and the unequal distribution of acidic and basic residues in the A- and B-chains (266,271). Exosite I, situated to the P1' side of the active site, is essential for binding Fg (272-274) and Fn (275-277), thrombin recognition of inhibitory HCII (278,279) and hirudin (280,281), binding cofactor precursors FV (282,283), and FVIII (282,284), and binding substrates thrombomodulin (56,285), PAR1 (61,62,286) and GPIba (27,287). Exosite II, situated "opposite" exosite I, is necessary for heparin binding and for facilitating AT and HCII inhibition by ternary complex formation (bridging mechanism) (288,289). Other studies have reported that exosite II also interacts with FV and FVIII (282), and platelet receptor GPIba (27).

1.3.3 <u>The 60s and γ-loop</u>

Access to the active site is sterically restricted by a 60s loop and a 147s (or the γ -loop) (290). The rigid 60s loop, comprised of hydrophobic side chains from Tyr-Pro-Pro-Trp residues (291), frames the upper portion of the active site interacting with the hydrophobic residues of the substrate/inhibitor that are N-terminal to the scissile P1-P1' bond (17). Reports on deletion experiments indicate that the 60s loop controls substrate specificity (258,291,292). The flexible γ -loop is more hydrophilic, interacting with substrate residues C-terminal to the scissile bond (17), and plays a role in substrate selection by partially blocking access to the active site (293).

1.4 Serpins and their Roles in Coagulation

1.4.1 <u>Serpin Structure</u>

Serpins are a superfamily (188,294,295) of structurally homologous, and functionally distinct serine and cysteine protease inhibitors identified in virtually all forms of life (1,296-298). Of the 37 human serpins currently identified (299), most function outside the cell to mediate protease activity (300), while others have protective intracellular roles (301). Through the course of evolution, some members have lost their inhibitory function, and are involved in hormone transport (corticosteroid binding globulin) (302), or have become precursor polypeptide hormones (angiotensinogen) (303).

A well-characterized subset of the 37 human serpins (i.e. AT, HCII, PCI, α_2 AP, PAI-1, PN-1) are involved in mediating the important process of hemostasis (304). Serpins share a highly conserved tertiary structure consisting of ca. 400 amino acids, 3 β -sheets, 8, or 9 α -helices and a flexible reactive center loop (RCL) of approximately 20 amino acids, but only share about 25% primary sequence homology (305). The RCL is essential for serpin specificity as the loop residues must precisely complement the active site amino acids in

order to act as "bait" for the protease (306). The loop residues range from the N-terminal hinge region (P17) to the C-terminal (P3'), based on the convention of Schecter and Berger (307), and link β -sheets A and C, respectively (304). The most important residue for serpin specificity is P1 because it binds the protease irreversibly during inhibition via an acyl intermediate bond (299). The flanking residues P4 to P3' are necessary for active site interaction while the other residues that interact with the protease are considered exosite contacts (307). The unique tertiary structure confers a high-energy (metastable, or S for Stressed) state to serpins in their native conformation as long as RCL integrity is maintained (188,308,309). Cleavage of the RCL, and loop insertion into β -sheet A (308,310,311), thermodynamically drives the inhibition reaction rendering the serpin hyperstable, or relaxed (312-315).

1.4.2 <u>Serpin Mechanism of Protease Inhibition</u>

Serine proteases like thrombin have 3 catalytically essential residues His57, Ser195 and Asp102 (using conventional numbering based on chymotrypsin) (316). The serpin RCL P1-P1' must interact precisely (317) with the active site to form a transient and reversible Progress toward a tetrahedral Michaelis (i.e. enzyme-inhibitor) complex (318). intermediate is associated with active site distortion facilitating the formation of an oxyanion hole (Gly193, Ser195) to accommodate the P1 carbonyl carbon (319). Decomposition occurs by acid hydrolysis, resulting in scissile P1-P1' bond cleavage and the formation of an acyl-enzyme intermediate between P1 and protease Ser-195 (1). Acylation triggers full RCL insertion, from P15 (the hinge region) to P1, into β -sheet A, along with active site stretching and oxyanion hole destruction as Ser 195 shifts circa 6 Å away from His 57 reducing the rate of deacylation and the possibility of enzyme An estimated -32 kcal/mol of energy is released with the regeneration (320). translocation of the protease some 70Å to the opposite pole of the serpin (304). Overall, the serpin transitions from a high energy to a low energy state while the protease, crushed at the base of the serpin, is rendered non-functional (321).

1.4.3 <u>The Serpin Branched Pathway Mechanism</u>

Most human serpins are protease inhibitors, and some function as protease substrates, while both types share common early intermediates in the branched pathway mechanism (see Figure 1.3) (322). These include: the reversible and non-covalent Michaelis complex (EI), the tetrahedral intermediate, and the acyl-enzyme intermediate (EI') (305). Beyond acylation, the fate of each serpin depends on the relative difference in the rate of RCL insertion (i.e. $k_4 \gg k_3$ leads to inhibitory serpin) compared to deacylation (i.e. $k_3 \gg k_4$ leading to substrate serpin), and both rates largely depend on the amino acid characteristics in the hinge region (323). Mutant serpins that act as substrates possess charged residues (i.e. Arg, Gln) at ~ P15, P14 near the hinge region of the RCL (188,324). Studies report that these amino acids interfere with loop insertion, and yield an inactive serpin with a cleaved RCL and regenerated protease (325). The parameter, stoichiometry of inhibition (SI), is a consequence of the branched pathway, and provides a quantitative measure between inhibitory (k₄) and substrate (k₃) serpins (see Figure 1.4) (305).

$$E+I \stackrel{k_1}{\longleftarrow} EI \stackrel{k_2}{\longrightarrow} [EI'] \stackrel{k_3}{\longleftarrow} \stackrel{E+I*}{\underset{k_4}{\longleftarrow}} EI*$$

Figure 1.3 - The serpin branched pathway mechansim.

The initial encounter between protease (E) and serpin (I) leads to the reversible, noncovalent Michaelis (EI) interaction, defined by forward, and back rate constants k_1 , and k_{-1} , respectively. The EI complex progresses via a tetrahedral intermediate to the acylenzyme EI' complex with a rate constant k_2 . The EI' intermediate, defined by RCL cleavage at the P1-P1' scissile bond, can proceed along one of two pathways, depending on the relative rates of RCL insertion into β -sheet A versus deacylation. The presence of non-charged residues in the hinge region (i.e. ~ P14), promotes RCL insertion with progress toward inhibition, and the formation of the serpin-enzyme complex (EI*) with a rate constant k_4 . If charged amino acids occupy the region, deacylation advances EI' toward protease regeneration (E) with rate constant k_3 , leaving the serpin (I) nonfunctional with a cleaved RCL (i.e. spent substrate). The rate constant k_5 refers to the decay of EI* toward a free but compromised proteinase (i.e. originates from a serpin inhibition reaction), and cleaved serpin.

$$SI = (k_3 + k_4)/k_4$$

Figure 1.4 - The stoichiometry of inhibition (SI).

The SI is a parameter used by scientists to provide a quantitative measure of the relative balance between inhibitory versus substrate serpin reactions. The stability of the covalent acyl-enzyme bond makes it possible to estimate SI directly from the reducing environment of SDS-PAGE gels. After protease challenge, the ratio of band intensities between inhibitory (i.e. complexed) and substrate (i.e. non-native) serpins can be used to approximate k_4/k_3 , and then SI. For example, when reactions are mostly inhibitory (i.e. $k_4 \gg k_3$), then SI ~ 1.

1.4.4 X-Ray Crystallography – Serpin Structure and Function

X-ray crystallography has played an essential role in solving the structure of many important macromolecules (326); approximately 90% of the 108,124 entries listed (April 21, 2015) in the protein data bank (PDB) were deciphered by this technique. Scientists have known for decades that crystallized proteins diffract x-rays in a pattern that reflects their unique tertiary structure (327). This property has been exploited to deduce the structure of several important biomolecules including DNA (328), myoglobin (329,330), and serpins.

API was the first serpin to have its crystal structure analyzed in 1980; it was revealed that RCL cleavage at P1-P1' resulted in a dramatic conformation change that led to a more thermodynamically stable inhibitor (308). By 1995 computer modelling predicted that the mechanism of inhibition involved: formation a covalent linkage between serpin and protease, relocation of the protease to the opposite pole, and insertion of the cleaved RCL into β -sheet A (331). While there was a general agreement with this proposed scheme, conflicting views remained as to whether the loop insertion was partial (332) or full (333). Importantly, the crystal structure analysis of the API-trypsin complex in 2000 confirmed the full insertion (P15 to P1), and the concomitant active site distortion prohibiting deacylation, and therefore, the possibility of protease re-generation (320).

While a general inhibitory mechanism was established, additional refinement was required for the serpins AT and HCII because of their dependence on GAG activation for optimal thrombin inhibition. Crystallographic structures revealed that GAG binding to AT occurs at helix D, resulting in its elongation, and the expulsion of the partially inserted residues P15, and P14 at the hinge region prior to full loop insertion (334,335). Although HCII shares a similar global mechanism for thrombin inhibition with AT, analyses have shown that the N-terminal tail is uniquely required for providing specificity toward thrombin (336).

1.4.5 GAG Cofactors Accelerate Serpin Inhibitory Activity

Glycosaminoglycans (GAGs) are essential for the existence of multi-cellular life (337). GAGs are involved in a broad range of biological processes including coagulation where they accelerate serpin inhibition of important proteases (e.g. thrombin) (338,339). GAGs are comprised of charged linear polysaccharide chains of repeating unique disaccharide sequences, and are localized to cell surfaces and the extracellular matrix (ECM) (340). One of the monosaccharides is often D-glucuronic acid (GU), or L-iduronic acid (IU), while the other is either N-acetyl-D-glucosamine (GA), or N-acetyl-D-galactosamine (GLA), and all monosaccharides can be N- or O- sulphated to varying degrees. In fact, GAG function is largely dependent on the degree of sulphation within the chain (341). Interestingly, deciphering the "sulphate code" is currently an area of research interest to scientists (342).

The GAGs most relevant to coagulation include heparin (H), heparan sulphate (HS), and dermatan sulphate (DS) (see Table 1.1; based on (343)). HS is comprised of GA, with an

amino group (-NH₂) at the C2 position that can be either sulphated or N-acetylated, linked to GU (β 1-4) or IU (α 1-4). The structure of DS is a combination of GLA, which can be sulphated at positions C4 or C6, linked (α 1-3) to IU. The structure for H is similar to HS but tends to be more sulphated. The linkages between disaccharide units are either α/β 1-4 (HS, H) or α/β 1-3 (DS).

GAG	Repeating Disaccharide Unit - Showing Carbon Positions	
H, HS	$[(GA) 2NY, 3X, 6X (\alpha 1, 4) (IU) 2X \text{ or } (GU) 2X (\alpha/\beta 1, 4)]_n$	
DS	$[(GLA) 2NY, 4X, 6X (\beta 1, 4) (IU) 2X \text{ or } (GU) 2X (\alpha/\beta 1, 3)]_n$	
X = sulphated or unsubstituted		
Y = sulphated, acetylated or unsubstituted		
Based on Ernst et al., (1995).		

Table 1.1 - Disaccharide composition of coagulation GAGs

Heparin, discovered around the time of WWI, remains one of the oldest and most important drugs still in clinical use: an anticoagulant employed in a broad range of medical procedures (344). Low molecular weight heparins (LMWH) are agents developed in response to an improved understating of coagulation biochemistry, and have the advantage of acting more precisely with reduced side effects (7,345). While heparin is found in human mast cells (346), it is not involved in coagulation; the role of physiologically relevant anticoagulant belongs to heparan sulphate localized to the lumen of vascular endothelial cells (347). Although DS is localized to the vascular sub-endothelium (i.e. ECM), and is known to accelerate HCII inhibition of thrombin, the physiological importance of this reaction is currently unknown (339).

1.4.6 Variant Thrombin Inhibitory Serpins

1.4.6.1 Alpha-1 Protease Inhibitor and the Pittsburgh Variant

Human α_1 -proteinase inhibitor (API) is a 394-residue (Mr = 51 kDa) single polypeptide (305) with 3 carbohydrate side chains (348) N-linked to Asn residues (349). API is synthesized in the liver, and circulates at a concentration of 25 μ M (350) with a half-life of 6 days (351). The RCL, with Met at the P1 (M358R) position (352), forms a 1:1 complex with the target protease (353), and shares approximately 29% structural homology with AT (354). Although API inhibits many serine proteases, it is most efficient at targeting leukocyte elastase (355-357). This preference is highlighted in individuals with a genetic deficiency in API, who suffer from severe pulmonary emphysema (358,359). Human API cleaved at its reactive centre was the first serpin to have its crystal structure resolved (308) and has since maintained its role as the prototypical serpin (188).

A mutant of API, discovered in a young patient with a chronic bleeding disorder (360), was characterized as an API Met (358) Arg substitution, and designated as API Pittsburgh (or API-M358R) variant (361). Recognition that API-Pittsburgh shared the same RCL P1

(Met358) - P1' (Ser359) as AT, explained in part the target protease switch from an elastase inhibitor to a potent inhibitor of thrombin (361). Two other cases of API-Pittsburgh have since been reported in the literature, although these patients presented with less severe bleeding tendencies (362,363). API (M358R) isolated and characterized from the original patient was also shown to be a potent inhibitor of the coagulation contact proteases FXIa, Kallikrein, and FXIIa_f (364). Collectively, these observations prompted consideration of API M358R as a potential treatment for septic shock, a multifactorial condition in which contact system activation is typical. A potential benefit over existing therapies is that API (M358R) does not require glycosaminoglycan (GAG) activation, and therefore patients would not be subjected to bleeding risks associated with heparin use (365).

Results from preliminary experiments were encouraging and showed that recombinant API variant (rAPI (M358R)) administered to piglets induced with septicemia was protective (366). Experiments conducted in larger primates were less encouraging as rAPI M358R was discovered to also be a potent activated protein C (APC) inhibitor, and this was thought to exacerbate the shock syndrome (367). Subsequent studies incorporated intra- and extra-RCL mutations, based on AT and HCII structures, with the objective of designing a new rAPI M358R variant with specificity for thrombin, but not APC. One of the first intra-RCL studies, by Hopkins et al., (2000), showed that substituting the API (M358R) RCL with AT Asn (393) at P3', in combination with AT P7 to P2', resulted in reduced APC inhibition (368). Similar results were observed by Filion et al., (2004) who replaced API (M358R) RCL P16-P3' residues with the corresponding amino acids from HCII L444R (369). Sutherland et al., (2006) showed that fusing the extra-RCL N-terminal 75 residues, or HCII "tail" to a1PI (M358R) increased the rate of thrombin inhibition (370). Moreover, if the same tail was combined with key API (M358R) RCL mutations, specifically those identified by Hopkins et al. (368) it was also possible to enhance specificity and increase the rate of thrombin inhibition (371). Collectively, these studies showed that the API (M358R) RCL can be genetically modified to enhance the activity and specificity of the resulting analogues. However, these directed mutagenesis studies have been time-consuming and their variant API products have proven to be only marginally improved over API M358R in animal model studies of thrombosis and endotoxemia (372).

1.4.7 Natural Thrombin Inhibitory Serpins

1.4.7.1 Antithrombin

Antithrombin (AT) is a 432 residue serpin Mr = 55 kDa (373,374), glycoprotein synthesized in the liver (375,376) that circulates at 150 mg/L (377) with a half-life of 3 days (378). AT also has six Cys residues that form three intermolecular disulphide bonds, and four N-linked carbohydrate chains (379). Upon inhibition, AT forms a 1:1 stoichiometric covalent complex with key coagulation proteases thrombin (7,380) and FXa (381-383) in addition to FIXa, (384), FXIa (380), FXIIa (385), and FVIIa (386,387) by a mechanism that involves the formation of an acyl-enzyme intermediate (388). AT

normally circulates in low activity form, and upon glycosaminoglycan (GAG) cofactor (i.e. heparin, heparan sulphate) activation, protease inhibition is accelerated by a factor of 10^3 (388,389). Early studies aimed at understanding the mechanism of interaction identified important GAG (390), and AT (391-393) binding regions, and that activation involves a change in conformation (394). The importance of AT is underscored by the therapeutic use of heparin and synthetic heparin as anticoagulants (289). A genetic deficiency in AT is associated with an increased risk of developing venous thrombosis (395) and AT knock-out mice are embryonically lethal (396). Standard sodium heparin is administered by injection into the bloodstream while the biosynthesis of the physiologically more relevant heparan sulphate is localized to the vascular endothelium (334). Both GAGs contain a unique pentasaccharide sequence that binds AT with high affinity resulting in its activation (389,397). About 30% of the heparin chains contain this sequence while a much smaller percentage of heparan sulphate binds with high affinity (321). The advent of AT crystal structures has helped to refine the details of GAG-associated conformational changes in AT-mediated protease inhibition (398-400).

GAG interaction with AT occurs primarily at helix D (334). The initial interaction is weak (K_D of 25 µM), but once bound it promotes the transition to a high affinity conformation (K_D of 50 nM) characterized by D helix extension, and hinge region expulsion (P15 and P14(401)) from β -sheet A (321,402). This allosteric mechanism (AM) results in a more favourable P1 orientation on the exposed RCL, leading to improved recognition of AT by FXa and FIXa (403). However, recent studies have reported that helix D extension results in modified Tyr (131) and Ala (134) side chain interactions with the hydrophobic serpin core and that this step, and not RCL expulsion (i.e. AM), is more critical for AT inhibition of target protease (404). The AM also does not explain AT activation for thrombin inhibition. Rather, AT and thrombin (through exosite II, or the heparin binding site) bind the same GAG chain to form a ternary complex in what is called the bridging or template mechanism (289,403).

1.4.7.2 Heparin Cofactor II

HCII is a single-chain glycoprotein, comprised of 480-residue with Mr = 65.5 kDa (405,406), synthesized primarily in the liver (407), and circulates at 90 µg/L (ca. 1 µM) (408) with a half-life of 2 to 3 days (409). HCII was first identified by Briginshaw and Shanberge (410), and later characterized by Tollefsen's group (408,411). Although it has three Cys residues, no intramolecular disulphide bridges have been identified (412). HCII inhibits thrombin and clot-bound thrombin (8), but no other coagulation protease, to form a 1:1 covalent complex with Mr ca. 96,000 (408,409) in a GAG-accelerated reaction (408) that uniquely includes dermatan sulphate (DS) (413). The HCII-thrombin complex is cleared within 2 to 3 min from circulation by binding low density lipoprotein receptor-related proteins located on hepatocytes (414).

In the presence of GAG cofactors heparin (H), heparan sulphate (HS) and DS, HCII is a potent thrombin inhibitor, accelerating the reaction by a factor of 10^3 , although more H and HS is required compared to AT (413). Heparin, when employed therapeutically as an

anticoagulant, preferentially activates AT due to a relatively low affinity for HCII (411). Although HCII can be activated by HS, which lines the vascular endothelium, it does not naturally protect against AT deficiency (415), and a deficiency in HCII does not lead to thrombosis (416). Some studies report that DS may be useful as a therapeutic option over H in the treatment of thrombosis (417) particularly with respect to clot-bound thrombin, against which H is ineffective (8). The localization of DS to the vascular subendothelium suggests that HCII may play a protective role in minimizing tissue damage at the injury site (418). Others have reported that HCII may play an important hemostatic role by protecting against atherosclerosis (419). Adding to the debate, two separate studies based on homozygous HCII knockout mice have reported that HCII is a residual component from an ancestral coagulation system that has been carried over (421).

Although the structure and mechanism of activation for HCII (i.e. allosteric mechanism) resembles that of AT, there are key differences between the two thrombin inhibitors (336). Unusually for a thrombin inhibitor, HCII contains Leu at P1 (405,406,422) of the RCL instead of Arg (AT) (373,374), resulting in a very low rate of inhibition in the absence of GAG activation (423). HCII is also unique in that specificity for thrombin is conferred by the action of an 80 amino acid N-terminal acidic tail (278), normally sequestered to the helix D region of native HCII (424).

Upon GAG binding to the helix D region, and activation, the tail is exposed (425) by a combination of partial displacement and conformational change (306). The tail, with repeat sequences resembling the C-terminal portion of hirudin, binds exosite I (fibrinogen binding site) of thrombin with high affinity (278). Like AT, HCII forms a ternary complex (bridging mechanism) with H and HS, but not with DS (321).

1.4.7.3 Protein C Inhibitor

Serpin protein C inhibitor (PCI) is a 387-residue (426), Mr = 57 kDa single chain glycoprotein serpin (427), synthesized in the liver (428), and circulates at 5.3 mg/L (429). PCI inhibits APC and other coagulation serine proteases, including thrombin and FXa (9), FXIa (430), and the thrombin-TM complex (431). Studies have indicated that PCI inhibition of APC is accelerated in the presence of H-like GAGs (9), and that the mechanism involves GAG binding to helix H (432,433) instead of helix D as in the case of AT (334) and HCII (425). Otherwise, PCI inhibition of APC and thrombin occurs through a similar mechanism of protease inhibition (320) with the formation of a ternary complex (434,435).

1.4.7.4 Protease Nexin-1

Serpin protease nexin-1 (PN1) is a 378-residue single-chain glycoprotein (436) with Mr = 43kDa (10). Unique amongst coagulation serpins, PN1 is a cell-produced protease inhibitor that binds H-like GAGs expressed on the cell surface (437-439). Although virtually undetectable in plasma (440), PN1 has been identified in various organ systems

including the brain, kidneys, lungs, spleen, and muscle including the heart (441). In vitro kinetic studies have reported that PN1 is a particularly efficient inhibitor of thrombin, in addition to tPA, uPA (10), FXa (442), and FXIa (443). The mechanism of inhibition is similar to the other coagulation serpins (319) and the reaction is accelerated in the presence of H-like GAGs through ternary complex formation with target protease (439).

1.5 Engineered Protein Display and Mutant Library Screening

Many investigators have sought to understand serpin structure and function by mutagenesis and recombinant protein expression, since cDNA clones became available for all human serpins in the 1980s and 1990s. Indeed, in 2004, De Taeye et al. mused, in the title of a review article, "The story of the serpin plasminogen activator inhibitor 1: is there any need for another mutant (444)? These authors went on to catalogue the over 600 mutations that had been introduced into this serpin. As described above in detail for API M358R in Section 1.4.6.1, some of these studies involved the deliberate substitution, or swapping of residues from the RCL of one serpin into another. However, in spite of the zeal with which investigators have designed and performed these mutagenesis studies, few laboratories have attempted an unbiased, genomic approach, in which all possible substitutions in a serpin RCL could be assembled and functionally probed with different proteases.

Biotechnological advances have rendered protein engineering by screening, rather than by design, a feasible alternative. The general approach, which has in some cases been applied to tailor proteins to specific therapeutic purposes, has been described as "directed evolution"; in this approach cDNA libraries coding for millions or trillions of recombinant candidates are created, expressed and/or displayed on a cell or particle surface, and then screened for the select few with the intended functional characteristics (445). Display technologies, in combination with screening methods, are powerful tools used to link the phenotype (i.e. the biochemical/binding properties of mutant protein candidates to the genotype (i.e. coding sequence)) (446).

The first report on surface display, in 1985, described the enrichment of a phageexpressed fusion protein comprised of a portion of the Eco RI restriction endonuclease (i.e. the minor M13 filamentous phage coat protein pIII) (447). Although the size of a phage-display library is potentially very large (10^{11}) , important disadvantages of such early approaches were the limitations on the size of cDNA and its encoded protein that could be expressed and the low copy number of the resultant fusion protein per virion (448). This limitation was solved with the development of cell-based display systems (bacteria, yeast, and mammalian) where a specialized anchoring domain is combined with the protein of interest as either an N or C-terminal fusion protein (449). Cell-based display systems also have the potential advantage of employing the powerful quantitative screening tool of fluorescence-activated cell sorting (FACS) (i.e. flow cytometry) (450-452). *E. coli.* is the most commonly employed host for bacterial display due to the ease of genetic manipulation, and rapid growth rates (453). Although most studies describe the targeting and anchoring of mutant protein candidates to the outer membrane (454-456), some have displayed fusion proteins successfully on the inner cytoplasmic membrane (457).

Yeast possess the post-translational machinery (proteolytic processing (458), disulphide bond formation (459), N and O-glycosylation (460)) required for the display of more complex protein molecules (461). Studies have reported that the screening of yeastdisplay libraries yields a more complete list of variants compared to phage display (462), and bacterial display (463). Some of the reported disadvantages of yeast display include the potential for a negative immune response due to hyperglycoslyation (464), and plasmid instability (465).

The creation of high affinity biomolecules for clinical use requiring a the full complement of post-translational modifications prompted the development of mammalian display (466). One advantage of this technique is the number of different cell lines (i.e. HEK 293 (467-469), COS (470-472), T24/83(473)) that are potentially available for transient transfection and protein display. The few studies that report on mammalian cell surface display are limited to the successful creation of antibodies for therapeutic purposes (466,474-476).

1.6 <u>Purpose of Investigation</u>

1.6.1 **Objectives and Hypotheses**

In spite of the advances in our understanding of the relationship between serpin structure and serpin function, with respect to inhibition of specific proteases, considerable gaps remain. The complexity of the serpin mechanism means that serpins must not only present an RCL that fits the active site of a specific protease with optimal complementarity, this RCL must also be optimally configured to insert into the serpin βsheet A rapidly and without steric clashes after reactive centre clearance. It must accomplish these two tasks in order to generate a serpin-enzyme complex in which cleaved serpin and active site-distorted protease remain stably associated. Directed mutagenesis approaches have generally failed to produce highly active serpins with specificity for novel targets, or resulted in the generation of serpins with enhanced rates of protease inhibition gained at the cost of increased substrate behaviour. Protein modelling approaches, while gaining in power with every passing year, are also not yet up to the task of simultaneously predicting optimal pre- and post-encounter RCL sequences in the context of an entire serpin.

The overall objective of this thesis work was to explore the possibility that display approaches could be employed to identify variant API proteins with novel RCL substitutions with enhanced activity and/or specificity against a protease target. At the

outset of my PhD research, display approaches had been applied only in a few studies all involving a single serpin, PAI-1. These studies had used bacteriophage systems, with or without surface display. It was not known if any serpin could transit the eukaryotic protein secretory pathway as a membrane protein and arrive at its intended plasma protein location in functional folded form. I selected this long-term goal because of the promise of mammalian cell display of providing selection of optimally configured serpins with native-like post-translational modifications. The original research presented in the following pages of this thesis in large measure followed this overall strategy, and tested at least in part the following hypotheses:

- 1. Serpins will be expressed as integral membrane proteins on the surface of mammalian cells i.e. when fused to an N-terminal combined signal/membrane-anchoring signal sequence, oriented with the N-terminus anchored to the plasma membrane and the C-terminus expressed on the outside of the cell;
- 2. Membrane-tethered serpins, including API M358R, AT, and HCII will retain the ability to form covalent serpin-enzyme complexes with thrombin when displayed on the surface of membrane-tethered cells;
- 3. Within the huge sequence space defined by twenty different amino acid possibilities at each of the twenty positions of the API RCL will exist variants that will inhibit serine proteases with enhanced rates of reaction compared to native serpins;
- 4. Membrane-tethered serpins will inhibit both exogenously added thrombin and endogenously generated thrombin in cells tested in plasma systems;
- 5. Thrombin will bind to a minority of transfected mammalian cultured cells in a library of surface-displayed API variants hypervariable at one or more RCL residues by forming serpin-enzyme complexes with functional variants, and serve as an affinity tool with which to select for functional API variants.

These hypotheses were tested in this thesis by achieving the following objectives:

- 1. To compare the ability of combined signal/membrane anchoring sequences derived from the human asialoglycoprotein receptor or the human transferrin receptor to direct API, AT, and HCII to the surface of transfected COS or HEK 293 cells using subcellular fractionation and immunofluorescent imaging;
- 2. To assess the ability of transfected serpins to form denaturation-resistant complexes with thrombin;
- 3. To compare the rates of reaction of a membrane-tethered serpin to its soluble counterpart;
- 4. To measure thrombin generation mediated by transfected mammalian cells displaying both membrane-tethered API M358R and tissue factor in the presence of human plasma;
- 5. To create and characterize hypervariable libraries encoding all or most amino acid possibilities at one or more RCL codons;

- 6. To devise and execute a fluorescence-activated cell sorting strategy to screen transfected HEK 293 cells transfected with a hypervariable codon 358 library for functional tethered API M358X variants;
- 7. To compare hypervariable library screening using bacterial expression of soluble API variants to the approach from Objective 6; and
- 8. To characterize kinetically any novel functional mutants arising from the screening work after re-expressing them in soluble form as necessary.
2 PAPER #1 – Retention of thrombin inhibitory activity by recombinant serpins expressed as integral membrane proteins tethered to the surface of mammalian cells

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Preface

I contributed to the design of this study, and conducted the bulk of the experimentation, data acquisition and analysis, and draft manuscript preparation.

Ms. Varsha Bhakta and Dr. Jason Sutherland contributed to the preliminary experimentation showing that mammalian display of serpins was feasible.

Ms. Varsha Bhakta also assisted with the design of plasmid manipulation, and helped with experiments in advance of manuscript re-submission.

Dr. Lisa Toltl and Dr. Patricia Liaw provided the expertise and instrumentation related to flow cytometry.

The concept and design of displaying serpins on the surface of mammalian cells originated with Dr. William Sheffield. Dr. Sheffield was also project supervisor as well as senior author, and responsible for final revisions to the manuscript prior to final submission and re-submission.

This research was supported by the Heart and Stroke Foundation of Ontario. I was a recipient of a Graduate Fellowship from Canadian Blood Services.

Journal of Thrombosis and Haemostasis, 9: 2424-2435

ORIGINAL ARTICLE

Retention of thrombin inhibitory activity by recombinant serpins expressed as integral membrane proteins tethered to the surface of mammalian cells

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Word count: Abstract 250; Text (excluding references and figure legends) 5660

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These studies were presented in preliminary form at the XXIInd and XXIIIrd Congresses of the International Society on Thrombosis and Haemostasis, Boston, USA, and Kyoto, Japan, August 2009, and July 2011.

Running Title: Serpins function as integral membrane proteins (46 characters)

Summary. Background: Serpins form a widely distributed protein superfamily but no integral membrane serpins have been described. *Objectives:* To anchor three serpins (α_1 proteinase inhibitor (α_1 PI M358R), antithrombin (AT) and heparin cofactor II (HCII)) in the plasma membranes of transfected mammalian cells and assess their ability to inhibit thrombin. Methods: Serpin cDNAs were altered to include N-terminal, non-cleavable plasma membrane targeting sequences from the human transferrin receptor (TR-serpin) or the human asialoglycoprotein receptor (AR-serpin), and used to transfect COS-1 or HEK 293 cells. Cells were analyzed for serpin expression by immunoblotting of subcellular fractions, by immunofluorescent microscopy, or by flow cytometry, with or without exposure to exogenous thrombin; AR- and TR-serpins were also compared to their soluble recombinant counterparts. Results: Both TR- and AR-a1PI M358R were enriched in the integral membrane fraction of transfected COS-1 or HEK 293 cells, and formed inhibitory complexes with thrombin, although less rapidly than soluble α_1 PI M358R. Thrombin inhibition was abrogated by an additional T345R mutation in AR- α_1 PI M358R. Surface-displayed AR-AT also formed serpin-enzyme complexes with thrombin, but to a lesser extent than AR-a1PI M358R; AR-HCII inhibitory function was not detected. Immunofluorescent detection and flow cytometric quantification of bound thrombin also supported the status of AR- α_1 PI M358R and AR-AT as thrombin inhibitors. Conclusions: Two of three thrombin-inhibitory serpins retained functionality when expressed as integral membrane proteins. Our findings could be applied to create and screen hypervariable serpin libraries expressed in mammalian cells, or to confer protease resistance on engineered cells in vivo.

Keywords: serpins, thrombin, membrane insertion, flow cytometry, protein expression

Introduction

The serpins are a large superfamily of protease inhibitors whose members typically, but not exclusively, regulate serine proteases. Thousands of serpins have now been described, primarily in eukaryotes but also in prokaryotes (297). While arguably the bestcharacterized serpins are extracellular plasma proteins such as α_1 -proteinase inhibitor (α_1 PI, also known as α_1 -antitrypsin or SERPINA1) or antithrombin (AT, also known as SERPINC1), the majority of the 36 human serpins exhibit an intracellular, nucleocytoplasmic localization (297). In spite of this predominantly intracellular distribution, no serpins have been described to date that are attached to phospholipid bilayers as integral membrane proteins.

Serpins exhibit a conserved structure of 3 β -sheets, 8 or 9 α -helices, and an exposed reactive centre loop (RCL) (305). Following attack by a cognate protease, in some cases appropriately oriented by exosite interactions between the reacting proteins (477), serpins undergo a dramatic rearrangement triggered by the cleavage of the serpin reactive centre bond (320,333). Bond scission unleashes stored energy in the serpin fold, driving RCL insertion and translocation of the attached protease to the opposite pole of the serpin. The scope and rapidity of the rearrangement distorts the active site of the protease, preventing completion of its catalytic reaction, and leaving it covalently attached to the serpin via an acyl-ester linkage (478). The sensitivity of the serpin fold to disruption is underscored both by observations showing that the speed of RCL insertion determines whether serpins act as protease substrates or protease inhibitors (479), and by the numerous single amino acid changes shown to promote serpin misfolding, some of which are linked to human disease (480).

In this study we sought to determine whether thrombin-inhibitory serpins could be expressed in functional form as membrane proteins comprised of both an extracellular serpin domain and a transmembrane anchoring domain. This was by no means certain, given that less extensive naturally occurring and engineered serpin mutations arrest and/or cause the destruction of nascent secreted serpins within the endoplasmic reticulum (481,482). We focused primarily on α_1 PI, the most abundant serpin found in human plasma, and specifically on its M358R "Pittsburgh" variant, which confers substantial thrombin inhibitory activity on the protein (483,484). We also produced analogous membrane-tethered forms of the larger, natural thrombin-inhibitory serpins AT and HCII. Our findings suggest that membrane-displayed serpins could be exploited to screen for novel variants in structure-function studies, or in cellular engineering applications to protect cells from undesirable proteolytic attack.

Materials and methods

DNA manipulations to obtain cDNA fragments encoding membrane targeting sequences Sequences encoding Met-Gly followed by codons 3-69 of the human asialoglycoprotein receptor (ASGPR H1 subunit, or AR) or Met-Gly followed by codons 3-98 of the human transferrin receptor (TR) were obtained by reverse-transcription Polymerase Chain Reaction (RT-PCR) of RNA isolated from human hepatoma cell line HepG2 as described (485). A single reaction for RT-PCR was carried out for each sequence, respectively, using a OneStep RT-PCR kit according to the manufacturer's instructions (Qiagen, Mississauga, ON), and the following primer pairs: for AR, ML 18459 and ML 18458; and for TR, ML 18457 and ML 18460. The DNA sequences of these and all other oligonucleotides used in this study can be found in Table 2.1. The resulting 248 bp AR and 342 bp TR PCR products were independently restricted with HindIII and NcoI. As shown schematically in Fig. 2.1A, these fragments were subsequently employed in three-part ligation reactions joining an A fragment (encoding a targeting signal), a B fragment (encoding a His-tagged mature serpin cDNA) and a C fragment (comprised of expression plasmid pCDNA3.1 (Invitrogen, Carlsbad, CA)).

DNA manipulations to join serpin cDNAs to targeting signals

DNA sequences encoding two forms of α_1 PI, AT, and HCII were next readied for ligation. Previously described plasmid pBAD-H₆-API (M358R) (486) was used as the source for α_1 PI codons, and modified using standard site-directed mutagenesis procedures to form pBAD-H₆-API (T345R/M358R), in which codon Thr345 was mutated from ACG to AGG (Arg) (487). Previously described plasmid pBAD-H₆HCII (488) was the source for HCII codons. An AT cDNA was obtained by PCR amplification using pC3-AT (481) as the template and oligonucleotides ML 9868 and ML 08-681. Serpin cDNA sequences were mobilized by NcoI-EcoRI restriction digestions, yielding restriction fragments of 1211 bp for α_1 PI M358R and T345R/M358R, 1458 bp for HCII, and 1334 bp for AT (in which the NcoI-compatible restriction enzyme AfIIII was employed).

Assembly of serpin expression plasmids

As shown schematically in Fig 2.1A, expression plasmids were assembled by joining DNA fragments of classes A, B, and C. Fragment C was the 5391 bp HindIII-EcoRI digestion product of pCDNA3.1 in all cases. For instance, ligation of the 248 bp HindIII-NcoI-restricted AR signal-encoding amplicon to the 1211 bp NcoI-EcoRI fragment encoding α_1 PI M358R and to Fragment C yielded plasmid pC3-AR-API M358R (6850 bp).

Generation of expression plasmids encoding soluble serpins

Two additional DNA constructs were produced by replacing sequences encoding a noncleavable membrane insertion signal with those encoding the cleavable 19 amino acid signal sequence of human HCII. Plasmid pC3-HCII (481) was PCR-amplified with oligonucleotides ML 08-358 and a generic T7 primer (Promega, Madison, WI) and the PCR product was restricted with HindIII and EcoRI. The resulting 133 bp fragment was substituted for its AR signal-encoding counterpart in pC3-AR-API M358R and pC3-AR vielding plasmids pC3-ssAPI (M358R), and pC3-ssAPI T345R/M358R, (T345R/M358R), which encoded singly- or doubly-mutated α_1 PI cDNAs equipped with a secretory signal sequence (ss). Both plasmids were 6735 bp. The coding and flanking regions of all constructs assembled for this study were verified by DNA sequencing (MOBIX Central Facility, Hamilton, ON); the same facility synthesized all

Table 2.1 - Sequence and purpose of oligonucleotides used in this study		
Oligonucleotide number	DNA sequence	Application
ML 18459	5'-TCAAAGCTTG	PCR AR signal
	ACCCTGAGCA	
	ATCCCAGGTC	
	CAGCGCCAGC	
	CCTATCATGG	
	GAACCAAGGA	
	GT-3'	
ML18458	5'-ATGGCCATGG	PCR AR signal
	CCTGCAGCTG	_
	GGAGTTTTGG	
	G-3'	
ML 18457	5'-TCAAAGCTTT	PCR TR signal
	CGAGCGGCTG	_
	CAGGTTCTTC	
	TGTGTGGCAG	
	TTCAGAATGG	
	GAATGGATCA	
	AGCTAGA-3'	
ML 18460	5'-ATGCCATGGC	PCR TR signal
	CTCAGTTTTT	_
	GGTTCTACCC-3'	
ML 9868	5'-GATCACATGT	PCR AT cDNA with N-
	CTCACCATCA	terminal His tag
	CCATCACCAT	
	CACGGGAGC	
	CCTGTGGAC-3'	
ML 08-681	5'-GATCGAATTC	PCR AT cDNA with N-
	TTACTTAACA	terminal His tag
	CAAGGGTTGG C-3'	
ML 08-358	5'-GCATCCATGG	PCR HCII secretion
	TACCCCACGC	signal
	AGATGTTATG-3'	





Panel A illustrates the cloning strategy that was employed, in which DNA encoding either a cleavable (SS) or non-cleavable signal sequence (AR or TR) was combined with that encoding one of four serpins, and the pCDNA3.1 expression vector (Invitrogen), in a 3-part ligation reaction facilitated by the presence of compatible restriction endonuclease-generated ends at the A/B, B/C, and C/A boundaries; full details are provided in Materials and methods. **Panel B** shows schematic diagrams of proteins in schematic, linear form, N- to C-terminal. Numbering refers to amino acid numbers; negative numbers refer to cleavable signal sequences. Black bars depict non-cleavable signal sequence/membrane insertion signals comprised either of residues 3-98 of the transferrin receptor (TR) or residues 3-69 of the asialoglycoprotein receptor (AR). Serpins are shown as open boxes; grey boxes relate to the 19 and 32 amino acid HCII and AT signal sequences. The black lines between targeting signals and serpin codons in all cases correspond to sequences encoding the nonapeptide MGSHHHHHH.

oligonucleotides. Prior to use in transfection experiments, high purity preparations of each plasmid described above were prepared using Plasmid Maxi Kits (Qiagen), as were preparations of pC3-AT and pC3-HCII, which encoded natural AT and HCII mature sequences under the secretory control of their native signal sequences, respectively (481).

Cell culture and transfection, and reactions with thrombin

HEK 293 cells and COS-1 cells were obtained from ATCC (Manassas, VA) and grown as monolayers in a humidified 5% CO₂ atmosphere, in T-25 flasks or on 6 cm plates, in DMEM/F12 media supplemented with 10% FBS, 1% L-glutamine, penicillin and streptomycin, as described (489). Cells were grown to 70% confluency, then switched to media lacking antibiotics. Twenty-four hours later they were transfected using 8 μ g of plasmid DNA and 20 μ L Lipofectamine 2000, in serum-free media (SFM) as directed by the manufacturer (Invitrogen, Carlsbad, CA). Following a recovery period of 36-48 hours in full media, cells were washed and switched to SFM with or without addition of human thrombin (Enzyme Research Labs, South Bend, IN) to 10 μ g/ml (270 nM) for 30 minutes. The medium was aspirated, the cells rinsed with PBS, and then harvested following exposure to citrate/saline (0.015 M trisodium citrate dehydrate pH 7.8 / 0.13 M potassium chloride) for 20 minutes at 37°C. Following centrifugation (5000 x g at 4°C for 5 minutes), cell pellets were stored at -80°C until further processing.

Subcellular fractionation

The method of Stagmeyer et al. was employed to generate cytosolic, peripheral membrane, and integral membrane fractions of transfected cultured cells (490). Transfected cell pellets were resuspended in 0.5 mL PBS/10% sucrose, then sonicated five times using a probe sonicator (Fisher Scientific, Nepean, ON, model 100, microtip attachment) for a total of 10 seconds at power setting 2. The sonicate was subjected to ultracentrifugation (Beckman LS-80) at 100,000 x g for 1 hour at 4°C, yielding the cytosolic supernatant fraction and the total membrane pellet. This pellet was resuspended in 250 µL freshly made 100 mM sodium carbonate pH 11.5, incubated for 1 hour at 4°C, and ultracentrifuged as described above. The second supernatant formed the peripheral membrane fraction, and the final pellet the integral membrane protein fraction (490,491), which was resuspended in 100 μ L 2% SDS or 160 μ L SDS-PAGE sample buffer. Portions of each fraction were combined with concentrated SDS-PAGE sample buffer and frozen prior to electrophoresis and immunoblotting. Mouse monoclonal antibodies to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and human anti-Na,K-ATPase subunit α -1 (both obtained from Abcam (Cambridge, MA)) were used as marker antibodies for cytoplasmic and integral membrane protein fractions, respectively. Total integral membrane protein preparations were enzymatically deglycosylated with Peptide: N-glycosidase F under denaturing conditions as directed by the manufacturer (New England Biolabs, Pickering, ON). In some experiments a rabbit antibody directed against custom synthetic peptide NH₂-KSPLFMGKVVNPTQK-COOH (Dalton Chemical Labs, Toronto, ON), corresponding to the C-terminal 15 residues of human $\alpha_1 PI$, was employed. Briefly, the peptide was conjugated to keyhole limpet hemocyanin (Sigma)

and injected into rabbits over a 3 month period; IgG from high-titer sera was then affinity-purified on peptide-Sepharose affinity column, as described (492).

Immunofluorescence microscopy

HEK 293 cells were grown as described above, except on sterile 22 X 22 mm microscope cover slips (Fisher) within 6 cm plates. Following Lipofectamine 2000-mediated transient transfection, they were reacted with 270 nM human thrombin in SFM for 30 minutes, then fixed with cold methanol. Fixed cells were rinsed three times with phosphate-buffered saline (PBS) for 3 minutes each, then incubated sequentially with: 1% BSA in PBS; affinity-purified sheep anti-human prothrombin IgG (reactive with thrombin) diluted 1:500 in 1% BSA/PBS (493); and finally with 2.0 µg/ml Alexa Fluor 488-conjugated donkey anti-sheep IgG (Invitrogen). Each step was for 45 minutes at 37°C, interspersed with PBS washes. Slides were prepared using ProLong Gold anti-fade mounting media as directed by the manufacturer (Invitrogen). Images of cell fluorescence were acquired using a Leica DMI 6000 microscope (20X objective, 10X eyepiece) in combination with a Hamamatsu Orca ER-AG camera and Volocity software (Perkin Elmer, Woodbridge, ON, version 5.0) with fluorescence (exposure time of 300ms) superimposed onto the bright field image. Semrock 3035B filters, with a center excitation/emission wavelengths of 472 and 520 nm, were employed.

Flow cytometry

Freshly harvested HEK 293 cells separately transfected with plasmids pC3-AR-API (M358R), pC3-AR-API (T345R/M358R), pC3-AR-AT, or pC3-AR-HCII were resuspended in PBS. A portion (0.1 ml) of the suspension was stored for subsequent determination of input serpin concentration on calibrated immunoblots (see below). The remaining suspension was heated to 37°C, and human thrombin was added to 270 nM final concentration for 1 minute. The reaction was halted by addition of D-Phe-Pro-Argchloromethylketone (PPACK) to 10 µM (494). Cells were then recovered by centrifugation at 2,000 X g at room temperature. Blocking and antibody reactions were performed as described above for fixed cells, using the same primary antibody to detect cell-surface thrombin, except that incubations took place at room temperature, and cells were pelleted and resuspended in 0.5 ml 1% BSA/PBS after incubation with 2.0 µg/ml Alexa Fluor 488-conjugated donkey anti-sheep IgG and characterized by flow cytometry in a BD FACSCalibur apparatus (BD Immunocytometry Systems, San Diego, CA). Instrument settings were optimized using freshly harvested unstained and non-transfected HEK 293 cells to establish gating ranges; data analysis for each cell population was performed using BD CellQuest Pro OSX VS.2.1 software.

Comparison of membrane-bound versus free serpin reaction rates

HEK 293 cell monolayers were transfected with either pC3-AR-API (M358R) or pC3ssAPI (M358R) plasmids. Cells from the former were isolated with citrate saline, resuspended in 0.1 ml PBS, divided into 20 μ l aliquots, and stored at -80°C. Conditioned serum-free media from the latter was concentrated to 150 μ l (Millipore, Mississauga, ON, Centricon centrifugal microconcentrators, 5K cutoff) and similarly stored. Aliquots

from either source were subsequently thawed, serially diluted, and electrophoresed, electroblotted onto nitrocellulose membranes, and subjected to immunoblotting using horseradish peroxidase-(HRP-) conjugated affinity-purified sheep anti- α_1 PI immunoglobulin (Affinity Biologicals, Ancaster, ON), and a series of diluted purified standards (Calbiochem, La Jolla, CA). Following immunoblotting as described (486,495,496), the blot image was captured using a Molecular Imager Gel Doc XR System (BioRad, Mississauga, ON) and recombinant α_1 PI M358R levels, free or tethered, were determined using the standard curve formed by plotting band intensity versus moles of input α_1 PI, using Quantity One software (BioRad). Concentrations of recombinant α_1 PI, either displayed on cell surfaces, or free in solution, were then adjusted to 33 nM, and thrombin was added to 3.8 nM final concentration to the parallel reactions. Reactions were terminated after 5, 15, 30 or 60 seconds by addition of concentrated SDS/PAGE sample buffer. Serpin-enzyme complex formation was followed by immunoblotting and quantified as described above using the Gel Doc system.

Reaction of thrombin with free or tethered recombinant AT or HCII

Either cells transfected with AT- or HCII-expressing membrane-tethered, or the corresponding conditioned media containing soluble serpins, were reacted with thrombin in the same manner as described above, with or without the addition of standard sodium heparin to 2 units/ml. In some reactions, immunoblotting was employed exactly as described for cells transfected with α_1 PI M358R constructs, except that either sheep anti-AT immunoglobulins (497) combined with alkaline phosphatase- (AP-) conjugated rabbit anti-sheep IgG or HRP-conjugated goat anti-HCII immunoglobulins were employed (Affinity Biologicals, Ancaster, ON). Purified AT or HCII preparations used as standards were obtained from Enzyme Research Laboratories or Affinity Biologicals. Other means of detecting serpin-enzyme complexes using antibodies directed against thrombin, either via immunoblotting or flow cytometry, were identical to those described above.

General

Protein electrophoresis (SDS-PAGE) under denaturing and reducing conditions was performed using a Mini Protean II system as recommended by the manufacturer (BioRad).

Results

Comparison of membrane targeting sequences

We initially compared two N-terminal, non-cleavable signal sequences: residues 3-98 of the human transferrin receptor (designated TR); and residues 3-69 of the human asialoglycoprotein receptor (designated AR). As shown in Fig. 2.1, these sequences were separately fused to an α_1 PI M358R cDNA identical to that we previously expressed in *E. coli* and kinetically characterized (486,498). Transfection of either HEK 293 or COS-1 cells with plasmids encoding AR- or TR- α_1 PI M358R led to the expression of 72 and 76 kDa full-length species, respectively (Fig. 2.1). In each case, an additional product of 67

and 70 kDa was also observed as a transfection-dependent polypeptide immunoreactive with anti- α_1 PI antibodies. The full-length mobilities corresponded to expected values, given the length of the engineered polypeptide chains, and the three known sites of Nlinked glycosylation of α_1 PI (N46, N83, and N247) (499). Incubation of cells with thrombin resulted in the formation of novel 105 and 109 kDa proteins consistent with the formation of denaturation-resistant thrombin- α_1 PI M358R complexes. Since the results did not differ substantively between the AR- or TR- α_1 PI M358R constructs, we arbitrarily selected the former for more detailed investigation of the subcellular localization of the membrane-targeted serpin.

We next examined all fractions that we obtained from application of a published subcellular fractionation protocol for cultured cells: cytoplasmic; peripheral membrane; and integral membrane. As shown in Fig. 2.2B, GAPDH antigen was found only in the cytoplasmic fraction, with the other two fractions being devoid of this cytoplasmic marker. Na,K-ATPase was found predominantly in the integral membrane fraction, as expected for this plasma membrane pump, but was also found in the peripheral and cytoplasmic fractions; densitometry quantified the cytoplasmic: peripheral: integral distribution as approximately 30:10:60 in HEK and 35:10:55 in COS. For AR-a1PI M358R, the two major polypeptide species of 72 and 67 kDa followed the same pattern of subcellular distribution as the Na,K-ATP, and were enriched in the integral membrane fraction. The extent of enrichment appeared greater in HEK 293 cells than in COS cells, and we therefore chose to focus on the former, rather than the latter cells, throughout the rest of this study. An additional minor anti- α_1 PI-reactive polypeptide species of low mobility, migrating between the 130 and 170 kDa markers on the immunoblot shown in Fig. 2.2B, was also observed in all fractions, although it was most noticeable in the cytoplasmic fraction.

Incorporation of T345R abrogates thrombin inhibition by AR- α_1 PI M358R

To confirm the status of the putative serpin-enzyme complexes observed in the experiment shown in Fig. 2.2, we repeated the experiment, examining only integral membrane fractions in all cases, and probed the resulting immunoblots not only with anti- α_1 PI, but also with antibodies reacting with thrombin. As shown in Fig. 2.3, several closely grouped thrombin-dependent species observed in the 105-100 kDa region of the blots, migrating less rapidly than control soluble thrombin- α_1 PI M358R complexes, reacted with both antibodies, strengthening their identification as SDS-stable thrombin-AR- α_1 PI M358R inhibitory complexes. We sought further support for the functionality of AR- α_1 PI M358R on the plasma membrane by incorporating an additional mutation: T345R. This substitution has been previously reported to convert α_1 PI into a substrate, rather than an inhibitor, of several serine proteases (487). Its incorporation into AR- α_1 PI M358R abrogated formation of thrombin- α_1 PI M358R and T345R/M358R could be detected.



Figure 2.2 - Subcellular fractionation of transfected mammalian cells.

HEK 293 or COS-1 cells (COS) were transfected with plasmids encoding either AR- α_1 PI M358R or TR- α_1 PI M358R and incubated with (+) or without (-) thrombin (IIa). Cell suspensions were either sonicated alone (Sonicate) or sonicated and fractionated into cytoplasmic (Cyto), peripheral (Peri), or integral (Integral, Int) membrane fractions. **Panel A** shows anti- α_1 -PI immunoblots in which 3.0% of the sonicate and 12.5% of the integral membrane fractions of HEK 293 transfected cells were electrophoresed, versus 3.75% and 20% of the corresponding fractions for COS cells. Thrombin-dependent putative serpin-enzyme (thrombin- α_1 -PI complex bands are identified with black The position of protein molecular mass markers, with their sizes in arrowheads). kiloDaltons, are shown at left; to allow magnification, only a portion of the blot is shown. In Panel B (upper panel), 6.0% of each of the subcellular fractions identified above the lanes was electrophoresed and immunoblotted using the antibodies identified at right; anti- α_1 PI represents the entire blot, while those for marker antibodies anti-GAPDH (cytosol) and anti-Na,K-ATPase (Anti-ATPase) represent portions of replicate blots M, prestained molecular mass markers, are shown (lane M) and identified, in kDa, to the left of all panels.

Similarly, when the additional T345R mutation was incorporated into soluble α_1 PI M358R, the doubly mutated soluble serpin lost the ability to form complexes with thrombin (Fig. 2.3A). For both AR- α_1 PI M358R and AR- α_1 PI T345R/M358R, doublet bands of approximately 150 kDa were also detected; in the case of the former, but not the latter, on exposure to thrombin, some of this material was shifted to a faint higher molecular weight band that cross-reacted with anti-thrombin antibodies. The slowly migrating minor form of AR- α_1 PI M358R therefore appears to be an aggregate, likely a dimer, that we reproducibly observed; however our data do not provide information as to whether it formed on the cell surface, or arose as a consequence of incomplete solubilisation of the integral membrane fraction in SDS. We also ensured that the putative serpin-thrombin SDS-stable complex was not an artefact carried-over from the thrombin preparation, by demonstrating that no bands in the 105 - 90 kDa were present in thrombin-only samples, even when probed at much higher concentrations of residual thrombin than were observed in the experimental samples (compare ~34 kDa thrombin B chain bands in Fig. 2.3A, lower panel, to those in Fig. 2.3B).

Heterogeneity in cell-surface $AR-\alpha_1 PI$ M358R largely arises due to differential glycosylation

We sought to better understand the nature of the two main polypeptides arising from AR- α_1 PI M358R expression, of approximate mass 72 and 67 kDa, by enzymatic deglycosylation. Peptide:N-glycosidase F treatment of transfected cell integral membrane preparations converted these two species (labeled 2 and 3 in Fig. 2.4, upper panel) to one main 60 kDa product (labeled 5) which reacted with both anti-hexahistidine antibodies and an antibody against a C-terminal peptide of α_1 PI. AR- α_1 PI proteins contain a hexahistidine tag between the AR and α_1 PI domains (see Fig. 2.1B). A 56 kDa minor product (band 6) was more evident in AR- α_1 PI T345R/M358R reactions than those involving AR- α_1 PI M358R; its absence from the anti- α_1 PI (C-terminal) blot suggests that it was proteolyzed at or near its reactive centre. The mobility of the putative dimeric species (band 1) was also increased by enzymatic deglycosylation (to band 4) as was the thrombin- α_1 PI M358R inhibitory complex (band 7 to band 8). Taken together, our results suggest that most of the heterogeneity observed in the AR- α_1 PI M358R polypeptides arose from incomplete glycosylation of the more rapidly migrating minor form at one or more of the 3 sites of α_1 PI N-linked glycosylation.

Acquisition of thrombin binding by HEK 293 cells correlates with AR- α_1 PI M358R expression

Having provided evidence by subcellular fractionation suggestive of functional cellsurface expression of α_1 PI M358R, we sought confirmation of this finding by independent techniques. As shown in Fig. 2.5C, immunofluorescent microscopy of HEK 293 monolayers expressing AR- α_1 PI T345R/M358R, using an antibody combination to



Figure 2.3 - Reaction of tethered or soluble recombinant API with thrombin.

Panel A: HEK 293 cells were transfected with plasmids encoding either membranetethered (AR- α_1 PI M358R or AR- α_1 PI T345R/M358R) or soluble (α_1 PI M358R or α_1 PI T345R/M358R) recombinant forms of α_1 PI, and incubated with (+) or without (-) thrombin (IIa). Equivalent portions of the carbonate-insoluble membrane protein fraction of each reaction were electrophoresed on duplicate blots and probed either with antibodies to α_1 PI (upper panels; Anti- α_1 PI) or antibodies to prothrombin (II) crossreacting with thrombin (Anti-II; lower panels). **Panel B** shows varying amounts of the commercial thrombin preparation used in all experiments (100, 10, and 1 ng) probed on an immunoblot with anti-II antibodies as in **Panel A**, lower blot M, prestained molecular mass markers, are shown (lane M) and identified, in kDa, to the left of all panels.



Figure 2.4 - Enzymatic deglycosylation of AR-API M358R and AR-API T345R/M358R.

As in **Figure 2.3**, HEK 293 cells were transfected with plasmids encoding either membrane-tethered AR- α_1 PI M358R or AR- α_1 PI T345R/M358R. Carbonate-insoluble membrane fractions were solubilised in SDS and enzymatically deglycosylated under denaturing conditions using Peptide: N-glycosidase F (PNGase F) with (+) or without (-) thrombin (IIa). Replicate blots were probed with different antibodies identified to the right of the panels, including an anti-hexahistidine (Anti-His) and anti- α_1 PI antibody directed against the C-terminal (C-term) 15 residues of the serpin (Anti- α_1 PI (C-term)). detect bound thrombin, resulted in only a diffuse, background staining. In contrast, applying the same treatment to HEK 293 monolayers expressing AR- α_1 M358R resulted in strong staining of approximately 25% of the cells in a field of identical size to that employed in Fig. 2.5C, consistent with expected rates of transient transfection of these cells (Fig. 2.5D). Conducting the same experiment with non-transfected cells (Fig. 2.5A) or leaving thrombin out of the AR- α_1 M358R transfection (Fig. 2.5B) yielded the same background staining as in cells transfected with non-functional AR- α_1 PI T345R/M358R (Fig. 2.5C).

Similar results were obtained using flow cytometry (Fig. 2.5E). Cells expressing AR- α_1 PI T345R/M358R exhibited only background fluorescence following exposure to thrombin and incubation with antibodies capable of detecting bound thrombin. The fluorescence profile of the population of cells transfected in this manner was superimposable on that of AR- α_1 PI M358R-transfected cells treated only with the secondary antibody. In contrast, cells transfected with AR-a₁PI M358R showed a substantial and significant increase in fluorescence related to thrombin binding. When this experiment was replicated four times each for AR- α_1 PI M358R and AR- α_1 PI T345R/M358R, the geometric means were found to be 76.0 \pm 1.6 arbitrary units versus 4.2 ± 0.3 units (mean of n=4 ± SD, p=0.029 by Mann-Whitney test) (Fig. 2.5F). When cells separately transfected with AR- α_1 PI M358R and AR- α_1 PI T345R/M358R were analyzed by flow cytometry without exposure to thrombin, using an anti- α_1 PI antibody and a Alexa-Fluor-conjugated secondary antibody, geometric mean fluorescence differed by less than 4% (data not shown), suggesting highly similar transfection and expression efficiencies for the functional versus non-functional tethered serpin constructs. While we did not titrate the thrombin concentration in flow experiments, we did repeat the experiment shown in Fig. 2.5E by mixing plasmids encoding AR- α_1 PI M358R and AR- α_1 PI T345R/M358R together prior to transfection. As the ratio of the former to the latter declined, the resulting peak, corresponding to the green peak in Fig. 2.5E diminished in both cell count and in fluorescent intensity, as least showing some responsiveness of the system to changes in a key reactant (data not shown).

Cell surface expression reduces the efficiency of α_1 *PI M358R as a thrombin inhibitor*

While our results were consistent with functional expression of α_1 PI M358R as an integral membrane protein of the mammalian cell plasma membrane, their semiquantitative nature left open the question of relative efficiency of the tethered serpin as an inhibitor compared to soluble counterparts. We therefore first sought conditions under which transfected cells could be frozen and thawed without affecting the ability of AR- α_1 PI M358R to form serpin-enzyme complexes with thrombin. We found that resuspension of transfected cells in either PBS, PBS/10% dimethylsulfoxide, or PBS/10% glycerol did not significantly affect the formation of thrombin- α_1 PI complexes by AR- α_1 PI M358R-expressing cells; freezing conditioned media similarly had no effect on the activity of soluble α_1 PI M358R (data not shown). It was therefore possible to transfect,



Figure 2.5 - Microscopic and flow cytometric detection of cell-surface bound thrombin.

Monolayers of HEK 293 cells subjected to mock transfection without DNA (**Panel A**), and those expressing either AR- α_1 PI M358R (**Panel D**) or AR- α_1 PI T345R/M358R (**Panel C**) were incubated with thrombin, then fixed and probed with anti-prothrombin IgG, followed by Alexa Fluor 488-conjugated secondary antibodies. **Panel B** shows the results of the same experiment as **Panel D** except that no thrombin was added to the cells. Fluorescence signals, overlaid on bright-field images (200X magnification) are shown. **Panel E** shows the histogram of the fluorescent signal (x axis) versus cell count parameter (y axis) from experiments in which unfixed cells were similarly exposed to thrombin and the same antibodies as in **Panels A** and **B**, then characterized by flow cytometry. Tracings correspond to: non-transfected cells (black); cells expressing AR- α_1 PI T345R/M358R incubated with both antibodies (green) or second antibody only (pink); or cells expressing AR- α_1 PI T345R/M358R incubated with both antibodies (blue). All transfected cells were exposed to thrombin. **Panel F** shows the geometric mean of the fluorescence intensity from multiple experiments like that shown in **Panel E** (n=4 ± SD) for cells expressing the indicated proteins; *indicates p = 0.029 by Mann-Whitney test.

store cells and media, determine serpin concentrations, and then subsequently make a fair comparison of thrombin inhibition by α_1 PI M358R in tethered or free form. As shown in Fig. 2.6, thrombin- α_1 PI M358R complex formation was slowed approximately 7- to 10-fold in AR- α_1 PI M358R compared to α_1 PI M358R, under conditions in which the initial rate of reaction could be compared. To ensure as fair a comparison as possible, non-transfected cells were added to the soluble α_1 PI M358R reaction at a count designed to match that of the tethered serpin reaction.

Thrombin complex formation by tethered AT or HCII

Having demonstrated that α_1 PI M358R could be expressed in functional form as a cellsurface tethered serpin, we sought to determine if other, natural thrombin-inhibiting serpins could similarly function in this novel location. Accordingly, AT and HCII cDNAs were modified by addition of the AR targeting signal (see Fig. 2.1). For AT, this led to an expected decrease in mobility of recombinant AR-AT on immunoblots versus soluble recombinant AT (Fig. 2.7) consistent with the additional 67 amino acids of the AR signal, as well as a shift to the carbonate-insoluble membrane fraction of the HEK 293 cell sonicated preparation. AR-AT migrated as a major 68 kDa and a minor 64 kDa band; on incubation with thrombin, a portion of AR-AT was converted into a 103 kDa complex reactive both with anti-AT and anti-thrombin antibodies. Addition of heparin to this reaction did not increase the intensity of this product, although it rendered more noticeable protein species intermediate in size between the 103 kDa complex and the 68 kDa AR-AT, which may be breakdown products of the thrombin-AR-AT complex; similar banding patterns were observed with the thrombin-soluble AT complexes. Addition of protamine sulphate to the reaction with thrombin that lacked heparin reduced complex formation (data not shown) suggesting that glycosaminoglycans were present in the cell suspension. Cells expressing AR-AT bound thrombin, as judged by flow cytometry experiments similar to those shown in Fig. 2.5E; however, since we lacked a control construct analogous to AR- α_1 PI T345R/M358R, we did not further pursue this observation (data not shown).

In the case of AR-HCII, expression levels were lower than for the other two serpins, and the protein products were more heterogeneous. Although transfection-dependent, anti-HCII-reactive protein species of approximately 90-95 kDa kDa were detected, their band intensities were faint (compare second lane of upper and upper left panels in Figs. 2.7 and 2.8 or the AR-HCII and HCII no thrombin lanes in upper panels of Fig. 2.8) and immunoblots required long developing periods. No evidence of AR-HCII-thrombin complex formation was obtained, either in the experiment shown in Fig. 2.8, in which the addition of dermatan sulphate did not change this negative result, or in a similar experiment in which heparin was employed.



Figure 2.6 - Comparison of initial rates of thrombin-API complex formation of tethered or free serpins.

Concentrations of conditioned media containing α_1 PI M358R and of cell suspensions expressing AR- α_1 PI M358R were adjusted using immunoblots calibrated with dilutions of plasma-derived α_1 PI, such that the input concentrations of the full-length proteins in the experiment shown in **Panels A** and **B** were 33 nM at time t=0. Reactions with soluble α_1 PI M358R were supplemented with the same numbers of non-transfected cells as were present in the reactions with cell surface-displayed AR- α_1 PI M358R. Following addition of 3.8 nM thrombin, aliquots were removed from the reaction mixture after the times shown, in seconds, above the lanes; molecular weight markers, in kDa, are shown at left. Formation of thrombin- α_1 PI M358R serpin-enzyme complexes (SEC, black arrowheads) was followed by immunoblotting (**Panel A**) and densitometry of the illustrated blots (**Panel B**); the thrombin- α_1 PI M358R reaction is shown in **Panel A**, upper blot, and the corresponding thrombin-AR- α_1 PI M358R reaction in **Panel A**, lower blot. M, prestained molecular mass markers, are shown (lane M) and identified, in kDa, to the left of all panels.



Figure 2.7 - Reaction of tethered or soluble recombinant AT.

The reaction of membrane-tethered (AR-AT) or soluble (AT) serpins was followed by immunoblotting as shown in **Figs. 2.2** and **2.3** for α_1 PI, in the presence (+) or absence (-) of thrombin. In some reactions thrombin was supplemented with 2 units/ml standard heparin (Hep). Replicate immunoblots were probed with anti-AT antibodies or the same thrombin-reactive anti-prothrombin antibody (Anti-II) employed in α_1 PI experiments. Putative serpin-enzyme complexes are identified by open (white) arrowheads M, prestained molecular mass markers, are shown (lane M) and identified, in kDa, to the left of all panels.



Figure 2.8 - Reaction of tethered or soluble recombinant HCII.

The reaction of membrane-tethered (AR-HCII) or soluble (HCII) serpins was followed by immunoblotting as shown in **Fig. 2.7** for AT, in the presence (+) or absence (-) of thrombin. In some reactions thrombin was supplemented with 3.0 μ M dermatan sulfate (DS). Replicate immunoblots were probed with anti-AT antibodies or the same thrombin-reactive anti-prothrombin antibody (Anti-II) employed in α_1 PI experiments. Putative serpin-enzyme complexes are identified by open (white) arrowheads M, prestained molecular mass markers, are shown (lane M) and identified, in kDa, to the left of all panels.

Discussion

In this study we sought to display thrombin-inhibitory serpins on the mammalian cell surface in functional form, as type II plasma membrane proteins (500,501). Such proteins are oriented with intracellular amino termini and extracellular carboxy termini ($N_{in}-C_{out}$), separated by a single-pass transmembrane domain, which also functions as an internal signal peptide/membrane insertion sequence. We selected this orientation over the opposite, N_{out} - C_{in} type III membrane protein geometry, in order to retain thrombin on the cell surface after formation of the serpin-thrombin complex. Because the serpin inhibitory mechanism leaves cognate proteases covalently bound to the N-terminal P1 residue of the scissile P1-P1' reactive centre peptide bond, it was therefore necessary to anchor the N-terminus of a displayed serpin in the membrane. Using the opposite orientation would have liberated the serpin-protease complex from the cell surface upon reactive centre cleavage, barring non-covalent interactions between the tethered serpin P1' - C terminus chain and the rest of the serpin in the serpin-protease complex.

Initially, we compared two type II targeting sequences: residues 3-98 of the human transferrin receptor; and residues 3-69 of the human asialoglycoprotein receptor H1 subunit. Both of these proteins are type II membrane proteins with large extracellular domains; these segments had been previously shown to target attached cytoplasmic proteins to microsomal membranes *in vitro* (502,503). Both the TR- and the AR-derived sequences functioned to target attached α_1 PI M358R to the plasma membrane. This conclusion is suggested by the presence of the anti- α_1 PI-reactive proteins of expected mobility in the carbonate-insoluble membrane fraction of sonicated transfected cells. Carbonate-insolubility of proteins associated with the >100,000 X g pellet of disrupted cells is a hallmark of integral membrane proteins (491,504). While we did not perform extensive experiments with TR- α_1 PI M358R, we observed that AR- α_1 PI M358R acquired the same subcellular distribution as the α_1 subunit of the Na,K-ATPase, a marker for the plasma membrane fraction.

In this novel subcellular location, multiple lines of evidence suggest that membranetethered $\alpha_1 PI$ M358R adopted a conformation that was at least partially functional. Firstly, incubation of cells expressing AR- $\alpha_1 PI$ M358R or TR-AR- $\alpha_1 PI$ M358R with thrombin led to the recovery of a serpin-enzyme protein complex of the expected mobility in the integral membrane fraction.

This complex was found to be enriched in the integral membrane compared to other subcellular fractions, consistent with thrombin's being prevented from interacting with any intracellular forms of α_1 PI M358R by the intact plasma membrane of the cultured cells. Indeed, AR- α_1 PI M358R exhibited a similar pattern of distribution through the subcellular fractions that we generated to the Na,K-ATPase, a well characterized plasma membrane protein, suggesting that cytoplasmic or peripheral AR- α_1 PI M358R was more of a consequence of limitations of the subcellular fractionation of relatively small numbers of cultured cells than of bona fide abnormal sorting in the cell. Secondly,

exogenous thrombin became bound to the cell surface of transfected cells expressing AR- α_1 PI M358R, but not to non-transfected cells. This property was detected in both fixed and non-fixed cells by immunohistochemical, or flow cytometric detection of thrombin. Importantly, repetition of any of the three types of experiments, changing only one variable, the introduction of the T345R mutation into AR- α_1 PI M358R, eliminated either complex formation or the ability to detect thrombin on the cell surface. The T345R mutation has been previously shown to convert α_1 PI into a protease substrate rather than a protease inhibitor (487).

Although α_1 PI M358R was functional when displayed on the surface of both mammalian cell lines that we employed, tethered via either the TR or AR type II membrane insertion sequence, its ability to inhibit thrombin was reduced compared to non-tethered $\alpha_1 PI$ M358R secreted from these cells. This reduction could have arisen due to the surfacedisplayed serpin's being in a suboptimal conformation, due to steric hindrance, or due to competing binding sites for thrombin on the cell surface. Although fusing the N-terminus of α_1 PI M358R to the transmembrane domain should have limited deleterious effects on the C-terminal RCL, a transmitted conformational change or partial increase in the rigidity of the serpin could have reduced its adequacy as an inhibitor. Alternatively, the protein could have been displayed too close to the membrane, complicating the approach of thrombin. It is possible that improvements in inhibitory efficiency could be achieved by including a more substantial spacer (e.g. an EGF domain) between the transmembrane domain and the serpin in future constructs. In this regard the distance of protein active sites above membrane surfaces, varied by spacer domains, has been shown to be an important parameter for both membrane-associated factor X and protein S (505,506). We can exclude the trivial explanation that the effective thrombin concentration at the tethered serpin location was diminished by low affinity binding elsewhere on the cell, because when we compared the rates of thrombin inhibition by tethered versus free $\alpha_1 PI$ M358, we included non-transfected HEK 293 cells in the latter reaction to account for this possibility.

Our finding that AR- α_1 PI M358R was capable of forming serpin-enzyme complexes with thrombin led us to ask whether functional cell-surface expression was limited to α_1 PI in this geometry or if other thrombin-inhibitory serpins could be similarly displayed. Although less effective than α_1 PI M358R when expressed on the cell-surface as a thrombin inhibitor, AT clearly retained function, although the partially protamine sulphate-sensitive nature of the reactivity indicated that heparin-like molecules were present in our experimental system, as has previously been suggested (507). In contrast, HCII was less efficiently expressed on the surface of HEK 293 cells than either AT or α_1 PI M358R; we are not certain whether or not this factor contributed to our inability to detect thrombin-AR-HCII complexes or if HCII was inactive when expressed in this novel cellular location. If HCII is less amenable to cell surface tethering via its Nterminus than α_1 PI or AT, this would not be surprising given its dependence for effective thrombin inhibition on its N-terminal, 75 residue acidic "tail' region. This domain is thought to interact internally with the body of HCII, and be fully mobilized for binding to thrombin exosite 1 in the presence of glycosaminoglycans (508-511).

In our work with transfected HEK 293 cells, it was a theoretical possibility that thrombin applied to the cells would engage other receptors, potentially compromising the approach. In this regard, we did not encounter a high degree of background binding, nor observable changes in cell phenotype due to cellular activation by thrombin. HEK 293 cells are originally derived from human embryonic kidney cells. Protease-Activated Receptor 1 (PAR-1), for instance, a high affinity thrombin receptor, is primarily expressed on endothelial cells and platelets (512). Several groups have transfected HEK 293 cells with PAR-1 cDNAs for structure/function studies, and have not reported a detectable background expression of PAR-1 (e.g.(513)).

While no integral membrane serpin has to our knowledge been previously found or engineered, one serpin, plasminogen activator inhibitor-1 (PAI-1), has been previously expressed on the surface of bacteriophage in functional form (514). In this setting, PAI-1 was displayed fused to the N-terminus of the pIII phage protein, which becomes embedded in the bacteriophage capsid, the protein-based outer shell of the phage. Phage-displayed PAI-1 libraries have been used to select for mutant PAI-1 proteins with enhanced stability (515,516). However, it is unclear why tPA-PAI-1 complexes did not diffuse away from the phage, but instead remained attached following reactive centre loop cleavage. This finding suggests that the PAI-1 P1'-C terminus remains non-covalently associated with the body of the serpin in the tPA-PAI-1 complex, which may not be a general feature of inhibitory serpins. Our approach employed the alternative geometry, in which serpins were N-terminally tethered to the biological structure containing the DNA that encodes them, and the resulting serpin-enzyme complexes could not diffuse away.

Apart from the demonstration that the serpin fold, in at least two cases, is compatible with expression as an integral membrane protein, there are two main applications of our findings from this study. Firstly, an application similar to that previously employed for PAI-1 can be envisaged, whereby hypervariable libraries of surface-displayed α_1 PI or AT molecules can be constructed and expressed in HEK 293, and probed using fluorescently labelled proteases to select for enhanced or diminished binding. Following cell sorting the identity of the mutation(s) of interest could be determined by PCR and sequencing of the selected mutant serpin cDNA. There are theoretical advantages to the use of mammalian cell surface display over that on phage surfaces, including appropriate post-translational modifications and the ability to employ cytometric sorting (517). Secondly, our approach can be applied in a cellular engineering context, in order to confer protease resistance to mammalian cells. For example, others have used analogous approaches to confer thrombin-inhibitory properties to transplanted endothelial cells (518-520).

In conclusion, our results demonstrate that $\alpha_1 PI$ M358R and AT retain thrombininhibitory activity when expressed on the surface of mammalian cells in culture. That no natural serpins have been found to be integral membrane proteins therefore derives not from conformational constraints but instead from the greater regulatory flexibility afforded to diffusible serpins. Future exploitation of this discovery may lead to greater understanding of structure-function relationships in these serpins and/or the ability to modulate protease activity on engineered cells.

Acknowledgements

We thank Dr. Murray Junop, Department of Biochemistry and Biomedical Sciences, McMaster University, and Dr. Dana Devine, Department of Laboratory Medicine, University of British Columbia, and Canadian Blood Services, for helpful discussions. This work was made possible by Grants-In-Aid T5597 and T6271 from the Heart and Stroke Foundation of Ontario to WPS. RFG and JSS received Graduate Fellowship Awards from Canadian Blood Services. LJT held a Canada Graduate Scholarship from the Natural Sciences and Engineering Research Council (NSERC).

Conflict of interest disclosure: The authors declare no competing financial interests.

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Expected completion date	Apr 2015	
Expected size (number of pages)	150	
Requestor Location	Richard Gierczak McMaster University 1200 Main Street West Hamilton, NOB 2KO	

3 PAPER #2 – Alpha-1 proteinase inhibitor M358R reduces thrombin generation when displayed on the surface of cells expressing tissue factor

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Preface

I contributed to the design of the study, and conducted the bulk of the experimentation, data acquisition and analysis, and draft manuscript preparation.

Laura Pepler, Dr. Vinai Bhagirath and Dr. Patricia Liaw provided the technical expertise and instrumentation related to continous TGA.

The concept and design of displaying serpins on the surface of mammalian cells for the purposes of gene therapy originated with Dr. William Sheffield. Dr. Sheffield was also project supervisor as well as senior author, and responsible for final revisions to the manuscript prior to final submission.

This research was supported by the Heart and Stroke Foundation of Canada. I received a Graduate Fellowship from Canadian Blood Services.

Thrombosis Research, 134: 1142 – 1149

REGULAR ARTICLE

Alpha-1 proteinase inhibitor M358R reduces thrombin generation when displayed on the surface of cells expressing tissue factor

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WORD COUNT: 4083 (Excluding abstract and references, including figure legends)

Running Title: Tethered API M358R and thrombin generation (37 characters)

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ABSTRACT

The M358R variant of alpha-1-proteinase inhibitor (API) is a potent soluble inhibitor of thrombin. Previously we engineered AR-API M358R, a membrane-bound form of this protein and showed that it inhibited exogenous thrombin when expressed on transfected cells lacking tissue factor (TF). To determine the suitability of AR-API M358R for gene transfer to vascular cells to limit thrombogenicity, we tested the ability of AR-API M358R to inhibit endogenous thrombin generated in plasma via co-expression coexpressing it on the surface of cells expressing TF. Transfected AR-API M358R formed inhibitory complexes with thrombin following exposure of recalcified, defibrinated plasma to TF on T24/83 cells, but discontinuously monitored thrombin generation was unaffected. Similarly, AR-API M358R expression did not reduce continuously monitored thrombin generation by T24/83 cell suspensions exposed to recalcified normal plasma in a Thrombogram-Thrombinoscope-type thrombin generation assay (TGA); in contrast, 1 μ M hirudin variant 3 or soluble API M358R abolished thrombin generation. Gene transfer of TF to HEK 293 conferred the ability to support TF-dependent thrombin generation on HEK 293 cells. Co-transfection of HEK 293 cells with a 9:1 excess of DNA encoding AR-API M358R to that encoding TF reduced peak thrombin generation approximately 3-fold compared to controls. These in vitro results suggest that surface display of API M358R inhibits thrombin generation when the tethered serpin is expressed in excess of TF, and suggest its potential to limit thrombosis in appropriate vascular beds in animal models.

KEYWORDS: alpha-1 proteinase inhibitor; tissue factor; thrombin; coagulation; thrombosis

Introduction

Alpha-1-proteinase inhibitor (α_1 PI or API, also known as α_1 -antitrypsin) is an abundant plasma protein belonging to the serpin superfamily (521,522). After secretion from hepatocytes, API is found in plasma at concentrations of 20 - 30 µM (523) and in lung epithelial lining fluid at 2 - 5 µM (524). There it inhibits neutrophil elastase (525), cathepsin G (526) and proteinase 3 (527). Individuals genetically deficient in API exhibit increased susceptibility to chronic obstructive pulmonary disorder (COPD) due to insufficient control of these serine proteases (528-530). Under ordinary circumstances, plasma API has minimal effects on coagulation, although it is capable of inhibiting coagulation factor XIa, albeit at reduced rates compared to other serpins like C1 inhibitor or α_2 -antiplasmin (531). However, a single amino acid substitution in the API reactive centre, M358R (532), dramatically increases its impact on coagulation, accelerating its rate of inhibition of thrombin, factor XIa, and activated protein C by 4,000- to 8,000-fold (533,534). Injection of recombinant API M358R attenuates thrombosis in mice (535) and improves survival in septicemia of pigs (536) but not baboons (537).

Like all serpins, API M358R inhibits proteases using a complex mechanism in which the attacking protease is translocated from one pole of the serpin to the other (538) and remains covalently linked to the serpin in the final inhibitory complex (539,540). Previously, we showed that API M358R remained capable of executing this mechanism and inhibiting exogenous thrombin even when anchored to the surface of mammalian cells in culture (541). This finding raised the possibility that API M358R tethered to vascular or tissue cells could reduce thrombosis more effectively than its soluble counterpart. As an initial step in investigating this idea, here we sought to determine if a tethered serpin could inhibit endogenously generated thrombin in plasma systems, using cells co-expressing tissue factor (TF) and tethered API M358R. We report that that membrane-tethered API M358R reduces endogenous thrombin generation, but only if it is present in excess of tissue factor (TF).

Materials and methods

Plasmids and DNA manipulations

Plasmids pC3-AR-API (M358R) and pC3-AR-API (T345R/M358R) (541), and a commercial plasmid (Genbank # NM_001993, Origene, Rockville, MD, USA) were used for gene transfer of cDNAs encoding functional and non-functional API and TF, respectively. Plasmid DNA for transfection was prepared from *E. coli* DH5α cultures using a Plasmid Plus Midi Kit (Qiagen, Valencia, CA, USA).

Culture and transient transfection of mammalian cell lines

Human bladder carcinoma T24/83 cells and human embryonic kidney HEK 293 cells were purchased from ATCC (Manassas, VA, USA) and grown as monolayers in an atmosphere of 5% CO₂ at 37° C in M199 and DMEM/F12 media, respectively, supplemented with 10% fetal bovine serum and 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine (Sigma-Aldrich, Oakville, ON, Canada). T24/83

cells at 75% confluency were transfected using 0.67 µg plasmid DNA/µL of transfection reagent (X-tremeGENE HP) DNA in serum-free medium, as directed (Roche Applied Science, Mississauga, ON, Canada). Forty-eight hours post-transfection, cells were removed from full M199 media, washed with phosphate-buffered saline (PBS), and harvested using exposure to GIBCO Cell Dissociation enzyme-free buffer (Invitrogen, Carlsbad, CA, USA) as directed. Transfected cells were recovered by centrifugation (200 x g for 5 min), suspended in PBS, and stored on ice until assay. Transfection of HEK 293 cells with API-encoding plasmids alone or in combination with the TF-encoding plasmid was performed as described, at 95% confluency, using 0.4 µg plasmid DNA/µL Lipofectamine 2000 reagent (Invitrogen) as directed (541). Washed transfected cells were reacted with 10 µg/mL (270 nM) human α -thrombin (Enzyme Research Laboratories, South Bend, IN, USA) for one minute at room temperature and the reaction was terminated using SDS-PAGE sample buffer. In some experiments diluted defibrinated plasma (see below) was substituted for thrombin.

Immunofluorescence microscopy of cell surface TF

T24/83 and HEK 293 cells were grown with or without transfection on cover slips and prepared for microscopy as previously described (541). After washing, fixed cells were: blocked in 1% (w/vol) BSA in PBS; reacted with purified monoclonal mouse anti-human CD142 IgG (1:1000) (BD Biosciences, Mississauga, ON, Canada); rinsed with PBS; and stained with goat anti-mouse IgG AlexaFluor 488-conjugated antibody (1:1000) (Thermo Fisher Scientific). Each step lasted 60 minutes at room temperature. Cells were preserved with ProLong Gold anti-fade mounting media (Thermo Fisher Scientific) and their fluorescent images were captured with a BX 60 microscope equipped with a DP25 camera (Olympus, Richmond Hill, ON, Canada). DAPI and FITC images were captured using filters for ultraviolet (330-385 nm) or blue light (460-490 nm) excitation, respectively, and superimposed using DP2-BSW software (Olympus).

SDS-PAGE and Immunoblot Analysis

Protein gel electrophoresis (SDS-PAGE) and immunoblotting using antibodies to API was carried out as previously described (541); immunoblotting for TF antigen was performed using the same protocol, except that purified monoclonal mouse anti-human CD142 IgG (see above) was employed at 1:2500 in 1% BSA in Tris-buffered saline, pH 7.4 (TBS) supplemented with 0.02% (v/vol) Tween 20. Cell lysates were prepared by suspending cells in SDS-PAGE sample buffer at approximately 2000 cells/µl. Purified soluble tissue factor (Sekisui Diagnostics (Tokyo, Japan) and plasma-derived API (EMD Millipore, Billerica, MA, USA) were employed as positive controls in some experiments.

Discontinuous assay of cell surface TF activity

Cell surface TF activity was measured as described, in a discontinuous assay in which factor Xa generation was measured (542,543). Briefly, this involved exposing washed 95% confluent T24/83 cells to 0.5 nM human recombinant factor VIIa (rFVIIa, (Niastase) Novo Nordisk, Mississauga, ON, Canada) and 15 nM human factor X (FX, Haematologic Technologies, Essex Junction, VT, USA) in the presence of 5 mM CaCl₂ for 30 minutes

at 37°C, then measuring factor Xa activity with 0.2 mM chromogenic substrate S-2765 (DiaPharma, West Chester, OH, USA) in a second step. In some reactions Thromborel S (Siemens AG, Berlin, Germany), a TF-containing prothrombin time reagent, was substituted for T24/83 cells.

Discontinuous assay of thrombin generation using T24/83 cells

Citrated normal human pooled plasma (NHPP) was defibrinated with ancrod snake venom (Arvin, Knoll Pharmaceuticals, Bonn, Germany) as described (544), which elevated its prothrombin time to > 400 seconds. Thrombin generation was assessed as previously described (541); this entailed exposing near-confluent T24/83 cells to 50% defibrinated plasma in calcified buffer and measuring thrombin generation over time by subsampling aliquots into 0.16 mM chromogenic substrate S-2238 (DiaPharma)

Continuous assay of thrombin generation

The Thrombin Generation Assay (TGA) was performed on transfected cell suspensions in 96-well plates using the SpectraMax M5e MultiMode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The 100 µL final volume of each test well consisted of 40 μ L NHPP, 5 μ L cell suspension (containing approximately 10,000 cells), 30 μ L PBS with or without addition of soluble proteins or sodium heparin, and 25 µL of 1 mM Z-G-G-R-AMC Technothrombin TGA fluorogenic substrate containing 15 mM CaCl₂ (DiaPharma). Any cellular autofluorescence was accounted for by subtracting values from adjacent control wells lacking fluorogenic substrate. Immediately following substrate addition, the sample plate was inserted into the microplate reader and maintained at 37° C and mixed automatically every 30 sec. Relative fluorescence readings were recorded once per minute per well for 60 minutes using the SoftMax Pro 5.4 software (Molecular Devices, USA). TGA parameters were determined using Technothrombin TGA software obtained from the manufacturer (Technoclone GmbH, Vienna, Austria). In some experiments soluble API M358R purified from a bacterial expression system (486), and soluble recombinant hirudin variant 3 (HV3) purified from a yeast expression system (545) were employed.

Statistical analysis

Values are reported as the mean \pm the standard error of the mean (SEM). Comparisons between three or more groups were performed using analysis of variance (ANOVA) with post-tests for normally distributed data, and non-parametric ANOVA (Kruskal-Wallis with post-testing by Dunn's Multiple Comparisons test) otherwise. Comparisons between two groups were performed using a two tailed student's t test for normally distributed data, or the Welch-corrected t test or the Mann-test otherwise. All calculations employed GraphPad InStat 4.0 (GraphPad Software, San Diego, CA). In all cases, p < 0.05 was taken to indicate statistically significant differences.

Results

Effects of tethered API M358R expression on T24/83 procoagulant activity

The T24/83 cell line was employed since it was reported to express functional TF on its cell surface abundantly and constitutively (542,546,547). There was only minimal FXa activity in calcium-containing buffers supplemented with FX and exposed to T24/83 monolayers, but supplementation with rFVIIa, led to a 25-fold increase in FXa-mediated amidolysis activity (Fig. 3.1A). Similar levels of activity were detected in 10 μ l of Thromborel S, a commercial prothrombin time reagent containing human TF (data not shown). TF antigen was also directly detected on T24/83 cells probed with anti-TF (anti-CD 142) antibodies, which demonstrated strong extranuclear staining by fluorescence microscopy, unlike negative control samples or micrographs employing an antibody unrelated to TF (Fig. 3.1B).

Having confirmed functional TF expression by T24/83 cells, we next transfected them with a plasmid encoding cell surface tethered API M358R. As previously observed in different cell types (541), T24/83 cells transfected with pC3-AR-API M358R expressed a major 72 kDa and a minor 67 kDa protein species undetectable in parallel total cell lysates from non-transfected cells (Fig. 3.1C). When exogenous thrombin was added to transfected T24/83 cells, the major species diminished in intensity, and a novel thrombindependent, anti-API antibody-reactive 105 kDa polypeptide was detected, consistent with the formation of denaturation-resistant thrombin-API M358R inhibitory complexes (Fig. Similarly, when transfected cells expressing tethered AR-API M358R were 3.1C). incubated with defibrinated recalcified plasma, a plasma-dependent band that co-migrated with the denaturation-resistant thrombin-API M358R inhibitory complex was observed (see asterisks in Fig. 3.1C and 3.1D); in contrast, extraneous bands were not dependent on the presence of both plasma and AR-API M358R (Fig. 3.1C and 3.1D). However, when thrombin generation was followed in a defibrinated plasma system, little or no difference in reaction profiles was noted when T24/83 cells expressing AR-API M358R were compared to non-transfected controls (Fig. 3.1E).

Continuous assay of thrombin generation on T24/83 cells with or without transfection

To explore the apparent discrepancy between detecting thrombin-inhibitory complexes and failing to find a reduction in thrombin generation, we applied the TGA. This permitted the use of recalcified NHPP without the need for removal of clottable fibrinogen from the plasma. When thrombin generation was directly assessed in the presence of suspensions of T24/83 cells and NHPP, a characteristic TGA profile was obtained, consisting of a brief lag, rapid thrombin generation to a peak, and a slower decline in thrombin levels (Fig. 3.2A). Substituting non-TF-bearing HEK 293 cells for T24/83 abrogated thrombin generation (Fig. 3.2A). Titration of anti-TF monoclonal antibody into the T24/83 TGA progressively lengthened the lag time and depressed peak thrombin generation; 15 μ g of anti-TF antibody (but not monoclonal anti- β -catenin



Figure 3.1 – TF and AR-API M358R expression by cultured T24/83 cells

1A. Change in optical density of T24/83 cells incubated with FXa-specific chromogenic substrate and FX alone or FX and FVIIa (mean \pm SEM of n = 4 to 8). **1B.** Fluorescence micrographs of T24/83 cells stained with Anti-TF monoclonal antibody and AlexaFluor 488-conjugated anti-mouse IgG secondary antibody (Anti-Tf, upper left) or secondary antibody alone (2nd only) or anti-β-catenin monoclonal antibody and AlexaFluor 488conjugated anti-mouse IgG secondary antibody (Anti-B-catenin Tf, lower). AlexaFluorrelated signals are green; DAPI-stained nuclei are blue. 1C. Anti-API immunoblot of total cell lysates from T24/83 cells without transfection (Non-Tf), transfected with AR-API M358R expression plasmid without (TF (AR-API)) or with (TF (AR-API) + IIa) exposure to thrombin (factor IIa); pd API indicates 25 ng of purified plasma-derived API. Markers (M) were pre-stained, with molecular weights of 170, 130, 100, 70, 55, 40, and 35 kDa, respectively. The asterisk to the left of the "TF (AR-API) + IIa" lane highlights the position of the thrombin-AR-API M358R complex. 1D. Anti-API immunoblot of total cell lysates from T24/83 cells combined with recalcified defibrinated plasma (DP) with or without transfection; "Tf (AR-API) + IIa" is the reaction product with exogenous thrombin rather than recalcified defibrinated plasma (same as 1C lane with the identical label); pd API is the same as in 1C. Asterisks (left of lanes) highlight the thrombin-AR-API M358R complexes. 1E. Thrombin generation by non-transfected T24/83 cells or cells transfected with AR-API M358R (+ AR-API M358R) without or with defibrinated plasma (+DP) (solid lines). Dotted lines show results of incubations of buffer and TF with or without DP. Optical density (405 nm) values relate to colour generated by cleavage of thrombin-specific chromogenic substrate. A single experiment representative of 3 is shown.

antibody, not shown) eliminated all activity. Soluble API M358R also progressively reduced thrombin generation (Fig. 3.2B) as it was increased from 200 to 1000 nM in the TGA; at 1000 nM it eliminated all activity. We next used TGA to determine if co-expression of TF and API M358R on the T24/83 cell surface would reduce thrombin generation. No significant differences were noted in any TGA parameter, including the time to thrombin peak (Fig. 3.2C) or in the area under the thrombin generation curve (Fig.3.2D) among non-transfected cells or those expressing either thrombin inhibitory AR-API M358R or inactive (541) AR-API T345R/M358R. In contrast, adding 1000 nM soluble recombinant leech thrombin inhibitor HV3 abrogated thrombin generation (Fig. 3.2C).

Continuous thrombin generation in HEK 293 cells following gene transfer of TF

The T24/83 data suggested that thrombin generation could be reduced by AR-API M358R co-expression on cells expressing lower levels of TF than T24/83. To gain control over the TF to API ratio, we chose to transfect HEK 293 cells with a TFexpressing plasmid. Such cells exhibited a reproducible thrombin generation profile that closely resembled that of T24/83 cells, while their non-transfected counterparts failed to support thrombin generation (Fig. 3.3A). Immunoblotting of total cell extracts from identical amounts of T24/83 or HEK 293 cells transfected with 8.0 µg of plasmid DNA, showed that the transfected cells produced similar patterns of several immunoreactive bands with a mobility of 50 - 55 kDa (Fig. 3.4B), consistent with previous reports of variability in TF polypeptide chain length and glycosylation (542,548). Intense staining of some, but not all transfected HEK 293 with anti-TF antibodies was noted by immunofluorescence microscopy (Fig. 3.3C), as expected in transient transfection; the intensity of those HEK 293 that were productively transfected exceeded that of equivalently tested T24/83 cells (compare Fig. 3.3C to Fig. 3.1B). The staining was most intense on the cell surface, dependent on the anti-TF monoclonal antibody, and differed in the pattern of staining from an unrelated monoclonal directed against β -catenin, which exclusively decorated cell-cell junctions (Fig.3.3C). Inclusion of the anti-TF monoclonal antibody in the thrombin generation assay diminished, and then abrogated, thrombin generation as the concentration was increased (Fig.3.3D).

Co-transfection of HEK 293 with TF and API

We next examined HEK 293 cells simultaneously co-transfected with plasmids expressing TF and API. Similar amounts of cell-bound TF were detected by immunoblotting whether or not HEK 293 cells were co-transfected with 4.0 μ g of TF-encoding plasmid and either the same amount of AR-API M358R-encoding plasmid or AR-API T345R/M358R-encoding plasmid; similarly, incubation of the co-transfected cells with thrombin prior to extraction and immunoblotting had no discernible effect on TF expression (Fig. 3.4A). The ability of AR-API M358R to form inhibitory complexes with thrombin, and the inability of AR-API T45R/M348R to do so, was unchanged by co-expression of TF (Fig. 3.4B). However, no difference in thrombin generation profile was


Figure 3.2 - Analysis of thrombin generation assay (TGA) of T24/83 cells.

2A - **2B.** TGA profiles of thrombin concentration (nM) versus time, for reactions involving recalcified normal pooled plasma with or without addition of cultured cells or anti-TF monoclonal antibody (**2A**) or soluble API M358R (**2B**). Conditions are indicated above the progress curves. **2C** – **2D**. Selected TGA parameters for T24/83 cells without transfection (Non-T) and with or without incubation with 10-1000 nM HV3, transfected with AR-API M358R (API M358R) or AR-API T345R/M358R (API RR) are shown, as indicated on the y axis (mean \pm SEM of 4-8 determinations).



Figure 3.3 - TF and API expression by transfected or co-transfected HEK 293 cells.

3A. TGA profile of thrombin concentration (nM) versus time, for reactions involving recalcified normal pooled plasma. Single determinations are shown for addition of no cells or non-transfected HEK 293 cells and the mean \pm SEM, n=2, for HEK 293 cells transfected with TF. **3B.** Anti-TF immunoblot of total cell lysates from T24/83 cells, non-transfected (Non-T HEK) HEK 293 cells or HEK 293 cells transfected with 8 ug per T-25 flask of plasmid DNA encoding TF (HEK/TF 5); sTF indicates 50 ng of purified recombinant soluble TF. Markers (M) are the same as those used in Fig. 3.1. 3C. Fluorescence micrographs of HEK 293 cells transfected with 8 µg per T-25 flask of plasmid DNA encoding TF T24/83 cells stained with secondary antibody alone (2nd only), Anti-TF monoclonal antibody and AlexaFluor 488-conjugated anti-mouse IgG secondary antibody (Anti-TF) or anti-β-catenin monoclonal antibody and AlexaFluor 488conjugated anti-mouse IgG secondary antibody (Anti-\beta-catenin). AlexaFluor-related signals are green; DAPI-stained nuclei are blue. 3D. TGA profile of thrombin concentration (nM) versus time, for reactions involving recalcified normal pooled plasma combined with HEK 293 cells transfected with TF with or without the amounts of monoclonal anti-TF antibody (MAb) indicated above the progress curves.

noted between cells co-transfected with TF and the functional tethered API M358R and those cells co-transfected with non-functional tethered T345R/M358R (data not shown).

TGA employing transfected HEK 293 expressing minimal TF

To maximize tethered API expression and reduce TF expression, we decreased the TFexpressing: API-expressing plasmid DNA mass ratio from 1:1 (Fig. 3.4) to 1:9 (Fig. 3.5). This approach led to a clear reduction in the amount of expressed TF (Fig. 3.5A, left panel) without reducing the amount or functionality of the expressed tethered API (Fig. 3.5A, right panel). Thrombin generation in HEK 293 cells was clearly reduced when 1:9 TF: API co-transfection was compared between cells expressing functional thrombininhibitory AR API (M358R) and cells expressing non-functional AR API T345R/M358R (Fig. 3.5B). Substitution of functional AR API M358R expression for non-functional AR API T345R/M358R expression: reduced peak thrombin from 180 ± 30 to 68 ± 7 nM (Fig. 3.5C); reduced the velocity index from 70 ± 25 to 8 ± 2 (Fig. 3.5D); reduced the AUC from 1400 ± 100 to 990 ± 25 nM-min (Fig. 3.5E); and lengthened the time to peak thrombin generation from 6 ± 1 to 14 ± 3 minutes (Fig. 3.5F) and the lag time from $3.3 \pm$ 0.6 to 5.3 ± 0.6 min (Fig. 3.5G).

DISCUSSION

To reduce the thrombogenicity of endothelial or smooth muscle cells, investigators have transferred a variety of genes, including cyclooxygenase (549), tissue plasminogen activator (550), hepatocyte growth factor (551), and ectonucleoside triphosphate diphosphohydrolase (552) in vitro and in vivo. Tethering a thrombin inhibitor to vascular cell surfaces cells has also been investigated. Hirudin, the most potent proteinaceous inhibitor of thrombin (553), was fused to a portion of the human membrane protein CD4 (518); immortalized porcine endothelial cells expressing the hirudin-CD4 chimera demonstrated reductions in clotting times when TF expression was induced and recalcified plasma was exposed to the transfected cells. Transgenic mice expressing the hirudin-CD4 chimera exhibited decreased neointimal expansion following injury to the carotid artery (554). However, recombinant hirudin administration to patients is immunogenic (555,556). Recombinant human protein administration should be less so. Accordingly, we adapted a variant human protein that inhibits thrombin, API M358R, for cell surface display via gene fusion to a portion of the human asialoglycoprotein receptor (AR-); the tethered fusion protein AR-API M358R inhibited exogenously added thrombin (541). Here we showed that it also inhibited endogenously generated thrombin in plasma when expressed on the surface of TF-expressing cells, a prerequisite for any future use of this protein to reduce thrombogenicity.

AR-API M358R-thrombin inhibitory complexes were formed on the exposure of transfected T24/83 cells to plasma, but AR-API M358R expression in these cells did not



Figure 3.4 - Immunoblot analysis of singly and doubly transfected HEK 293 cells.

3.4A. Anti-TF immunoblot of total cell lysates from T24/83 cells, non-transfected (Non-T) HEK 293 cells or HEK 293 cells transfected with the amounts of TF-encoding plasmid DNA (8.0 or 4.0 µg per T-25 flask) indicated above the lanes, with or without co-transfection with 4.0 µg per T-25 flask of plasmid DNA encoding AR-API M358R (API R) or AR-API T345R/M358R (API RR), without or with thrombin (+ IIa); sTF indicates 50 ng of purified recombinant soluble TF. **3.4B.** Replicate anti-API immunoblot of that shown in Fig.3.4A; pd API indicates 25 ng of plasma-derived API. Markers (M) are the same as those used in Fig. 3.1.



Figure 3.5 - Immunoblot and thrombin generation of singly and doubly transfected HEK 293 cells.

3.5A. Anti-TF or anti-API immunoblots of total cell lysates from non-transfected (Non-T) HEK 293 cells or HEK 293 cells transfected with the amounts of TF-encoding plasmid DNA (8.0 or $0.8\mu g$ per T-25 flask) indicated above the lanes, with or without co-transfection with 7.2 μg per T-25 flask of plasmid DNA encoding AR-API M358R (API R) or AR-API T345R/M358R (API RR) without or with thrombin (+ IIa); sTF indicates 50 ng of purified recombinant soluble TF and pd API indicates 25 ng of plasma-derived API. **3.5B.** TGA profile of thrombin concentration (nM) versus time, for reactions involving recalcified normal pooled plasma combined with HEK 293 cells co-transfected with 0.8 TF μg per T-25 flask of TF-encoding plasmid DNA and 7.2 μg per T-25 flask of plasmid DNA encoding AR-API M358R (API R). **3.5C** – **3.5F.** TGA parameters for repeated experiments of the kind shown in **3.5B** (mean \pm SEM, n=3 determinations). Markers (M) are the same as those used in Fig. 3.1.

result in an overall reduction in thrombin generation. This discrepancy was likely related to the abundance of TF expressed on T24/83 cells and its provision of a strong trigger for thrombin generation. Any modest reduction in thrombin output due to protease inhibition by AR-API M358R was likely insufficient to impact the mean TGA profile, lying within the range of variation in replicated TGA assays. Using HEK 293 cells allowed us to increase the ratio of expressed AR-API M358R to co-expressed TF by lowering the TF-expressing plasmid mass used for co-transfection. The success of this strategy supported the idea that the T24/83 procoagulant stimulus was too great to be impacted by AR-API M358R expression. Others have noted that peak thrombin concentrations could be decreased 2.5-fold by decreasing TF in TGA from 30 to 3 pM (557). Although we did not rigorously quantify recombinant TF expression levels, our results are consistent with this report, in that we decreased the thrombin generation into the AR-API-inhibitable by decreasing the amount of TF-encoding plasmid DNA in transfection.

Although API M358R is a promiscuous serine protease inhibitor capable of forming inhibitor complexes with other coagulation factors such as factor XIa (534), we did not detect covalent complexes between AR-API M358R and any other protease, at least electrophoretically. This observation is consistent with the much greater rate of reaction of API M358R with thrombin than other serine proteases (534); as well, the reduction in thrombin inhibition rate that accompanied membrane tethering of API M358R further diminished the likelihood of forming AR-API M358R inhibitory complexes with any other activated coagulation factor (541).

In this study, we adapted the TGA to incorporate intact nucleated mammalian cells as the source of TF necessary to conduct the assay. Others have incorporated platelets (557) or intact nucleated cells into this assay (558), but for different purposes. Similarly, tumor cell line lysates, but not intact tumour cells, have been used as the requisite source of TF for TGA (559). To our knowledge we are the first to employ intact cultured cells to fulfill this requirement. Although this was not our goal, our adaptation of this system could therefore be exploited by investigators wishing to test the functionality of TF mutant proteins, by transfection of HEK 293 with plasmids expressing these variants followed by TGA.

Our current results with cell-surface displayed AR-API M358R make reasonable further investigations of this likely minimally immunogenic human thrombin inhibitor in animal models of thrombosis. The effectiveness of AR-API M358R in limiting thrombosis would be expected to depend on the strength of the TF signal in a particular vascular bed. Gene transfer of AR-API M358R to tumour cells is another translational possibility, since TF expression on tumour cells likely increases thrombotic risk and may also be linked to tumour progression (560). Improved efficacy of AR-API M358R as an anticoagulant could also ensue if more powerful promoters were employed than the CMV intermediate-early promoter used in gene transfer in this study.

ACKNOWLEDGEMENTS

This work was made possible by Grants-In-Aid T6271 and 000267 from the Heart and Stroke Foundations of Canada and Ontario to WPS. RFG received a competitive Graduate Fellowship Program award from Canadian Blood Services (CBS) in support of his doctoral research at McMaster University. Because CBS receives research support from Health Canada, a department of the federal government of Canada, as a condition of funding this article must contain the statement, "The views expressed herein do not necessarily represent the views of the federal government [of Canada]."

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Licensed content date	November 2014
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Estimated size (number of pages)	150

4 PAPER #3 – Comparison of mammalian and bacterial expression library screening to detect recombinant alpha-1 proteinase inhibitor variants with enhanced thrombin inhibitory capacity

Submitted for publication, Journal of Biotechnology.

Preface

I contributed to the design of the study, conducted the bulk of experimentation, data acquisition and analysis, and draft manuscript preparation.

Ms. Varsha Bhakta contributed to the project design in the original article upon which this research was based, and performed some of the experiments in this study.

Mr. Michael Xie conducted some of the experiments in this study.

The concept and design of displaying serpins on the surface of mammalian cells for the purposes of screening RCL mutant libraries for enhanced protease inhibitors originated with Dr. William Sheffield. Dr. Sheffield was also project supervisor as well as senior author, and responsible for final revisions to the manuscript prior to final submission.

This reaserch was supported by the Heart and Stroke Foundations of Ontario and Canada. I received scholarship support in the form of a Graduate Fellowship from the Canadian Blood Services.

Journal of Biotechnology

REGULAR ARTICLE

Comparison of mammalian and bacterial expression library screening to detect recombinant alpha-1 proteinase inhibitor variants with enhanced thrombin inhibitory capacity

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[#]Financial support: This work was made possible by Grant-In-Aid 000267 from the Heart and Stroke Foundation of Canada (Ontario Provincial Office) to WPS. RFG received a competitive Graduate Fellowship Program award from Canadian Blood Services (CBS) in support of his doctoral research at McMaster University. Because CBS receives research support from the federal government of Canada, this article must contain the statement, "The views expressed herein do not necessarily represent the views of the federal government [of Canada]."

Conflict of interest: The authors declare that they have no conflicts of interest, financial or otherwise, in this work.

Abstract

Serpins are a widely distributed family of serine proteases. A key determinant of their specificity is the reactive centre loop (RCL), a surface motif of ~ 20 amino acids in length. Expression libraries of variant serpins could be rapidly probed with proteases to develop novel inhibitors if optimal systems were available. The serpin variant alpha-1 proteinase inhibitor M358R (API M358R) inhibits the coagulation protease thrombin, but at submaximal rates compared to other serpins. Here we compared two approaches to isolate functional API variants from serpin expression libraries, using the same small library of API randomized at residue 358 (M358X): flow cytometry of transfected HEK 293 cells expressing membrane-displayed API; and a thrombin capture assay (TCA) performed on pools of bacterial lysates expressing soluble API. No enrichment for specific P1 residues was observed when the RCL codons of the 1% of sorted transfected 293 cells with the highest fluorescent thrombin-binding signals were subcloned and sequenced. In contrast, screening of 16 pools of bacterial API-expressing transformants led to the facile identification of API M358R and M358K as functional variants. Kinetic characterization showed that API M358R inhibited thrombin 17-fold more rapidly than API M358K. Reducing the incubation time with immobilized thrombin improved the sensitivity of TCA to detect supra-active API M358R variants and was used to screen a hypervariable library of API variants expressing 16 different amino acids at residues 352-357. The most active variant isolated, with TLSATP substituted for FLEAI, inhibited thrombin 2.9fold more rapidly than API M358R. Our results indicate that flow cytometric approaches used in protein engineering of antibodies are not appropriate for serpins, and highlight the utility of the optimized TCA for serpin protein engineering.

Keywords: serpins, alpha-1-proteinase inhibitor, thrombin, recombinant proteins, bacteria, microtiter plate assays, fluorescence-activated cell sorting, expression library screening

Introduction

Members of the serpin superfamily that act as serine protease inhibitors contain a scissile bond called the reactive centre (reviewed in (561,562)). When a cognate protease attacks the reactive centre, its cleavage releases stored energy (563,564) which powers a massive conformational change, one that results in translocation of protease to the opposite pole of the serpin (565-567). The distortion of the protease active site traps it in acyl intermediate form, covalently attached to the cleaved serpin (568). The reactive centre is found on the serpin surface, housed within a region known as the reactive centre loop (RCL), of approximately 20 amino acids in length (569). Aligned serpin RCL sequences show little consensus (569). Nevertheless, crystallized encounter complexes of serpins with active site-mutated proteases confirm that, for those serpins lacking binding exosites, the RCL is the first point of contact between serpin and protease (510,570-572). As such, it represents a compelling target for serpin protein engineering; by varying the sequence of the RCL, serpins of novel specificity could be generated.

The serpin alpha-1 proteinase inhibitor (API, also known as alpha-1 antitrypsin) is the most abundant serpin found in mammalian plasma (523). Its chief physiological role as an inhibitor of neutrophil elastase in lung tissue is supported by the increased risk of emphysema borne by individuals genetically deficient in API (525,530). The API reactive centre is M358-S359 (573), or P1-P1' using the Schechter and Berger conventional numbering scheme (574). The naturally occurring M358R Pittsburgh mutation re-directs API from inhibiting elastase to inhibiting thrombin and other activated coagulation proteases (484,575). While this change elicited a bleeding tendency in the index case (361), it sparked interest in using the API scaffold to generate novel protease inhibitors by protein engineering. Directed mutagenesis approaches have met with some success in enhancing the specificity of API for non-physiological targets, but typically at the cost of elevating the reaction stoichiometry (486,576-578). Random mutagenesis approaches involving the screening of hypervariable serpin expression libraries has until recently been employed only in the case of one serpin, plasminogen activator inhibitor 1 (PAI-1) (514-516,579).

We previously described the expression of antithrombin and API M358R as membrane proteins tethered to the surface of mammalian cells in culture, and the retention of thrombin inhibitory activity by the tethered serpins (541). This finding suggested a strategy for selection of novel API variants by cell sorting of transfected cells acquiring the ability to bind thrombin (541). We also described a thrombin capture assay and its use to select functional serpin variants from pools of candidates expressed in libraries expressed in *E. coli* (580). Our objectives in the current study were: (1) to adapt the mammalian cell expression of tethered API M358R for library screening; (2) to screen the same API P1 expression library in both mammalian cell and bacterial systems; and (3) to optimize the more efficient system of the two to favour the selection of variants inhibiting thrombin with improved kinetics compared to API M358R. We report that the bacterial, but not the mammalian expression strategy, enriched for thrombin-inhibitory API P1 variants in a single round of gene transfer, and the isolation of a novel API M358R

variant hyper-reactive with thrombin through optimization of the screening of the bacterial expression library.

Materials and methods

Construction of pCEP4 constructs directing the expression of AR-API proteins Plasmid DNA from plasmids pC3-AR-API (M358R) and pC3-AR-API (T345R/M358R) (541) was restricted with HindIII and XhoI, and the 1492 bp minor fragment was inserted between these sites in pCEP4 (Invitrogen/Life Technologies, Carlsbad, CA), forming pCEP4-AR-API M358R and pCEP4-AR-API T345R/M358R, respectively (both 11,661 bp).

Construction of hypervariable API expression libraries in pCEP4-AR-API background

Overlap extension was used to position unique restriction sites upstream and downstream of RCL codons in pCEP4-AR-API M358R, using PCR mediated by Phusion HF heatstable DNA polymerase under conditions recommended by the manufacturer (Finnzymes/Fisher Scientific, Ottawa, ON). Deoxyribonucleotide primer sequences are shown in Table 4.1. Amplicons generated by PCR using primers A and B (430 bp) and primers C and D (177 bp) were denatured, annealed, and extended to yield a 583 bp product ABCD. ABCD was restricted with PmlI and XhoI and the resulting 523 bp fragment was inserted between these sites in pCEP4-AR-API M358R, yielding pCEP4-AR-API T339Stop, which contained unique restriction sites BsiWI and AfIII between codons P23 and P6'. Restriction of pCEP4-AR-API T339Stop with BsiWI and AfIII allowed insertion of annealed oligonucleotides to restore RCL codons; the BsiWI and AfIII sites were not regenerated, in order to restore wild-type residues at P21 (L340) and P6' (V364). Annealing and insertion of degenerate oligonucleotide pairs E and F and G and H produced pCEP4-AR-API-based hypervariable plasmid libraries with (a) all possible codons at M358 (P1) (in pCEP4-AR-API M358X) or (b) all possible codons between P7 and P2 (ran for randomized), and a subset of possible codons not encoding Arg (in pCEP4-AR-API (P7-P2ran-P1 not Arg).

Construction of hypervariable API expression libraries in pBAD-H₆-API background

The hypervariable region in pCEP4-AR-API (P7-P2-ran/P1 not Arg) was transferred into the pBAD-H₆-API background by inserting the 559 bp PmII-EcoRI M358R restriction endonuclease digestion fragment of pCEP4-AR-API (P7-P2-ran/P1 not Arg) between the corresponding sites of pBAD-H₆-API T345R/ M358R, replacing all RCL codons and forming pBAD-H₆-API (P7-P2 ran-P1 no Arg). A second hypervariable library for bacterial expression screening was constructed *de novo*, via PCR using primers A and I. The resulting amplicon was digested with PmII and Bsu36I to yield a 458 bp digestion product, which was inserted between these sites in pBAD-H₆-API M358R, replacing the RCL codons and forming pBAD-H₆-API (P7-P2 ran No Stop/M358R). In this API hypervariable library, the first codon position encoding P7-P2 residues inclusive was limited to A, C, or G. This limitation imposed an absence of Phe, Tyr, Trp, Cys, and termination codons at P7-P2 inclusive among the possible API mutant proteins encoded

Table 4.	1 - Sequence of deoxyribonucleotide pr	imers employed in this study.
Primer	Primer sequence	Purpose
Α	5' CAAGGACACC AGGAAGAGG	Overlap extension and API
	ACTT 3'	cDNA excision
В	5' GAACTTAAGC TCCTACCGTA	Overlap extension
	CGGCCTTATG CACGGCCTTG	
	GAGAGCTT 3'	
С	5' GCCGTACGGT AGGAGCTTAA	Overlap extension
	GTTCAACAAA CCCTTTGTCT	
	TCTTAATG 3'	
D	5' ATCATGTCTG GATCCGCCCT	Overlap extension and API
	TCCC 3'	cDNA excision
Ε	5' GTACTGACCA TTGATGAGAA	PCR and cassette mutagenesis,
	AGGGACTGAA GCTGCTGGGG	sense, M358X (NNN)
	CCATGTTTTT AGAGGCCATA	
	CCCNNNTCTA CTATCCCCCC	
	GGAGG 3'	
F	5' TTGACGTCCG GGGGGATAGA	PCR and cassette mutagenesis,
	NNNGGGTATG GCCTCTAAAA	antisense, M358X (NNN)
	ACATGGCCCC AGCAGCTTCA	
	GTCCCTTTCT CATCAATGGT	
	CA 3'	
G	5' GTACTGACCA TTGATGAGAA	PCR and cassette mutagenesis,
	AGGGACTGAA GCTGCTGGGG	sense, P7-P2 randomized,
	CCATGNNNNN NNNNNNNNN	M358 not Arg (where D is A,
	NNNNDNTCTA CTATCCCCCC	G, or T)
	GGAGG 3'	
Н	5' TTGACGTCCG GGGGGATAGA	PCR and cassette mutagenesis,
		antisense, P/-P2 randomized,
	NCATGGCCCC AGCAGCTTCAG	M358 not Arg (where H is the
	TCCCTTTCTCA TCAATGGTCA 3'	complement of D)
I	5' TGACCTCAGG CGGGATAGAT	PCR, partial randomization of
	CINNVNNVNN VNNVNNVNNV	P/-P2 codons, no Stop codons
	CATGGCCCCA GCAGCTTC 3	(where V is A, C, or G)
J	5' TGACCTCAGG CGGGATAGAN	PCR, randomization of P1
	NNGGGTATGG CTCTAAAAAC	codons
17		
K	5 CCGGAATICT TATTTTGGG	PCK, sequencing of pJE11.2
	IGGGATICAC 3	constructs

in the library. A third library was constructed via PCR using primers A and J by analogous steps as described above, forming pBAD-H₆-API M358X.

Transfection and cell sorting of HEK 293 cells

HEK 293 cells were transfected with pCEP4-based AR-API expression plasmids as previously described for pCDNA 3.1-based AR-API constructs, using Lipofectamine 2000 reagent at 90 - 95% confluency (581). Following transfection, cells were resuspended in serum-free culture medium and reacted with thrombin, affinity-purified sheep anti-human prothrombin IgG, and Alexa Fluor488-conjugated donkey anti-sheep IgG as previously described (541). They were then characterized by flow cytometry and 0.5 to 1.0 X 10⁶ transfected cells were sorted using a BD LSR II bench-top flow cytometer operated by the McMaster Flow Cytometry Facility powered by FACSDiva 6.0 software (BD Biosciences, San Diego, CA). The resulting data was analyzed using FlowJo version 10.0.7 (FlowJo LLC, Ashland, OR). In some experiments, subpopulations ($\sim 5 - 10,000$ sorted cells) with the lowest or highest fluorescent signals were recovered and re-plated in cell culture flasks or pooled and subjected to DNA extraction (Qiagen Miniprep kit, Qiagen, Chatsworth, CA). Recovered plasmid DNA was amplified by PCR using oligonucleotides AX and K, and the resulting 592 bp amplicon was purified (Qiaquick PCR Purification Kit, Qiagen) and inserted into pJET1.2 via bluntended ligation as directed by the manufacturer (Thermo/Fisher Scientific, Ottawa, ON). The DNA sequence of inserted DNA]in pJET1.2 subclone plasmids was determined using commercial pJET1.2 forward and reverse primers at the Molecular Biology Institute (MOBIX) Central Facility, McMaster University. Other constructs were also verified analogously.

Screening of pBAD-H₆-API-based expression libraries using a thrombin capture assay

Screening of libraries of bacterial colonies harbouring pBAD-H6-API plasmids varying at one or more API codons was performed as previously described (580). Briefly, single bacterial colonies or pools of ten colonies were used to inoculate archive plates or liquid cultures; the latter were lysed by sonication and tested by incubation with thrombin immobilized onto the wells of microtiter plates. Bound recombinant API variants competent for thrombin binding were subsequently detected by anti-API IgG binding and quantified by optical density following colour generation by horseradish-peroxidase coupled sheep anti-human API IgG (580). In some experiments the time of incubation of the lysates with bound thrombin, previously set at 60 minutes, was varied.

Expression, purification, and kinetic characterization of recombinant API

Purification of recombinant hexahistidine-tagged API M358R and variants from bacterial cell lysates using nickel-chelate affinity chromatography followed by ion exchange on DEAE-Sepharose (GE Health Care, Baie d'Urfe, Canada)(486). Recombinant API proteins were characterized kinetically as thrombin inhibitors by determination of their pseudo-first order rate constant under appropriate order conditions (\geq 10-fold molar excess of API over thrombin in a two-stage assay) and division of that value by the initial API concentration to yield the second order rate constants (k₂) (486,535). Reaction

stoichiometries (i.e. stoichiometries of inhibition, SI) were also determined as previously described (582).

Statistical analysis

Statistical analysis was facilitated through the use of GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA). For multiple comparisons, an analysis of variation (ANOVA) was employed (with Tukey's post-tests). A two-tailed student's t test was used for pairwise comparisons.

Results

Reaction of episomal plasmid-encoded serpins with thrombin

To facilitate the propagation of transiently transfected mammalian cells after selection by cell sorting, we separately transferred the cDNAs encoding membrane-tethered AR-API M358R and AR-API T345R/M358R into episomal expression vector pCEP4. The T345R mutation eliminates the ability of API M358R to form denaturation-resistant complexes with thrombin (541). Sonicates (Fig. 4.1A) or whole cell HEK 293 lysates (Fig. 4.1B) were found to contain two anti-API-reactive protein species of 72 kDa and 67 kDa in both cases. On sub-cellular fractionation of sonicated cell preparations, these proteins were found predominantly in the integral membrane fraction (Fig. 4.1A, compare three right panels). Incubation of cells expressing AR-API M358R, but not AR-API T345R, with thrombin led to the appearance of a novel 105 kDa polypeptide product, consistent with the formation of a thrombin-API serpin-enzyme complex (Fig.4.1A and B, SEC). Transfected cells selected in culture medium containing 15 μ g/ml hygromycin demonstrated retention of AR-API-expressing plasmids without a noticeable reduction in their growth rate (data not shown).

Cell sorting of transfected HEK 293 cells following reaction with thrombin

HEK 293 cells transfected with pCEP4-AR-API M358R or pCEP4-AR-API T345R/M358R plasmids were reacted with thrombin and immunofluorescence was used to follow thrombin binding by flow cytometry. As shown in Fig. 4.2A, the flow cytometric profile of pCEP4-AR-API M358R-transfected cells was right-shifted compared to that of cells transfected with pCEP4-AR-API T345R/M358R; the former population contained cells with greater reactivity with thrombin than the latter. In contrast, HEK 293 cells transfected with a plasmid library of pCEP-4-AR-API containing all possible nucleotides at codon 358 displayed a flow cytometric profile overlapping that of pCEP4-AR-API T345R/M358R, except for a small shoulder of higher fluorescence (Fig. 4.2A). One percent of the total sorted cells with the lowest (L) or highest (H) fluorescent intensity was recovered by cell sorting (Fig.4.2A, circles) and lysates were subjected to PCR amplification and DNA sequencing to determine if the cell populations differed in API P1 codons.



Figure 4.1 - Subcellular fractionation of transfected mammalian cells.

HEK 293 cells were transfected with pCEP4-based plasmids directing the expression of AR-API M358R or AR-API T345R/M358R and incubated with (+) or without (-) thrombin. Transfected cell suspensions were either sonicated alone (Sonicate) or sonicated and subjected to subcellular fractionation into Cytosol or Peripheral or Integral Membrane fractions. (A) 3% of the Sonicate and 12.5% of the other three fractions was electrophoresed and immunoblotted with anti-API antibodies. SEC denotes serpinenzyme (thrombin-API M358R) complexes, whose position, and that of full-length, membrane-tethered API is highlighted by arrows (at right). Asterisks within the panels also identify SEC bands. (B) As in **panel A**, except that transfected cells were recovered by microcentrifugation and lysed in SDS sample buffer without sonication to form whole cell lysates; 10% of the lysate was immunoblotted. In both panels only the relevant portions of the immunoblots are shown.



Figure 4.2 - Flow cytometric detection of bound thrombin.

Transfected HEK 293 cells were reacted with thrombin followed by anti-thrombin antibodies and Alexa Fluor 488-conjugated secondary antibodies. (**A**) The histogram of the fluorescence-activated cell sorting is shown, with the cell number (Count) shown on the y-axis and the corresponding fluorescent signal on the x-axis. Tracings correspond to cells transfected with pCEP4-based plasmids expressing AR-API T358R/M358R (blue), AR-API M358R (orange), or a library expressing AR-API with all possibilities at codon 358 (AR-API M358X). Circles highlight the 1% of cells expressing AR-API M358X with the lowest fluorescence (L) and the corresponding fraction with the highest fluorescent signal (H). (**B**) A similar histogram to **panel A** is shown, for transfected cells expressing AR-API T358R/M358R (purple) or AR-API M358R (salmon) or AR-API M358R diluted with increasing ratios of AR-API T358R/M358R (1:5, blue; 1:20, orange; 1:50, green; 1:100, deep purple; 1:500, magenta) identified on the panel.

Table 4.2 shows the expected abundance of P1 variants based on genetic code degeneracy, versus the abundance observed in 19 H and 7 L clones, and in 8 clones from the unsorted library. No striking enrichment was discernible for any P1 codon among those expressed in AR-API M358X form in cells reacting strongly with thrombin; indeed 15 of the possible 21 amino acids and stop codons were found among the 19 subclones examined (see Table 4.2). Arg codons were not enriched. The recovered AR-API-M358X-encoding plasmids were less diverse in cells reacting less strongly with thrombin (Table 4.2).

We next mixed pCEP4-AR-API T345R/M358R and pCEP4-AR-API M358R DNA in different ratios and performed transfection and cell sorting. As shown in Fig. 4.2B, the population of cells transfected with pCEP4-AR-API M358R bound thrombin more effectively than those transfected with pCEP4-AR-API T345R/M358R, as judged by immunofluorescence intensity per cell. As the ratio of DNA encoding AR-API M358R: AR-API T345R/M358R was decreased from 1:5 to 1:100, the flow cytometric profile shifted from one partially overlapping with the pCEP4-AR-API M358R-only transfected cells to one fully overlapping the pCEP4-AR-API T345R/M358R-only transfected cells. At 1:50 the profile resembled that obtained after transfection with the M358X library (compare Fig. 4.2A to 4.2B).

Thrombin capture assay screening of E. coli expressing API randomized at P1

Having observed no selection for known thrombin-inhibiting P1 variants such as M358R in the mammalian cell sorting experiments, we transferred the P1 library to an arabinose-inducible pBAD-based bacterial API expression plasmid. Fig. 4.1A shows the results of screening sixteen pools of ten bacterial colonies each harbouring pBAD-API M358X plasmids using a thrombin capture assay (TCA) previously employed to screen an API P12 variable library (i.e. pBAD-API A347X) for thrombin-inhibiting variants (580). Six of sixteen pools exhibited optical density values indicative of thrombin-complexing activity at levels exceeding the background by at least 3-fold. Individual candidates from the pool were re-probed with thrombin (e.g. pool C (Fig. 4.3B) and pool E (Fig. 4.3C)). DNA sequencing revealed that the single active P1 variant in five pools was API M358R, and that the single active P1 variant in the sixth was API M358K.

In contrast to the facile selection of API M358R and API M358K from the P1 hypervariable library, when we substituted a library hypervariable in the P7-P1 RCL residues, but without the possibility of Arg at P1, we found no "hits" above background in a screen of 1000 candidate colonies by TCA (data not shown).

Gel and kinetic characterization of purified API P1 variants

The TCA included a relatively lengthy 60 minute incubation of bacterial lysates containing recombinant API M358X which could have allowed two API variants

Table 4.2: Abundance of P1 codons subcloned from transfected HEK 293 cells						
P1	Theoretical	Observed abundance (%)				
residue	abundance		10/	10/ 6		
		Unsorted	1% of transfected	1% OI		
		погагу	cens (nignest	transfected		
			nuorescence)	fluorescence)		
Ala	6.3	_	5.3	-		
Arg	9.4	12.5	5.3	_		
Asp	3.1	-	5.3	-		
Cys	3.1	12.5	5.3	-		
Gly	6.3	-	5.3	28.6		
Gln	-	12.5	-	-		
Glu	3.1	-	5.3	-		
His	3.1	-	10.5	-		
Ile	4.7	-	5.3	28.6		
Leu	9.4	25	10.5	14.3		
Lys	3.1	25	-	-		
Pro	6.3	-	5.3	14.3		
Ser	9.4	12.5	5.3	-		
Tyr	3.1	-	5.3	14.3		
Trp	1.6	-	5.3	-		
STOP	4.7	-	15.8	-		
Val	6.3	-	10.6	-		



Figure 4.3 - Screening of a bacterial expression library of API randomized at residue 358 (P1).

(A) Thrombin capture assay readings (Optical Density (450 nm)) are shown for 16 pools of 10 independent clones, coded A through P (Pool). Duplicate results (\pm SEM) are shown for positive pools with a signal > 300% of the negative control (NEG) (filled bars) or negative pools (open bars). Control lysate values are also shown for control reactions (Con, grey bars), including LYS (lysate of empty vector colonies), NEG (lysate of API T345R/M358R-expressing cells) and M358R (lysate of known API M35R-expressing cells. (**B and C**) As in **panel A**, except archive plates for pools C and E were used to grow individual colony cultures for thrombin capture assay screening of the ten independent clones comprising each pool; bars reflect the mean of duplicate assay values \pm SEM.

differing in their kinetics of thrombin inhibition to yield similar signals. Since the screen shown in Fig. 4.3 identified two of the three basic amino acids (Arg and Lys) as functional P1 substitution variants, we constructed the third (His) and purified API M358R, M358K, and M358H proteins. Like the previously characterized, bacterially expressed API M358R, the K and H variants formed SDS-stable complexes with thrombin (Fig. 4.4A and 4.4B, SEC) and were also cleaved by thrombin at their reactive centres (Fig. 4.4A and 4.4B, cleaved serpin, CS). Complex formation was less extensive and less rapid for the H and K variants than M358R, as judged by visual inspection of the band intensity. As expected, API T345R/M358R did not form complexes with thrombin but was cleaved (Fig. 4.4A, at right).

Kinetic analysis of the three P1 variants revealed that the K and the H variants were on average 17-fold and 20-fold less rapid inhibitors of thrombin than API M358R (Fig. 4.4C), as evidenced by their reduced second order rate constants of 8.5 ± 0.4 and $7.4 \pm 0.5 \times 10^3 \text{ M}^{-1} \text{sec}^{-1}$ versus $1.5 \pm 0.9 \times 10^5 \text{ M}^{-1} \text{sec}^{-1}$ for API M358R. The reduction in k_2 values was statistically significant (p < 0.001) for both H and the K variants but the difference between the rates of thrombin inhibition mediated by these variants was not.

Effect of time of incubation with thrombin on the thrombin capture assay

The time of bacterial lysate incubation with thrombin bound to the well of a microtiter plate well in the TCA was varied to generate the product versus time graph shown in Fig. 4.5. Lysates containing API M358R exhibited a gradual increase in thrombin-bound API reactivity over 60 minutes, while two API M358R variants with additional mutations previously shown to enhance their rate of thrombin inhibition by 2-(583) to 19-fold (582) exhibited more rapid rates of thrombin binding that plateaued over time, and API T345R/M358R exhibited minimal, background levels of binding (Fig. 4.5). The optical density ratio of the HAPI M358R or API (P7-P3) AAFVS M358R lysates to those containing API M358R was found to be greater at 10 minutes (5-8) than at 60 minutes (3-4), the incubation time used in the thrombin capture assays shown in Fig. 4.3.

Thrombin capture screening of a P7-P2 hypervariable API library lacking stop codons

We reduced the time of incubation of bacterial lysates with bound thrombin to 10 minutes and screened 1300 colonies from a pBAD-API M358R library which P7-P2 residues had been randomized, but without the possibility of generating termination, Cys, Phe, Trp, or Tyr codons. Pools of ten candidates with positive thrombin capture assay values 3-fold or higher than background were re-probed at the single candidate level. Replicated thrombin capture assay values for the eight candidates are shown in Fig. 4.6A (solid bars); the wild-type API M358R values are also shown (open bar). The P7-P2 codons present in each candidate were identified by DNA sequencing; note that the candidate with the highest thrombin capture assay value, TLSATP, was found twice in the screen. This variant (API (P7-P2 TLSATP) M358R) was purified and its k₂ and stoichiometry of



Figure 4.4 - Immunoblot and kinetic characterization of thrombin reactions with recombinant API variants.

(A) Purified API M358R or API T345R/M358R was reacted with thrombin for times (t) ranging from 0 to 60 seconds (sec) and reactions examined by electrophoresis and immunoblotting with anti-API antibodies. SEC indicates serpin-enzyme (thrombin-API) complex, S denotes serpin (API variant) and CS denotes cleaved serpin. M is a prestained molecular weight marker, with bands corresponding to 180, 130, 95, 72, 55, 43, 34, and 26 kDa, respectively. (B) Same as **Panel A**, except for API M358K and API M358H. (C) The second order rate constant of thrombin inhibition (k₂, in units of M⁻¹sec⁻¹) is shown for the API P1 variants identified on the x-axis. Note that the y-axis is logarithmic. The mean \pm SD for 5 determinations in each group is shown. Arrows above the columns indicate groups under comparison: *** denotes p < 0.001 by ANOVA with post-tests; n.s. denotes "not significant" (p > 0.05).



Figure 4.5 - Thrombin capture assay time course.

Thrombin immobilized on microtiter plates was used to capture API from bacterial lysates containing known API variants. The time of incubation of lysate and bound thrombin is indicated on the x axis and the assay readout (Optical Density (450 nm)) is given on the y axis. Results are the mean and range of duplicate determinations. API variants are identified on the graph by arrows to the corresponding reaction progress curve.

inhibition values were compared to those of API M358R. K2 values were increased 2.9-fold over those of API M358R (to $4.2 \pm 0.4 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ versus $1.4 \pm 0.1 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ for API M358R, p < 0.001, Fig. 4.6B), while SI values remained unchanged (2.9 \pm 0.2 versus 3.1 ± 0.3 , Fig. 4.6C, p > 0.05, Fig. 4.6C).

Discussion

This laboratory showed previously that API M358R retained thrombin inhibitory function when expressed as a membrane protein displayed on the surface of mammalian cells in culture (541). This finding suggested that expression screening of libraries of mammalian cell-displayed API variants could be productively used for protein engineering of API, provided that a powerful means of selection of variants of interest could be found. Flow cvtometric cell sorting was employed to separate API-expressing cells hypervariable at codon 358R using thrombin-linked fluorescence as a surrogate marker for denaturationresistant, API-thrombin serpin-enzyme complex formation. Other investigators have shown that both single-chain Fv antibodies (584) and, more recently, complete immunoglobulin molecules (585), may be expressed on the surface of transfected cells. In the first instance, single pass fluorescent cell sorting was employed to select a high affinity variant of an anti-CD22 surface-displayed ScFv library randomized at two residues within a complementary-determining region (CDR) hotspot (584). In the second, an IgG library was constructed from PCR-amplified germline sequences from normal donor lymphocytes, and was used to select anti-nerve growth factor antibodies by flow cytometry (585). In both instances, HEK 293 cells were used for transfection. These studies provided an impetus for attempting similar applications of API libraries displayed on the surface of HEK 293 cells.

However, surface display of API was complicated by several factors that likely contributed to our failure to enrich specific functional P1 variants in a single pass. Thrombin immunofluorescence was not solely indicative of formation of a tethered APIthrombin covalent complex. We observed both thrombin binding to non-transfected HEK 293 cells (541) and to transfected cells displaying API T345R/M358R. This variant cannot form covalent complexes with thrombin (487,541,580) but it is a thrombin substrate (541), suggesting that transient binding of thrombin to this and other nonfunctional API derivatives takes place. The flow cytometric profile of the AR-API M358X transfected cell library overlapped with that of AR-API T345R/M358R, which was expected if most of the candidates did not express a functional thrombin-inhibitory serpin, but was indicative of an unfavourable signal to noise ratio. While the final steps in the inhibition pathway of proteases by serpins involve irreversible complex formation, analogous to very high affinity binding, the initial step is the formation of a reversible encounter (or Michaelis) complex. Competition for binding of a fluorescent probe to active site-mutated S195A thrombin by API M358R is relatively low affinity, with a reported inhibitory constant of ~ 3.0 μ M (586), suggestive of binding affinities three to four orders of magnitude less tight than displayed by many antibodies.



Figure 4.6 - Chracterization of API variants identified by screening of a bacterial expression library of API partially randomized at residues P7-P2 (residues 352 - 357, no termination codons, all amino acids except F, C, W, or Y).

(A) Thrombin capture assay readings (Optical Density (450 nm)) are shown for lysates from individual colonies from the library (filled bars) and from API M358R (FLEAIP, open bar). The mean of 4 - 8 determinations is shown \pm SD. P7-P2 codon identity shown below the columns was determined by DNA sequencing. (B) The second order rate constant of thrombin inhibition (k₂, in units of M⁻¹sec⁻¹) is shown for API M358R (open bar) and for API (P7-P2) TLSATP variant (closed bar); the mean \pm SD of 12 determinations is shown. Arrows above the columns indicate groups under comparison: *** denotes p < 0.001 by student's t test; n.s. denotes "not significant" (p > 0.05). (C) The stoichiometry of inhibition is shown for API M358R (open bar) and for API (P7-P2) TLSATP variant (closed bar); the mean \pm SD of 6 determinations is shown. Arrows above the columns indicate groups under comparison arrows above the columns indicate groups under comparison indicate groups under comparison indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison is shown. Arrows above the columns indicate groups under comparison indicate groups und

Although we did not observe enrichment for any specific P1 variant, including M358R, in the cell-sorting screen, we cannot exclude the possibility that such enrichment might have emerged following multiple rounds of HEK 293 display-mediated screening. However, since we have already described a multiple round method for selection of thrombin inhibitory variants of API M358R, T7 phage display (583), we did not pursue this possibility, but instead compared bacterial expression screening of the same hypervariable P1 library.

In contrast to the lack of enrichment in the mammalian cell approach, a single pass screen of sixteen pools of ten bacterial lysates yielded six hits, all of which proved to be true positives for thrombin inhibitory activity. Most were confirmed to be API M358R on single lysate testing but API M358K also showed strong activity in this non-kinetic assessment. On kinetic testing of these basic variants, as well as API M358H, we found a 17- to 20-fold rate advantage of API M358R over the Lys or His variants. This finding was consistent with our phage display screen of an API library hypervariable at P2-P1, which yielded only Pro-Arg (583), and consideration of serpin RCL sequences (561). Of the 27 human serpins known to function as protease inhibitors, 13 contain Arg at P1 and two Lys; of the serpins that inhibit thrombin, only heparin cofactor II lacks a P1 Arg (561). This preference matches thrombin peptide cleavage specificities determined using both peptide (587) and phage-displayed substrate sequences positioned between two thioredoxin polypeptides (588). Indeed, in the latter study, five selection rounds of a P4-P4' randomized substrate library, followed by random plaque sampling from the enriched population, yielded only Arg at P1. Deliberate substitution of Lys into the LTPR-GVRL consensus sequence reduced the rate of thrombin cleavage by approximately 10-fold, similar to our findings with API M358K. Although P1 variants such as API M358V(589), M358A and M358C(590) have been previously described, the effects of API M358K or M358H on thrombin inhibition are novel findings of this study.

Having demonstrated the superiority of the bacterial lysate approach to cell sorting of transfected mammalian cells, we sought to improve its efficiency for selecting API variants with enhanced rates of thrombin inhibition. Reducing the time of incubation of the lysates with immobilized thrombin increased the sensitivity of the assay for identification of API variants inhibiting thrombin more rapidly than API M358R, and eliminating thymidine from the first position of otherwise hypervariable codons eliminated uninformative candidates with a truncated RCL. This strategy preserved 16 of 20 possible amino acids as possibilities between P7-P2 in our "BNN" API M358R library, at the cost of not sampling Phe, Tyr, Trp, or Cys residues at these positions. The variant with the strongest signal in the thrombin capture assay that was found on screening of this library, TLSATP, inhibited thrombin 2.9-fold more rapidly than API M358R, surpassing P7-P3 AAFVS or DITMA as the most rapid API variant obtained from expression library screening efforts to date (583). TLSAT fit the "P7-Not Aromatic/P6-Hydrophobic/P5-T S/P4-Hydrophobic/P3-Not or Aromatic" loose consensus previously defined in our P7-P3 screening efforts (583). Like DITMA or AAFVS, the TSLATP improvement in the rate of thrombin inhibition did not come at the

cost of an increased reaction stoichiometry. That all API M358R variants selected as a consequence of hits in the screened lysate pools contained Pro at P2 was fully consistent with our previous serpin phage display study(583) and with the strong preference of thrombin for this residue noted in the P4-P4' phage display substrate study(588).

In this study we found that the binding affinity of thrombin for functional API variants tethered to the surface of HEK 293 cells was likely insufficient to allow for their separation from non-functional variants, possibly due to non-specific binding of thrombin to both cellular and serpin determinants on the transfected cells. Precedents for the use of mammalian cell sorting to capture surface displayed proteins have been in the immunoglobulin area, and the higher affinity initial binding of antibodies to their antigen targets, as compared to the formation of the initial serpin-enzyme Michaelis complex, may explain the observed, current inadequacy of this approach for serpins. In contrast, the screening of pooled lysates expressing the same small library of P1-randomized API M358R was shown to be rapid and sensitive. Although the bacterial lysate or TCA approach is currently capable of sampling only a fraction of library diversity, generated by randomizing, or greatly varying multiple residues of the API RCL, in optimized form it was sufficient to detect novel supra-active variants. Future improvements in the bacterial lysate approach would ensue if the number of candidates in a pool could be substantially increased, or if direct methods for detecting functional serpins in bacterial colonies could be developed.

Acknowledgements

We thank Dr. Murray Junop, Department of Biochemistry and Biomedical Sciences, McMaster University, Dr. Patricial Liaw, Department of Medicine, McMaster University, and Hong Liang, Facility Operator, McMaster Flow Cytometry Facility, for expert advice and helpful discussions.

5 SUMMARY & PERSPECTIVES

The principal aim of this thesis was to examine the functional consequences of expressing serpins, especially API M358R, as membrane proteins tethered to the surface of mammalian cells. In this novel location, the functionality of tethered API M358R and AT as inhibitors of exogenous thrombin was established. This finding suggested two testable hypotheses: that tethered API would inhibit thrombin generation in the more complex biological setting of plasma; and that functional tethered API variants could be selected from a population made up primarily of non-functional tethered API variants, for instance using flow cytometry. The first hypothesis was supported by studies in plasmacontaining systems, in which inhibition of endogenous thrombin generation was demonstrated by varying the ratio of tissue factor to tethered API. The second hypothesis was tested but not supported by studies in which a small plasmid library of API cDNAs hypervariable at residue 358 was transfected, cells were sorted on the basis of fluorescence related to their thrombin-binding capacity, and plasmid DNA was recovered and sequenced. Screening of several API variant libraries in a bacterial expression system, in contrast, led to the discovery of novel API variants that inhibit thrombin faster than API M358R.

This general discussion is subdivided into three sections. The first section describes how the mammalian cell displayed serpins API (M358R), and AT maintained inhibitory function toward thrombin; however, the double mutant (i.e., API (M358R/T345R)) as expected, and HCII did not. The second section expands on this characterization by showing that tethered API (M358R) was capable of inhibiting endogenous thrombin formation in human plasma systems when the level of TF expression was appropriately tempered. The third section describes the difficulty in achieving serpin RCL mutant candidate enrichment with mammalian cell display, and reports on the successful application of an alternate technique based on bacterial screening. The sectional investigations undertaken in this thesis are then unified in a discussion of potential future directions.

5.1 <u>Rationale for Investigation</u>

An original and persistent experimental direction in this work was the development of mammalian cell display of serpins in combination with fluorescence activated cell sorting (FACS) flow cytometry. If possible, the strategy was to capitalize on this technical advance to screen serpin RCL mutant libraries, and identify candidates with high affinity for coagulation proteases, including thrombin. This outcome would have had two applications: increased understanding of serpin structure and function; and the potential identification of lead candidates for novel antithrombotic protein drugs. Prior to the commencement of this thesis work, phage-display had been used to screen randomized RCL libraries of the serpin PAI-1 (591-593). During this work, phage display of API was described (594). However, difficulties in translating phage display findings to the humanized protein context have been noted, in part due to the lack of post-translational modifications in bacterial and bacteriophage systems (595). Moreover, while mammalian display has advantages over other techniques, it has only been applied toward the display

of immunoglobulin G-related libraries (466,474). Due to the uncertainty of displaying serpins on the surface of mammalian cells, it was essential to establish whether this strategy could lead to the expression of functional inhibitors, be amenable to RCL library screening, and to sorting by FACS flow cytometry.

5.2 Achieving Serpin Display on Mammalian Cells

Our strategy was to express serpins as single-pass type II membrane proteins (596) based on either the partial N-terminal asialoglycoprotein receptor (ASGPR 1, isoform H1a, or AR) (597) codons 3 to 69 (preceded by ATG GGA) or the partial N- terminal transferrin receptor (TR) (598) codons 3 to 98 (preceded by ATG GGA). The partial N-terminal segments of these two well characterized membrane proteins had been previously employed successfully to target passenger proteins to microsomes, which are vesicles derived from the endoplasmic reticulum (597,599). In their native settings, both sequences code for a 21 residue hydrophobic trans-membrane "signal-anchor" domain, responsible for protein insertion and anchoring, sandwiched between sequences for an Nterminal cytoplasmic domain, and a C-terminal extracellular domain (600). We reasoned that this orientation would be more likely to leave the protease, serpin, and the transfected cell and DNA encoding the serpin all linked together after serpin-enzyme complex formation than other geometries. The portion of the serpin polypeptide chain C-terminal to the reactive centre is not covalently linked to the rest of the protein after bond scission in API or HCII; in API it remains linked via a disulphide bridge. The alternative type I orientation would have required the engineering of both a cleavable N-terminal signal sequence and a separate membrane insertion signal, and at least for API and HCII, could have resulted in separation of the serpin-enzyme complex and the transfected cell after bond scission.

5.3 <u>Serpin Targeting to the Plasma Membrane</u>

As described in Chapter 2, several lines of evidence suggested that AR- or TR-fusion achieved its intended aim of localizing serpins to the plasma membrane. These included: the detection of API, AT, and HCII in the carbonate-insoluble, ultracentrifuge-pelleted sub-cellular fraction enriched in the Na+/K+-ATPase, a plasma membrane marker (601-603); the decreased electrophoretic mobility of the fused versus unfused serpins; and the accessibility of membrane-tethered API and AT to exogenous thrombin, demonstrated both by the formation of denaturation-resistant SEC and by the increase in immunofluorescent above background of cells exposed to thrombin and fluorescent anti-thrombin antibodies. Taken together, they indicated that the AR- or TR- strategy was successful in directing at least some of the expressed serpin to the cell surface as an integral membrane protein, with the serpin moeity oriented on the outside of the cell.

Could some of the expressed API, AT, or HCII have been misrouted to other subcellular locations? It should be noted that the subcellular fractionation protocol that was employed was designed for small numbers of cultured cells, and did not employ density-gradient centrifugation or other tools possible with the use of cells harvested through organ homogenization. This question was addressed in the most detail for API M358R.

Sonicates from HEK 293 or COS cells expressing AR-API-Pittsburgh or TR-API-Pittsburgh, were centrifuged to yield the cytoplasmic fraction, and a pellet subsequently incubated in highly alkaline sodium carbonate. Additional centrifugation of the carbonate-treated sample generated the peripheral (supernatant), and integral membrane (pellet) fractions. Cytosolic marker enzyme GAPDH (604) was distributed as expected. Plasma membrane marker Na^+/K^+ APTase (plasma membrane) (605) was enriched in the integral membrane fraction, but was also detected in the peripheral membrane fraction. This pattern matched the distribution of AR- and TR-API. The simplest explanation for these results is that our cytoplasmic fraction was free of contamination with membrane fractions, but the peripheral membrane fraction was partially contaminated with integral membrane proteins. The observed carry-over can be rationalized by the method sensitivity (602).

There was no discernible difference between the efficiency of AR- or TR- membranetargeting signals in experiments with AR-API and TR-API. The AR- signal was therefore arbitrarily chosen for fusion to AT and HCII cDNAs, and AR-API constructs were employed in the rest of the experimental work presented in Chapters 4 and 5.

Thrombin-challenged AR-API-expressing HEK 293 cells were probed with an antithrombin antibody detected by reaction with fluorescently tagged secondary antibodies as an alternate means of probing SEC formation. Non-transfected HEK 293 cells gave low but detectable fluorescent signals on subsequent flow cytometry, with a range of signals within the cell population. Cells expressing AR-API T345R/M358R double mutant (606) showed a right shift on fluorescence-activated cell sorting (FACS) profiles, as did cells expressing AR-API M358R, except that in the last case, the right shift was greater than in all other conditions. These results were consistent with SEC formation between thrombin and AR-API M358R, but also with lower affinity binding events. These could have included non-specific binding of either antibody to cell surfaces, thrombin binding to non-PAR-family receptors, and thrombin binding, but not SEC formation, with imperfectly folded AR-API M358R. Nevertheless, the cells with the strongest fluorescent signals, of those compared, were those expressing AR-API M358R. Accordingly, these favourable immunofluorescence data provided the requisite incentive for pursuing FACS as a potential method for screening and sorting serpin cDNA libraries.

5.4 <u>Measuring Inhibition Efficiency: Tethered vs. Soluble Serpins</u>

Satisfied that API-Pittsburgh displayed as an AR type II membrane protein retained some function, we sought to compare protease inhibition efficiency with its soluble recombinant counterpart. Kinetic studies were performed using equimolar amounts of the Pittsburgh variant displayed on the surface of 293 cells, or in soluble form mixed with non-transfected cells, and then incubated with protease. The results unequivocally showed that soluble API-M358R was the more efficient protease inhibitor; we estimated that the initial rate of thrombin inhibition was reduced by 7- to 10-fold by membrane tethering. This is likely because untethered API M358R is free to diffuse, increasing the probability of RCL alignment with the active site, a necessary precondition for serpins to

act as bait for proteases (306). Serpins and coagulation proteases are large protein molecules of ca. 400 amino acids (305), and their normal interaction (i.e., AT inhibiting thrombin) occurs in plasma, where both are free to diffuse, or on glycosaminoglycan chains of proteoglycans attached to cells, where both reactants are partially constrained but in a way likely to lead to productive interaction and inhibition.

How could engineered serpins be further tailored for cell surface expression? The performance of displayed serpins could be enhanced by extending the serpin outward from the cell surface to facilitate protease access at the RCL in a way that is analogous to previous studies reported with recombinant FX variants (505). This enhancement may be achievable by incorporating a "spacer" polypeptide domain immediately C-terminal to the signal-anchor domain. A number of possible domain candidates exist, based on the structure of the coagulation factors, including the EGF-like domain (present in TM, PS, FVII, FIX, FX, FXII, PC, tPA, and uPA). This domain is comprised of ca. 40 amino acids, in the form of two β -sheets, and stabilized by three intra-molecular disulphide bonds (607). Importantly, although the main function of the EGF-like domain is to facilitate protein-protein interactions, it has also been reported to function as a spacer for certain cell-surface proteins (608).

The concept of elevating the serpin above the membrane could also be applied to tethered AT, but not to tethered HCII. Unlike API and API M358R, AT and HCII require GAG interaction for both activation and ternary complex formation in order to achieve efficient plasma protease inhibition (321). GAG interaction at helix D (321) induces a conformational change that includes RCL extension resembling API, while contact with thrombin exosite II facilitates a bridging mechanism (289). In addition, activation of HCII involves N-terminal tail expulsion also from the helix D region (413), and with specificity for thrombin exosite I, it too promotes ternary complex formation (321). Consequently, there was never any certainty that AT or HCII, with far more complicated mechanisms of inhibition, could perform like AR-API.

Soluble AT inhibited thrombin compared to their respective displayed forms. Importantly, tethered AT maintained approximately equal activity regardless of whether heparin was included in thrombin inhibition assays. In the case of HCII, there was no compelling data to indicate that surface-displayed HCII retained any inhibitory activity. Neither of these findings was unexpected considering that unhindered access to these tethered serpins is paramount for optimal performance.

Extending AT outward from the cell surface by including single, or multiple EGF-like spacer domains may improve access to both the RCL, and to the helix D region, resulting in enhanced inhibition. However, improving HCII display poses some additional challenges. The requirement for tail release during GAG activation of HCII implies that "fixing" the N-terminal tail to the plasma membrane will almost certainly restrict proper serpin folding, activation, and overall function. Even the inclusion of multiple EGF-like

spacer domains would be unlikely to significantly improve tail dynamics and confer functionality on tethered HCII.

In Chapter 2, serpins were displayed on the surface of HEK 293 cells as type II membrane proteins with an outward-oriented C-terminal that facilitated RCL interactions with the serine protease active site to form SEC. Cell-displayed API-Pittsburgh and AT retained inhibitory function while HCII and the double mutant API (T345R/M358R) - as expected, did not. Collectively, these observations provided the incentive to explore the use of tethered serpins in a more physiological milieu. Work in Chapter 3 provided initial insights that could eventually be translated into a gene therapy scheme to modify the phenotype of diseased cells/tissue.

5.5 Inhibition of endogenous thrombin generation by AR-API M358R

The study presented in Chapter 2 described how the HEK 293-displayed AR-API M358R and AR-AT maintained thrombin inhibitory activity when exogenous, purified thrombin, was added to cells suspended in buffer solutions. It provided the incentive to explore whether tethered serpins would inhibit endogenous thrombin generation when transfected cells were suspended in recalcified plasma solutions, a precondition for any future exploitation as a gene therapy to modify the phenotype of diseased cells/tissue.

Preliminary experiments, performed to confirm the TF-dependent pro-coagulant activity (PCA) in WT T24/83 bladder carcinoma cells as a function of FXa generation, employed a discontinuous assay. This two-step assay used a protease-specific chromogenic substrate in combination with OD measurements, and had previously been used to measure TF PCA in monocytes (609), platelets (610), smooth muscle cells (611), T24/83 cells thrombin (612), epithelial cells (613), and microparticles from pancreatic cancer blood samples (614). Because optical means are used to detect thrombin activity, conditions must be employed that prevent fibrin polymerization, such as snake protease treatment of plasma to remove clottable fibrinogen.

We first established that WT T24/83 cells expressed abundant TF. We performed the assay to compare differences in thrombin generation between WT and AR-API M358R transfected T24/83 cells in culture. While no convincing differences were observed between the two profiles, the presence of SEC on immunoblots confirmed that surface-displayed AR-API M358R maintained some inhibitory activity against endogenously generated thrombin, as previously observed with exogenous thrombin (615). We initially rationalized that the assay simply lacked the sensitivity required for our analysis one of several limitations previously described (543). Consequently, a more sensitive fluorescence-based continuous thrombin generation assay (TGA) replaced the discontinuous assay.

TGA employs a fluorogenic substrate, and has been used to measure thrombin in human plasma (616,617), and hybrid EA.hy926 cells expressing TM and the PC receptor (618). It provides a thrombin concentration profile as a function of time throughout coagulation:

initiation (i.e., extrinsic coagulation; TF/FVIIa), propagation (i.e., intrinsic coagulation; FVIIIa/FIXa), and termination (i.e., AT and Fg to Fn) (245). Importantly, modeling the thrombin profile provides useful quantitative parameters: lag time, peak thrombin, peak time, velocity index (VI; thrombin generation slope), area under the curve (AUC), and endogenous thrombin potential (ETP; AUC x peak height x time to peak), making TGA a robust assay (246). At the time we employed it, no studies had reported on TGA performed with suspensions of TF-expressing mammalian cells.

Unexpectedly, there were no significant differences observed between the TGA profiles for WT T24/83, T24/83 expressing AR-API M358R or AR-API M358R/T345R negative control, in spite of our detection of SEC between AR-API M358R and endogenously generated thrombin. We postulated that the TF expression generated high levels of thrombin, such that the small amount of serpin-complexed thrombin did diminish this total significantly. To test this idea, we performed two additional experiments.

In the first experiment soluble hirudin, a highly specific and potent non-serpin thrombin inhibitor was included in the reaction mix. Hirudin, a 65 residue polypeptide (Mr=7kDa), is naturally produced by the medicinal leech *Hirudo medicinalis* (619) with a tertiary structure comprised of a compact hydrophobic N-terminus preserved by three disulphide bridges approximating a "figure 8", and a loose chain-like acidic C-terminus (620). The mechanism of thrombin inhibition differs dramatically compared to coagulation serpins. The N-terminus of hirudin forms a tight, irreversible, and non-covalent interaction with the active site without the requirement for GAG activation, while the C-terminus binds with exosite I (620,621). Moreover, hirudin is not cleaved by thrombin action (622).

In the second experiment, we replaced hirudin with soluble API-M358R in the reaction mix. The results revealed that complete thrombin inhibition required up to 1000 nM of either hirudin or soluble Pittsburgh, supporting our working hypothesis. Strictly speaking, it was essential to reduce the levels of TF expression in order to observe measureable tethered Pittsburgh inhibition by TGA. Consequently, HEK 293 cells were transiently transfected with plasmid coding for TF in order to "adjust" thrombin generation, and replace T24/83 cells in TGA. HEK 293 cells are derived from primary cultures of human embryonic kidney cells transformed by an adenovirus (467).

Importantly, immunoblot and TGA experiments confirmed that WT 293 cells did not possess TF-dependent PCA, and therefore would not contribute background thrombin. Co-transfection experiments were performed with TF:serpin plasmid DNA ratios of 1:10, and the AR-API M358R TGA profile was compared with the negative control AR-API M358R/T345R.

The TGA profiles clearly showed measureable differences. AR-API M358R-bearing cells (0.8 μ g TF/7.2 μ g serpin) displayed an overall depressed thrombin profile at all stages of coagulation: a reduced peak height; velocity index; and AUC relative to the double mutant (0.8 μ g TF/7.2 μ g serpin). Only the differences in serpin function can
rationalize these results (i.e., inhibitor vs. substrate); the potential for thrombin generation would have been the same because the co-transfection ratios were identical.

Recently, we reported that 293-displayed API (M358R), and AT maintain Thrombin inhibition activity, and hypothesized that the *de novo* expression of unique surface proteins, as part of a gene therapy scheme, could favourably alter a cells' phenotype (615). In the present study, we show by TGA that mammalian cells expressing surface TF generate thrombin profiles suppressed by the tethered Pittsburgh variant.

TF is a 263 residue, Mr = 46 kDa integral membrane glycoprotein that is normally localized to the adventitia (623,624). Upon exposure to circulating blood, TF rapidly binds FVIIa to form the extrinsic tenase complex (TF/FVIIa) initiating several reactions that culminate in the formation of thrombin: the key enzyme of coagulation. The physiological regulation of thrombin generation occurs directly by GAG activated serpins (i.e. AT), and indirectly by APC (41,166) and TFPI (42).

It was during the late 19th and early 20th centuries that clinicians first reported that thromboembolic disease (TED) was as an early sign of cancer development in patients (625-627). Over the years, support has grown for the notion that tumour cells activate local or systemic blood coagulation, which favours metastasis (628).

Currently, attention is being directed to the link between the over-expression of TF in tumour cells and tumour progression (560). Significantly, a recent study suggests that tumour cell-derived pro-coagulant TF-expressing micro-particles are released into circulation and may represent the mechanism responsible for the development of TED in cancer patients (629). In light of the current findings, it is tempting to propose that tethered serpins be used therapeutically to suppress extrinsic TF/FVIIa-dependent thrombin generation in cancer patients. Conceivably, displayed serpins could be part of a gene therapy protocol where plasmid DNA targeting a particular organ system (e.g. the pancreas) reduces TED and metastasis.

5.6 Mammalian Display Screening of Mutant RCL Libraries

Based on the results described in the chapters 2 and 3, we postulated that mammalian cells displaying the Pittsburgh variant could serve as a platform for screening large RCL mutant libraries by FACS/flow cytometry, providing the appropriate multiplicity, with the goal of identifying a better thrombin inhibitor.

Mammalian cell display evolved in response to the clinical requirement for high-affinity monoclonal antibodies (mAb), and is an important technical improvement over the use of phage display which is often restrictive due to inaccurate expression of human proteins (595). The successful affinity maturation of mAb was achieved with the powerful screening of cell displayed peptide libraries by fluorescence activated cell sorting (FACS) flow cytometry as recently described (466,474). Briefly, these studies detail the enrichment of single-chain variable fragment (scFv) libraries, fused to human platelet derived growth factor receptor (PDGFR), and transiently displayed on the surface of

mammalian cells. Ho *et al.*, (2006) describe how a single-sort of the top 0.1% of anti-CD22 scFv, expressed on the surface of human embryonic kidney (HEK 293) cells, resulted in the isolation of rare mutants with high affinity for CD22. In the approach by Beerli *et al.*, (2008), anti-nicotine scFv displayed on the surface of baby hamster kidney (BHK) cells, using the viral-based Sindbis mammalian expression system, were subjected to one-time sorting. The desirable scFv mutants were isolated then used to prepare recombinant human IgG, and/or Fab for *in vivo* evaluation in mice.

Although mammalian display is suitable for the development of improved mAb, it was uncertain whether the technique was applicable to other displayed randomized protein combinations, including serpin mutant RCL libraries. In preliminary experiments, we tested mammalian cell display with FACS flow cytometry by screening a modest RCL mutant library degenerate only at P1 rationalizing that successful sorting would generate the expected Arg candidate (i.e., the WT API (M358R)). Anticipating favourable P1 results, a second library degenerate at P7 to P1 (where P1 is always Arg) was prepared with the objective of identifying unique RCL mutant candidates with affinity for thrombin that was equal to or greater than WT. While we confirmed library degeneracy at P1, by applying PCR to plasmids extracted from 293 cells transiently expressing the library, we were unable to claim successful enrichment after repeated sorting attempts.

We concluded that mammalian display is not effective for screening serpin libraries, and that this is likely due to an important difference between the two protocols; the identification of high-affinity serpin RCL mutant candidates requires the addition of the protease thrombin prior to fluorescence staining, as opposed to non-enzymatic antigens in the antibody library screening. Thrombin is an important and well-characterized coagulation enzyme (17) with a number of non-haemostatic functions (25). These include mitogenic activity, although this is typically mediated via PAR family receptors missing from HEK 293 cells. Nevertheless, thrombin was observed to bind to nontransfected HEK 293 cells in our studies, albeit to a lesser extent than to transfected cells. Thrombin has some affinity for glycosaminoglycans and other charged molecules, and this could underlie our observation. Operationally, great care had to be exercised preparing the HEK 293 cells for flow cytometry following exposure to thrombin and antithrombin antibody, in that they tended to aggregate if not resuspended in 1% BSA/PBS prior to analysis.

While the exact nature of these interactions is unclear, studies that report on the origin and characterization of HEK 293 cells help to provide clues. HEK 293 were originally derived from human embryonic kidney cells (467). More recent studies, based on the analysis of mRNA, report that 293 cells have characteristics similar to neurons, and express a wide variety of protein receptors (469,630). Collectively, these observations suggest the possibility of non-specific thrombin interaction.

Another difference between the two protocols concerns the relative binding affinity between the weaker tethered serpin-enzyme compared to the more efficient Ab-Ag. This

is an important consideration because strong interactions translate into higher fluorescence signals; a greater difference in geometric mean between binders and nonbinders within a library population facilitates sorting. While we have semi-quantitatively established that tethered API M358R maintains function, it is probable that the binding affinity (K_A) for thrombin is several orders of magnitude less (e.g., API M358R and anhydrotrypsin ~ 10¹⁰ M⁻¹ (631)) than the mAb to Ag interactions (e.g., up to 10¹⁴ M⁻¹) (632) that occur during antibody library screening by FACS.

Overall, the combination of non-specific thrombin-cell surface interactions, and the weaker SEC may have combined to reduce the effective fluorescence signal leading to the lack of serpin RCL library enrichment we observed with mammalian display.

5.7 <u>Thrombin Capture Screening of Degenerate RCL Libraries</u>

The lack of HEK 293-displayed library enrichment combined with our desire to screen the degenerate P1 RCL library, and identify novel mutant combinations required that we resort to an alternate screening approach. To this end, we employed a protocol based on the bacterial expression system previously developed in our laboratory (633). Lysates, containing soluble serpin mutants, were prepared from individual colonies derived from bacterial cells transformed with plasmids coding for an API RCL library degenerate at P1, and then incubated in the presence of thrombin immobilized on the surface of micro-titer plates. Screening was performed employing a chromogenic substrate in combination with OD_{450} measurements, and was based on differences in optical density, where a greater intensity reflected a stronger interaction with thrombin. Although this manual technique limited the number of mutant colonies that could be screened at once, the implementation of a "kinetic stringency" based on a shorter substrate incubation time than was used in our original publication on the technique made it more robust.

As expected, the method identified the WT candidate (Arg) in several screened lysates (frequency of 5 in ~ 150 lysates), and encouragingly it detected the P1 mutant Lys as well (frequency of 1 in ~ 150 lysates). Arg and Lys are two of three basic amino acids (His being the other) characterized by relatively large hydrocarbon side-chains containing side chains that act as proton acceptors at physiological pH, and bestow these residues with an overall positive charge. The importance of a basic amino acid at P1 of the RCL is underscored by the presence of Arg in the API Pittsburgh variant, and in AT, the most relevant thrombin inhibitor in human plasma (373,634). It seems reasonable to suggest that P1 Arg (and Lys, His to a lesser extent) of the scissile peptide bond has preferentially evolved by providing optimal size and charge distribution; it promotes the precise interaction with Ser-195 (i.e., S1) in the thrombin active site during Michaelis complex formation (317).

The successful identification of both the Pittsburgh and API (M358K) mutants provided us with an incentive to compare measured rates of thrombin inhibition between all three variants with basic residues at P1: API (M358R), API (M358K), and API (M358H). The kinetic analysis revealed that the P1 Arg mutant is approximately two orders of magnitude more potent as a thrombin inhibitor ($k_2 \sim 10^5 \text{ M}^{-1} \text{sec}^{-1}$) compared to both Lys and His P1 mutants (both with $k_2 \sim 10^3 \text{ M}^{-1} \text{sec}^{-1}$).

Although the bacterial expression method limited our screening to a modest library due to multiplicity requirements, we were intrigued at the possibility of applying the technique to the more expansive P7 to P1 (always Arg) library. Interestingly, the method was able to identify mutant candidates (frequency: 5 of ~ 100 lysates screened) comprised of non-polar P2 residues (i.e., 2 x Pro, 2 x Leu, and Met) comparable to the Pro at P2 of the WT, API-(M358R), and to the API degenerate P2-P1 library screens recently reported by serpin phage display (594). Moreover, the lysate with Met at P2 (and Arg at P1) showed a relatively high optical density that was comparable to the WT. These observations confirm that although the flanking RCL residues (e.g., P4 to P3' excluding P1) are not directly involved in SEC formation, they still play an important ancillary role in directing the serpin interactions at the active site during inhibition (307).

Importantly, screening ~ 1000 colonies from the P7 to P1 (never Arg) library did not result in identifiable candidates above the background optical density, again confirming the importance of Arg at P1 position (data not shown).

The library was then modified to include only the repeated nucleotide sequence VNN (i.e. no Cys, Tyr, Trp or Phe) at P7 to P2, while maintaining P1 (Arg), in order to eliminate the possibility of displaying candidates with a truncated RCL. After screening ~ 1300 candidate colonies, the assay returned 7 mutants all with Pro at P2, supporting the findings from the P7 to P1 (always Arg) library screening. Interestingly, one mutant candidate TLSATPR, registered the largest kinetic response ($k_2 \sim 5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$), exceeding the API M358R by a factor of ~ 3 ($k_2 \sim 1.5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$), and surpassing the API P7-P3 mutant library candidates AAFVS, and DITMA previously identified by serpin phage display (594), all within the anticipated stoichiometry of inhibition (SI) range.

In this study, we displayed serpins with degenerate RCL libraries on the surface of mammalian cells, and then attempted to sort for mutants with the highest affinity for thrombin by employing FACS flow cytometry. Unfortunately, repeated screening attempts of the modest degenerate P1 library failed to provide the anticipated enrichment. had previously achieved affinity maturation While flow cvtometrv with immunoglobulins, we rationalized that thrombin addition interfered with successful enrichment; the protease contributed to non-specific binding, and excessive background resulting in sorted cell distributions that could not distinguish sufficiently between strong and weak binders for this purpose. Alternatively, we employed a thrombin capture technique based on a bacterial expression system where lysates containing soluble mutant serpin RCL libraries interacted with immobilized thrombin; variant candidates with the greater affinity presented with higher rates of change in optical density. Importantly, implementation of this approach resulted in the confirmation of previously reported trends as well as the identification of novel serpins with hyperactivity towards thrombin.

5.8 <u>Future directions</u>

Importantly, the work described in this dissertation affirmed the functional versatility of mammalian cell displayed API and AT; the inclusion of a non-cleavable N-terminal combined signal/stop transfer sequence (597) does not interfere with proper folding to the metastable conformation. This is significant because correct folding is crucial to the serpin inhibitory mechanism, and there was never any certainty that this would work. Under normal physiologic conditions, the biosynthesis of secreted serpin involves a spontaneous yet extraordinary folding mechanism to a thermodynamically unstable (i.e. metastable or stressed (S)) native conformation highlighted by the presence of a long, flexible, and mobile RCL (635). The process is initiated in the cytoplasm where the N-terminal signal sequence of the nascent polypeptide is recognized by a signal recognition particle (SRP) (636) which then binds the docking protein (DP) (637) located on the endoplasmic reticulum (ER). As the polypeptide chain grows it extends through the ER bilayer into the lumen where the signal sequence is cleaved, and the polypeptide undergoes additional modification (597).

Throughout the course of my PhD I have conducted numerous experiments and while many have been successful, some have yielded results that fell short of expectation. Occasionally, the findings were traceable to the technical limitations related to experimental design, and these were not always obvious in advance of proving a hypothesis. In the following, I briefly describe a few specialized recommendations that I think will likely improve future chances of success.

By far the biggest challenge to the research was the consistently low level of serpin expression and the adverse impact this had on both the sorting of API mutant libraries by FACS flow cytometry (chapter 4), and the inhibition of TGA thrombin generation by the API Pittsburgh variant (chapter 3). Both areas of the research would greatly benefit from enhanced expression. One powerful option might be to replace the existing plasmid transfection with the transduction of virus-based vectors. Viruses have evolved to efficiently infect an organism and take over the hosts replication machinery in order to propagate their own existence (638). Currently, vectors based on third generation of HIV-1-based lentiviruses have been engineered for maximum biosafety (639) are now commercially available for mammalian cell transduction (e.g., ViraPower™, Invitrogen). Lentivirus based vectors, compared to plasmids used with transfection, have the advantage that virtually any type of mammalian cell can be transduced (640,641) with higher efficiency, and can achieve improved levels of protein expression (642).

In chapter 2, I describe in detail another potential solution that I think should be used in combination with viral transduction. Briefly, it involves the use of spacer domains to raise the N-terminus of serpins above the cell surface to accommodate the dramatic mechanism of inhibition. While the approach has been successfully employed with FX (643), the number of domains required would have to be determined empirically by estimating the rate of protease inhibition on a per domain basis.

In chapter 4, I describe how 293 cells become "sticky" during the thrombin incubation protocol. I postulated that this could be due to the non-specific binding of thrombin to the PM of 293 cells. However, it may also be due to the pro-thrombin antibody cross-linking two or more cells prior to the addition of secondary antibody. Therefore, it may be possible to resolve this by using a single GFP-tagged thrombin FAb. Another option might be to investigate the use of buffer fortified with BSA and Ca⁺², or Mg⁺² in advance of thrombin addition to neutralize binding. Alternatively, I have added EDTA to stained cells suspended in BSA just prior to sorting by flow cytometry, and this appeared to help minimize "clumping" (unpublished observations). Clearly, more work needs to be done to understand how proteases (i.e. thrombin) interact non-specifically with mammalian cell surfaces.

Surprisingly, I had good success with the bacterial screening method where the secreted mutant serpin libraries were harvested, and then incubated in the presence of immobilized thrombin. Currently, the selection of transformed bacteria is due to antibiotic resistance. It may be possible to make the protocol even more robust by replacing/augmenting antibiotic resistance with a plasmid that constitutively expresses a protease lethal to bacteria (i.e. granzymes (644)).

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Feb 09, 2015

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