Elucidating the anticoagulant properties of the DNA aptamer HD1

A STUDY OF THE SPECIFICITY AND EFFECTIVENESS OF HD1, A THROMBIN-DIRECTED DNA APTAMER, AS AN INHIBITOR OF COAGULATION

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Abstract

The coagulation system is initiated when subendothelial tissue factor is exposed to circulating blood, and culminates in the production in the enzyme thrombin, which catalyzes clot formation. However, the failure of built-in controls to limit thrombin generation to sites of vascular damage, leads to pathological clot formation, termed thrombosis. Anticoagulants, such as heparin and warfarin, are used to prevent thrombosis; however, these can cause bleeding. As a result, there is a need for novel anticoagulants that limit clot formation without the risk of bleeding. DNA aptamers are small oligonucleotides designed to bind a specific target with high affinity. HD1 is a DNA aptamer that was developed by Griffin and Leung in 1993 to bind thrombin. The focus of this work was to better characterize the anticoagulant properties of HD1.

We demonstrate that HD1 binds a subdomain of exosite 1 that is fully developed in prothrombin, unlike other exosite 1-directed ligands, such as $Hir^{54\text{-}65}(SO_3^-)$ and fibrin, which do not bind prothrombin. As a result, HD1 binds prothrombin with high affinity and inhibits prothrombin activation by prothrombinase with an IC_{50} value of 115 nM. HD1 competes with fVa for binding to prothrombin. Based on its capacity to inhibit both thrombin and prothrombin, HD1 is more potent than $Hir^{54\text{-}65}(SO_3^-)$ at inhibiting standard plasma clotting assays, but not in the thrombin clotting time. Furthermore, HD1 inhibits thrombin generation by prolonging the lag time, reducing peak thrombin, and reducing the ETP to a greater extent than $Hir^{54\text{-}65}(SO_3^-)$. Based on thrombin generation assays conducted in cofactor-deficient plasma, we demonstrate that HD1 inhibits thrombin feedback activation of fV, but not fVIII, and that inhibition of prothrombinase accounts

for the reduction in both peak thrombin and prolongation of the lag time. HD1 is neutralized in buffer-based and plasma-based coagulation assays by an antisense oligonucleotide, antiHD1. AntiHD1 preferentially targets free HD1 because HD1 bound to either thrombin or prothrombin is protected from neutralization by antiHD1. Based on our data, HD1 may be a useful anticoagulant due to its capacity to inhibit both prothrombin and thrombin. Positioned at the junction of the intrinsic and extrinsic pathways prothrombin could be a useful target for aptamer development into anticoagulant applications.

Acknowledgements

My interest in science began when I was a child, when my parents bought me a small microscope. I spent hours in my bedroom dissecting bees, house flies, and looking at computer chips that my dad brought home from work. However, I was always more interested in those things constructed by nature, and not those designed by man. In elementary school I was fascinated by properties of water. The fact that the physical state of water could be altered depending on the kinetic energy applied to the system fascinated me. I believe that this was the point in my life where I started to think of things from a reductionists' point of view. The world is made up of small atoms, and the speed of their movement dictates their physical state. I was hooked!

Despite my apparent interest in science, I was not a very good student. As a result, my future as a scientist was never guaranteed. I almost failed grade 9 science class, just getting by with a 56% average, and was promoted to grade 10 general science class. However, long discussions between my parents and my science teacher resulted in me being promoted to grade 10 advanced science on an interim basis. I pulled up my socks, and in that year I achieved a 71% average in science class. I do not attribute this sudden improvement to my interest in science; this was already well-established. Instead it was my developing study habits that allowed me to improve my marks. I wholly credit my parents to seeing this potential in me, and pushing me to aspire to a higher level of education.

Now, during my final days as a student, I find myself reflecting on those who have guided me through graduate school. First and foremost I wish thank my supervisor

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To my bride, Lisa Nancekivell, you are the love of my life and the incarnation of all my dreams. I dedicate this thesis to you, and to the family that we will raise.

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Abbreviations:

ANOVA – analysis of variance

AntiHD1 – anti-sense oligonucleotide of HD1 (CCAACCACACCAACC)

APC – activated protein C

aPTT – activated partial thromboplastin time

b – biotin

C6PS – Phosphatidylserine variant with 6 carbon chain (soluble PS)

$CaCl_2$ – Calcium chloride

CAT – calibrated automated thrombography

Chz-Th – chromozym thrombin

Ct# - chymotrypsin numbering system

CTI – corn trypsin inhibitor

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

dEGR - 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone

DNA – deoxyribonucleic acid

ε – extinction coefficient

EDTA - ethylenediaminetetraacetic acid

ETP – endogenous thrombin potential

f- – fluorescein-

f - factor

F1 - prothrombin fragment 1

F2 - prothrombin fragment 2

FPRck or PPAck - D-Phe-Pro-Arg chloromethyl ketone

FITC - fluorescein isothiocyanate

fVdp/+fVa – defibrinated factor V-depleted plasma containing 10 nM fVa

fVIIIdp/+fVIIIa – defibrinated factor VIII-depleted plasma containing 1 nM fVIIIa

fVfVIIIdp/+fVa+fVIIIa – defibrinated factor V- and VIII-depleted plasma containing 10 nM fVa and 1 nM fVIIIa

Gla - γ-carboxylated glutamic acid

h - hours

HBS – 20 mM Hepes, pH 7.4, 140 mM NaCl

HCII – heparin cofactor 2

HD1 – thrombin exosite 1-binding aptamer (GGTTGGTGTGGTTGG)

HD22 – thrombin exosite 2-binding aptamer (AGTCCGTGGTGGTAGGGCAGGTTGG GGTGACT)

HD23 – a non-specific oligonucleotide (AGTCCGTAAAGCAGGTTAAAATGACT)

Hepes – N-[2-hydroxyethyl]piperazine-N'-[2-athanesulfonic acid]

$Hir^{54-65}(SO_3^-)$ - Tyr^{63}-sulfated COOH-terminal hirudin peptide amino acids 54-65

I_o – baseline fluorescence intensity

I – fluorescence intensity following injection

II – prothrombin

IIa – thrombin

IC_{50} - concentration at 50% inhibition

K^+ - potassium ion

k_1 – first-order rate constant of inhibition

k_2 – second-order rate constant of inhibition

k_{cat} – rate of substrate turn-over

K_D – Dissociation constant

kDa - kiloDalton

K_m – Michaelis-Menten constant

$MgCl_2$ – magnesium chloride

mIIa – meizothrombin

mIIa(-F1) - meizothrombin des F1

min – minutes

OD – optical density

PCPS - L-α-phosphatidyl-choline and L-α-phosphatidyl-L-serine

PEG – polyethylene glycol

PPP – platelet-poor plasma

pre1 - prethrombin 1

pre2 - prethrombin 2

PRP – platelet-rich plasma

PS – Phosphatidylserine

PT – prothrombin time

RB006 – factor IXa-directed RNA aptamer

RB007 – antisense oligonucleotide to RB006

Req - response units at equilibrium

RNA – ribonucleic Acid

rTAP – recombinant tick anticoagulant peptide

s - seconds

SA – streptavidin

SDS-PAGE – sodium doedecyl sulfate polyacrylamide electrophoresis

SEM – standard error of the mean

SPR - surface plasmon resonance

TAFI – thrombin-activatable fibrinolysis inhibitor

TBS – 20 mM Tris-HCl, pH 7.4, 140 mM NaCl

TF – tissue factor

TFPI – tissue factor pathway inhibitor

TGA – thrombin generation assay

TRIS – Tris-(hydroxymethyl)-aminomethane

V_H-Pro – a peptide derived from the variable domain of a prothrombin-binding antibody

WT – wild-type

Chapter 1: General Introduction

Overview of hemostasis

The hemostatic system has evolved to react to and repair damage to the circulatory system. In the simplest terms, the fluidity of blood must be maintained in order to properly distribute oxygen and nutrients to the various organs in the body for the purposes of cellular aerobic respiration and metabolism. Endothelial cells line the luminal face of blood vessels and maintain the blood in a fluid state because they express anticoagulant agents, such as thrombomodulin and heparin sulphate (Preissner, 1988). However, when the blood vessel is damaged, either by physical trauma or as a result of disease, the endothelial layer is perturbed allowing circulating blood to come in contact with the subendothelium (Mackman *et al.*, 2007). This procoagulant surface initiates a series of reactions involving cells and circulating enzyme precursors that culminate in the generation of the enzyme thrombin, which catalyzes the formation of a blood clot (Butenas and Mann, 2002). This blood clot protects the circulatory system from blood loss until the wound is repaired. Blood flow is then re-established as the damage to the blood vessel is healed by cellular repair mechanisms.

The traditional model of hemostasis describes two distinct but overlapping mechanisms: (a) accumulation of a platelet plug and (b) fibrin clot formation. In this model, coagulation was seen as a linear cascade of events culminating in the generation of thrombin and the formation of a blood clot. A recently proposed mechanism attempts to integrate knowledge about platelets and coagulation into a unified cell-based model of

hemostasis in which coagulation is viewed as a non-linear system of reactions (Roberts *et al.*, 2006) (Figure 1.1).

Following vascular damage, sub-endothelial collagen is exposed to circulating blood and induces platelet activation. Platelets are anucleate cells, whose membranes undergo a chemical and morphological change upon activation. Activated platelets release the contents of their α-granules and dense granules, express phosphatidylserine (PS) on their membrane surface and release membrane fragments called microparticles. These processes all contribute to the promotion of coagulation. The activated platelets adhere to the damaged vessel and aggregate to form an initial platelet plug (Roberts *et al.*, 2006, Walsh, 2004).

Along with collagen, subendothelial tissue factor is also exposed to circulating blood following vascular damage. Tissue factor binds the circulating activated factor VII (fVIIa) and localizes it to the subendothelial tissue, forming a complex called extrinsic tenase, which catalyzes the activation of zymogens fX and fIX. In the absence of essential cofactors, the resultant fXa slowly converts prothrombin into thrombin. This minute amount of thrombin serves to amplify the coagulation system by activating the procofactors fVIII and fV. Thus, fVIIIa and fIXa assemble on the surface of activated platelets to form the intrinsic tenase complex, which serves to more rapidly activate fX. Similarly, fVa and fXa assemble to form the prothrombinase complex, which rapidly converts prothrombin to thrombin. The formation of these macromolecular complexes results in a burst of thrombin generation (Roberts *et al.*, 2006).

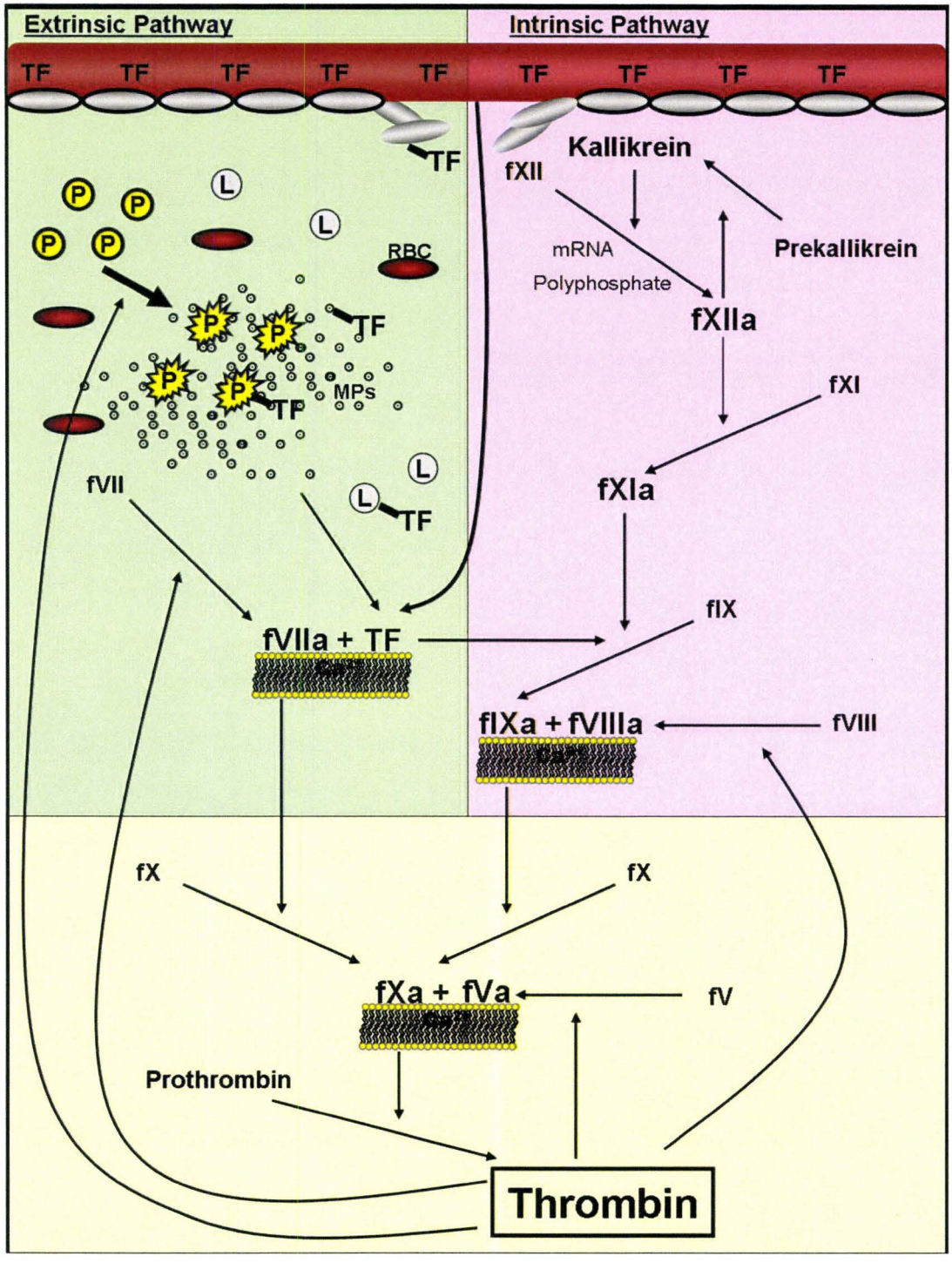

Extrinsic Pathway
Intrinsic Pathway
TF
TF
TF
TF
TF
TF
TF
TF
TF
TF
Kallikrein
fXII
mRNA
Polyphosphate
Prekallikrein
fXIIa
fXI
fXIa
RBC
MPs
fVII
fIX
fVIIa + TF
fIXa + fVIIIa
fVIII
fX
fX
fXa + fVa
fV
Prothrombin
Thrombin

Figure 1.1: Schematic of the coagulation system

Coagulation involves a series of steps that convert inactive zymogens into active serine proteases. Ultimately, the enzyme thrombin is produced that converts soluble fibrinogen into insoluble fibrin and activates platelets (P). Coagulation can be initiated by either the extrinsic (green box) or intrinsic (pink box) pathways. The extrinsic pathway is initiated by tissue factor (TF) from the vascular bed, leukocytes (L), activated platelets, or microparticles (MPs). The intrinsic pathway can be initiated upon release of mRNA or polyphosphate from damaged endothelial cells and platelets, respectively. The common pathway (yellow box) represents the convergence of the intrinsic and extrinsic pathways, which both generate factor (f) Xa that converts prothrombin into thrombin. Thrombin acts in positive feedback to generate the activated cofactors fVIIIa and fVa, which assemble into intrinsic tenase (with fIXa) and prothrombinase (with fXa) respectively on activated platelet surfaces in the presence of Ca^{2+}. Assembly of these activation complexes is critical to rapid thrombin generation.

Thrombin catalyzes the conversion of fibrinogen to fibrin, which polymerizes into an insoluble mesh-like network at the site of injury that helps to stabilize the platelet plug. In addition, the fibrin mesh traps cells, including red blood cells and white blood cells. As a result, a stable blood clot is a complex organization of insoluble fibrin supporting aggregated platelets and other hematopoietic cells (Roberts *et al.*, 2006). A more in-depth discussion of the enzymes and cofactors of the coagulation system, and their regulation, will follow below.

Coagulation System

Coagulation can be stimulated extrinsically (by the exposure of extravascular tissue factor), or intrinsically (by autoactivation of circulating fXII). Although the distinction between intrinsic and extrinsic pathways is somewhat archaic, it is useful for organizing a discussion of the coagulation system.

The rapid response of the coagulation system to vascular injury is in part due to the assembly of enzyme and cofactor complexes on activated membrane surfaces. Cell membranes are typically composed of an asymmetrical phospholipid bilayer. The outer-leaflet of the membrane contains phosphatidylcholine (PC) and sphingomyelin, whereas the inner-leaflet of the membrane contains negatively charged phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) (reviewed by (Devaux *et al.*, 2008)). However, following activation, platelets and endothelial cells lose this phospholipid asymmetry and expose PS and PE on their outer surfaces. Many of the enzymes of the coagulation system contain a domain that mediates binding to PS-

containing membranes in a Ca^{2+}-dependent manner. This domain is composed of glutamic acid residues that have been γ-carboxylated in a vitamin K-dependent reaction, forming the so called Gla-domain. A recent crystal structure has demonstrated that a Ca^{2+}-induced conformational change in the Gla-domain exposes hydrophobic residues that integrate into the membrane bilayer (Huang *et al.*, 2003). In general, *in vitro* buffer-based assays used to study coagulation reactions are designed to try to recapitulate the coagulation response that occurs *in vivo*. Therefore small vesicles composed of PC and PS (called PCPS vesicles) are included in these assays to act as a surrogate activated cell membrane, to aid in the assembly of the activation complexes.

The extrinsic tenase complex

The extrinsic tenase complex is widely acknowledged as the initial activator of the coagulation system. This complex forms when damage to the endothelium, or activation of leukocytes, exposes membrane-bound tissue factor to circulating fVIIa. Tissue factor and fVIIa combine on an anionic membrane. Extrinsic tenase activates fX and fIX, thereby initiating thrombin generation.

Tissue factor

Tissue factor (factor III) is a 43 kDa transmembrane glycoprotein that is expressed or exposed at sites of vascular injury. Tissue factor is present in the subendothelial layers of blood vessels, and in hematopoietic cells, such as platelets and leukocytes. Vascular wall tissue factor is thought to be the main contributor to clot and thrombus formation in vivo (Wang *et al.*, 2009a), whereas hematopoietic cell-derived

tissue factor may serve as a mobile pool of tissue factor that supplements clot formation or growth (Chou *et al.*, 2004, Hrachovinova *et al.*, 2003). Recent evidence suggests a role for circulating tissue factor in thrombus formation, although the extent of this contribution is controversial and likely depends upon the extent of vascular damage.

Deletion of the tissue factor gene in mice is embryonic lethal, highlighting the importance of tissue factor for survival (Toomey *et al.*, 1996). As the initiator of the coagulation system, tissue factor acts as a cofactor for fVIIa to catalyze the activation of fX and fIX. TF binds fVIIa and induces a conformational change in the enzyme that results in maturation of (a) the active site (Persson *et al.*, 2001), and (b) a macromolecular substrate binding exosite that is distinct from the active site and endows fVIIa with its substrate specificity (Baugh *et al.*, 2000). Thus, when fVIIa binds to TF, the rate of fIX and fX activation is accelerated 1000-fold.

In order to maintain fluidity in the intact vascular system, circulating tissue factor is suspected to exist in an inactive or encrypted state. De-encryption of tissue factor may involve the formation of a disulfide bond between Cys^{186} and Cys^{209}, resulting in the maturation of the fVIIa binding site on tissue factor (Chen *et al.*, 2006). Recently, protein disulfide isomerase (PDI) has been identified as a potential physiological activator of encrypted tissue factor (Cho *et al.*, 2008a, Reinhardt *et al.*, 2008). Other possible mechanisms for the de-encryption of tissue factor include: (a) tissue factor dimerization (Donate *et al.*, 2000), and (b) exposure of PS membranes (Wolberg *et al.*, 1999).

Factor VII

FVII, originally termed proconvertin, is a 50 kDa Gla-containing protein that is synthesized in liver. It circulates in plasma at a concentration of 10 nM. Activation of single chain fVII occurs by the limited proteolysis of the Arg^{152}-Ile^{153} peptide bond, resulting in the formation of two-chain fVIIa. FVII can be activated either by autoactivation by fVIIa itself (Nakagaki *et al.*, 1991), by RNA (Nakazawa *et al.*, 2005) or by other coagulation proteases, including fXa (Bajaj *et al.*, 1981, Wildgoose and Kisiel, 1989), fIXa (Wildgoose and Kisiel, 1989), or thrombin (Broze, Jr. and Majerus, 1980). Although there is substantial evidence of a dedicated fVII-activating protease, its role in coagulation is still debated (Kanse *et al.*, 2008). Positive feedback in the activation of fVII occurs when fVII binds to tissue factor and is activated by fXa (Rao and Rapaport, 1988). However, studies by Butenas *et al* demonstrate that thrombin is the most physiologically relevant activator of fVII (Butenas *et al.*, 1997), suggested that activation of fVII occurs after clot formation. Additional fVIIa is thought to be generated so that the coagulation system can respond to subsequent vascular injuries. FVIIa activates fX and fIX slowly when it is not bound to tissue factor. In the presence of tissue factor, however, the rate of fX activation by fVIIa is enhanced over 1000-fold (Chen *et al.*, 2006). Although deletion of the fVII gene is not embryonic lethal in mice, most die prematurely because of severe perinatal bleeding (Rosen *et al.*, 1997).

Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) is a 40 kDa single chain polypeptide that contains three Kunitz-type proteinase inhibitor domains. TFPI is synthesized in

endothelial cells and functions to regulate the extrinsic pathway of coagulation by inhibiting the tissue factor/fVIIa complex (Ameri *et al.*, 1992). Upon stimulation by thrombin, TFPI is released from endothelial cells, but may remain localized to the vascular wall because of its capacity to bind glycosaminoglycans (Iversen *et al.*, 1996). TFPI reversibly binds to fXa, and the TFPI/fXa complex then inhibits fVIIa bound to tissue factor, thereby forming a quaternary complex. Like the tissue factor gene, deletion of TFPI is embryonic lethal, suggesting that regulation of tissue factor activity is essential for development (Huang *et al.*, 1997). TFPI is an important inhibitor of the coagulation system because it limits clot formation to regions of massive tissue factor exposure caused by sufficient vascular damage (Butenas *et al.*, 1997). Thus, TFPI contributes to the so-called threshold theory of coagulation (van't Veer and Mann, 1997). This theory states that TFPI can control coagulation in the presence of low tissue factor exposure because the rate of activation is slower than the rate of inhibition by TFPI and other serine protease inhibitors (van't Veer and Mann, 1997). However, once thrombin generation has reached a "threshold" value, TFPI cannot effectively down-regulate coagulation, and the typical burst of thrombin generation proceeds.

Contact system

The contact system of coagulation is initiated when blood or plasma comes into contact with a negatively charged surface. Initially, this system was only thought to be relevant *ex vivo* when blood contacted glass, plastic, or medical devices, such as stents or catheters (Blat and Seiffert, 2008, Luscher *et al.*, 2007, Pai and Crowther, 2007). However, recent findings suggest that the contact system plays an important role in thrombosis (Kannemeier *et al.*, 2007).

The components of the contact system include fXII, prekallikrein and high molecular weight kininogen. Contact of fXII with negatively charged surfaces results in its autoactivation to fXIIa (Citarella *et al.*, 1997). FXIIa can amplify its own generation by feedback activation of fXII. In addition, fXIIa converts prekallikrein to kallikrein and kallikrein also can feed back to activate fXII. FXIIa initiates coagulation by activating fXI. FXIa activates fIX and the resultant fIXa then binds to fVIIIa to form the intrinsic tenase complex, which can generate fXa of the prothrombinase complex.

The importance of the contact factors to hemostasis was questioned because deficiencies of fXII, prekallikrein or high molecular weight kininogen are not associated with a bleeding diathesis (Kitchens, 2002). However, recent evidence reveals a physiologically relevant role for fXIIa in thrombosis (reviewed in (Renne and Gailani, 2007)). In 2007, Kannemeier *et al* demonstrated that free RNA released from damaged or necrotic endothelial cells contributed to the growth of arterial thrombi in mice, and that administering RNAse significantly delayed occlusive thrombus formation (Kannemeier *et al.*, 2007). Furthermore, RNA was found to bind fXII with high affinity and to accelerate

its autoactivation in vitro (Kannemeier *et al.*, 2007). Thus, extracellular RNA represents a novel signal for the initiation of blood coagulation at sites of extensive cellular damage. This mechanism may be relevant in thrombotic diseases such as cancer and sepsis, where cellular debris can be exposed to circulating blood (Kopreski *et al.*, 1999, Saukkonen *et al.*, 2008). Recently, polyphosphate has also been implicated as an activator of the contact system (Smith *et al.*, 2006). Since polyphosphates are found within the dense granules of platelets (Ruiz *et al.*, 2004), they may be released upon platelet activation and contribute to thrombosis and hemostasis.

The intrinsic tenase complex

The intrinsic tenase complex is composed of the enzyme fIXa and its cofactor fVIIIa assembled on an anionic membrane surface in the presence of Ca^{2+}. Intrinsic tenase catalyzes the conversion of fX to fXa at a rate that is 10^6-fold more efficient than that of fIXa alone (Duffy and Lollar, 1992). Computer modelling of the coagulation system suggests that fX activation by intrinsic tenase is the rate limiting step for robust thrombin generation (Lawson *et al.*, 1994, Rand *et al.*, 1996). Because fX activation by extrinsic tenase is inhibited by TFPI once fXa accumulates, intrinsic tenase is required to supplement fXa generation. Thus, deficiencies of fIX or fVIII lead to hemophilia B and hemophilia A, respectively. Furthermore, the importance of intrinsic tenase to coagulation is shown by the development of novel anticoagulants that specifically target fIXa (Chan *et al.*, 2008b, Dyke *et al.*, 2006, Nimjee *et al.*, 2006, Rusconi *et al.*, 2002).

Factor IX/IXa

FIX, also known as Christmas factor, is a 56 kDa protein that circulates in plasma at a concentration of 90 nM (Thompson and Chen, 1993). FIX activation is achieved by proteolysis of two peptide bonds, at Arg^{145} and Arg^{180}, which releases an 11 kDa activation peptide. Initial cleavage at Arg^{145} generates the intermediate fIXα, which has reduced procoagulant activity compared with the fully active fIXa (also called fIXaβ) (Lawson and Mann, 1991). Conversion of fIX to fIXa can be catalyzed by either intrinsic or extrinsic components.

Activation

TF/fVIIa activates fIX with a 10-fold lower catalytic efficiency than it activates fX owing to differences in the respective k_{cat} values (Zur and Nemerson, 1980). Recent work has demonstrated that the Gla and epidermal growth factor (EGF)-1 domains of fIX interact more weakly with TF/fVIIa than do the corresponding domains of fX, providing a structural explanation for differences in the rates of activation of these zymogens by extrinsic tenase (Ndonwi *et al.*, 2007). Because extrinsic tenase is an inefficient activator of fIX, a second activator is required. This comes from the intrinsic pathway. Thus, FXIa generated via the contact system also activates fIX. However, because fXIa does not possess a Gla-domain, it activates fIX in a membrane-independent fashion (Mannhalter *et al.*, 1984). Instead of a membrane surface, fIX initially binds to an exosite on fXIa prior to gaining access to the active site of the enzyme. This exosite is localized to both the heavy and light chains of fXIa (Ogawa *et al.*, 2005) (Sinha *et al.*, 2007), and binding of fIX to this exosite promotes its activation by lowering the K_m.

Factor VIII/VIIIa

Factor VIII (fVIII) is a 330 kDa glycoprotein that circulates in plasma at a concentration of 300 pM. The primary sequence of fVIII shares homology with fV and ceruloplasmin, a copper-binding plasma protein. The linear domain organization of fV and fVIII is identical, and consists of a heavy chain (A1-A2 subunits) and a light chain (A3-C1-C2 subunits) separated by an intervening B domain. Although fVIII is synthesized as a single chain molecule in endothelial cells, it circulates in tight association with von Willebrand factor where it exists as a heterodimer formed by limited proteolysis between the B-A3 domains; cleavages effected by an intracellular protease (Saenko *et al.*, 1999). Von Willebrand factor stabilizes fVIII in the circulation (Butenas *et al.*, 2009). Although, thrombin and fXa activate fVIII at similar rates (Monkovic and Tracy, 1990, Neuenschwander and Jesty, 1988), the 10-fold higher plasma concentration of prothrombin over fX suggests that thrombin is the more physiologically relevant activator (Butenas *et al.*, 1997).

Activation

Factor VIII is converted to fVIIIa by removal of the B domain and separation of the A1 and A2 domains. Thrombin cleaves fVIII within the heavy chain at Arg^{372} between the A1-A2 subunits and at Arg^{740} which separates A2 from the B domain (Figure 1.2). This results in the formation of the 50 kDa A1 and 40 kDa A2 subunits of fVIIIa, while the B domain is degraded into small peptide fragments. The cleavage at Arg^{372} is responsible for exposing the fIXa binding site within the A2 domain that is necessary for proper cofactor activity (Fay *et al.*, 2001). The light chain of fVIII is cleaved at Arg^{1689}

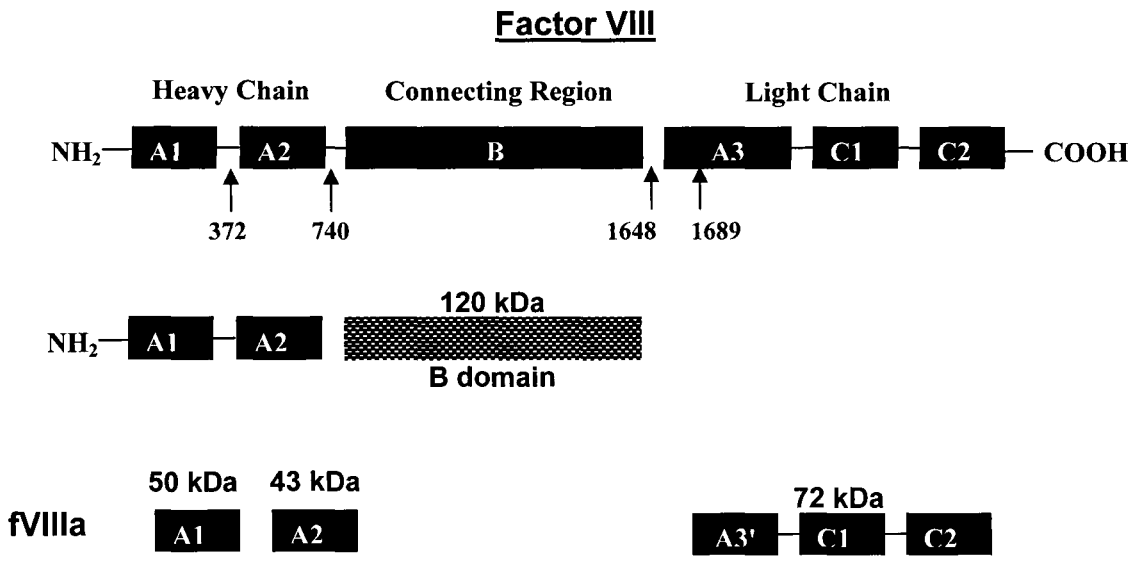

Factor VIII
Heavy Chain
Connecting Region
Light Chain
NH2
A1
A2
B
A3
C1
C2
COOH
372
740
1648
1689
120 kDa
NH2
A1
A2
B domain
50 kDa
43 kDa
fVIIIa
A1
A2
72 kDa
A3'
C1
C2

Figure 1.2: Models of the structure of factor VIII and factor VIIIa

The domain structures of fVIII and fVIIIa are shown with the heavy and light chains and the connecting region identified. The A, B, and C domains that make up the chains are indicated. Sites of cleavage by thrombin are identified by the amino acid residue numbers. The shading of the B-domain after initial proteolysis by thrombin reflects its digestion into small fragments.

in the A3 subunit, releasing a ~40 residue acidic peptide and the A3-C1-C3 subunit. Of these cleavages, proteolysis at Arg^{372} and Arg^{1689} appears to be most important for cofactor activity, although the latter is responsible for releasing fVIII from von Willebrand factor and, therefore, may not contribute to cofactor activity *in vitro* (Pittman and Kaufman, 1988).

Recent work has focussed on delineation of the order of cleavages during fVIII activation by thrombin. Thus, thrombin initially cleaves fVIII at Arg^{740}, which facilitates subsequent cleavages at Arg^{372} and Arg^{1689} (Newell and Fay, 2007). However, cleavage at Arg^{1689} facilitates the cleavage at Arg^{372} (Newell and Fay, 2009). FVIII cofactor activity is not fully developed until the final cleavage at Arg^{372}, which exposes the fIXa binding site on the A2 domain.

Assembly of intrinsic tenase

As mentioned, the presence of a negatively charged membrane surfaces is essential for the assembly of the intrinsic tenase complex and the subsequent conversion of fX to fXa. However, fIXa is a poor activator of fX, even in the presence of PCPS vesicles and calcium, despite a reasonably low K_m value of 100 nM that is comparable with the plasma fX concentration of 130 nM (Fribourg *et al.*, 2006). In the presence of fVIIIa, there is a 3000-fold increase in the k_{cat} for fX activation by fIXa, and a modest 2-fold reduction in the K_m. Consequently, the cofactor activity of fVIIIa is primarily required to optimize the cleavage of fX, and not the binding of fX to fIXa. Previous experiments have shown that the fluorescence anisotropy and the changes in fluorescence intensity of fluorescein-Glu-Gly-Arg-fIXa in the presence of fVIIIa are indicative of a

conformation change at the active site of fIXa (Duffy *et al.*, 1992). Thus, fVIIIa binds to fIXa and induces a conformational change at its active site, which drastically enhances the rate of fX activation.

A computer-generated three dimensional model of the fIXa/fVIIIa complex has been developed to predict and validate experimental data (Autin *et al.*, 2005). This model confirms experimental data and shows that (a) the Gla domain of fIXa interacts with the light chain of fVIIIa (Blostein *et al.*, 2003), (b) the heparin binding site of fIXa interacts with the A2 domain of fVIIIa (Yuan *et al.*, 2005), and (c) regions of fVIIIa that do not bind fIXa (Chang *et al.*, 2003) are absent at the interface between fIXa and fVIIIa (Autin *et al.*, 2005). In addition, this model confirms fluorescence resonance energy transfer (FRET) experiments, using dansyl-Glu-Gly-Arg-fIXa and rhodamine-labelled PCPS vesicles, that the catalytic domain of fIXa is situated ~85 Å above the surface of the membrane, even in the presence of fVIIIa (Mutucumarana *et al.*, 1992). The position of the active site of fIXa within intrinsic tenase is optimized to interact with the protease-sensitive sites on fX that lead to its conversion into fXa.

Stability

Once assembled, the activity of intrinsic tenase is primarily controlled by the instability of fVIIIa; a "self-dampening" process. In fact, the A2-subunit rapidly dissociates from fVIIIa because of charge repulsion between the A2 and A3 subunits (Wakabayashi *et al.*, 2008), which results in a 90-fold reduction in the k_{cat} of fX activation (Fay and Koshibu, 1998). When fVIIIa is assembled within intrinsic tenase, the rate of A2 dissociation is reduced 10-fold (Fay *et al.*, 1996).

Attempts to increase the stability of fVIIIa focussed on addition of a disulfide linkage between the A2 and A3 domains. This resulted in a 5-fold prolongation of the half-life of fVIIIa in whole blood clotting assays and similar activity compared to wild-type (WT) fVIIIa (Radtke *et al.*, 2007). The authors hypothesized that this more stable fVIIIa variant would have advantages over currently available recombinant fVIII that is used for treatment of patients with hemophilia A.

In addition to A2-dissociation, the activity of intrinsic tenase also is controlled by APC-catalyzed inactivation of fVIIIa as well as inhibition of fIXa by antithrombin, reactions that will be discussed in detail below.

The prothrombinase complex

The prothrombinase complex consists of the enzyme fXa and its cofactor fVa assembled on an anionic phopholipid membrane in the presence of Ca^{2+}. Its role is to convert the zymogen prothrombin into the serine protease thrombin, which is the final effector of the coagulation system.

Prothrombinase is the cornerstone of the common pathway of coagulation because fX is positioned at the convergence of the extrinsic and intrinsic pathways, and both pathways lead to its activation. Because of its central role in coagulation, bleeding disorders resulting from deficiencies in the components of prothrombinase are relatively uncommon. For example, parahemophilia, which is due to congenital fV deficiency, is characterized by mild to severe bleeding. Mice completely lacking fV (FV-/-) die during embryogenesis (Yang *et al.*, 2000), unlike fVIII-/- mice which are fully viable (Bi *et al.*, 1995), validating the importance of prothrombinase for life.

Factor X/Xa

FX, also known as thrombokinase, is a 59 kDa Gla-containing glycoprotein that circulates in plasma at a concentration of 170 nM. Following synthesis in the liver, fX is secreted as a 2 chain polypeptide linked by a disulfide bond, and circulates with a half-life of 36 h (Biggs and Denson, 1963). FX can be activated by either intrinsic or extrinsic tenase. FXa converts prothrombin into thrombin, activates fVII (Wildgoose and Kisiel, 1989), and activates fV (Monkovic and Tracy, 1990) and fVIII (Lollar *et al.*, 1985). FXa possesses a Gla domain, which, in the presence of Ca^{2+}, allows it to bind to PS-containing membranes with a K_D value of 15 nM (Stenflo, 1999). FXa interacts with its cofactor, fVa, with a K_D value of 30 nM (Camire *et al.*, 1998). FXa also possesses two endothelial growth factor (EGF) domains that contribute to its role in cell-signalling, and may contribute to fXa binding to prothrombin (Kittur *et al.*, 2004).

Activation

Activation of fX is achieved when either TF/fVIIa or fIXa/fVIIIa cleaves fX at Arg^{194}, which releases an activation fragment from the protease domain. The activated protease domain remains tethered to the Gla domain, thereby allowing fXa to interact with PS-exposed membranes (Camire *et al.*, 1998, Wang *et al.*, 2003).

Factor V/Va

FV is a 330 kDa glycoprotein that is synthesized in the liver and is present in circulation at a concentration of 30 nM (Tracy *et al.*, 1982). It shares sequence homology with fVIII and ceruloplasmin, and has a domain structure identical to that of fVIII; A1-A2-B-A3-C1-C2. Even though it does not contain a Gla domain, fVa binds to anionic

phospholipid membranes with a K_D value in the range of 0.01-1 nM, (Bloom *et al.*, 1979, van de *et al.*, 1983). The membrane-binding portion of fVa appears to be localized to hydrophobic residues in the C1 and C2 domains (Peng *et al.*, 2005), consistent with the proposed interface of fVa and the membrane surface (Stoilova-McPhie *et al.*, 2008). In addition to binding fXa and membranes, fVa also binds prothrombin with a K_D value of 1 μM (Mertens *et al.*, 1999). The importance of prothrombin/fVa interaction to prothrombinase activity is one of the topics addressed in this thesis.

FV is also found in the α-granules of platelets (Tracy *et al.*, 1982). Recent evidence suggests that megakaryocytes, the cells from which platelets are derived, take up plasma fV by endocytosis prior to their differentiation into platelets (Gould *et al.*, 2005). Although the exact role of platelet-derived fV is controversial, it may contribute to thrombus growth in a manner similar to platelet-derived tissue factor. Regardless of the source of fVa, its role within prothrombinase has been extensively studied and is well-defined.

Activation

Thrombin first cleaves fV at Arg^{709}, which liberates the A1-A2 heavy chain from the B domain. This is followed by proteolysis at Arg^{1018} within the B domain (Figure 1.3). Cleavage at Arg^{1018} may disrupt an inhibitory sequence within the B-domain that masks its cofactor activity (Zhu *et al.*, 2007). Finally, cleavage at Arg^{1545}, which releases the remaining portion of the B domain from the A3 subunit of the light chain, is the most important for expression of cofactor activity (Bloom *et al.*, 1979, Kalafatis *et al.*, 2003).

The activation of fV by thrombin results in a 50-fold increase in its affinity for fXa, likely reflecting exposure of an extensive fXa-binding site on the A2 domain (Gale *et al.*, 2007, Steen and Dahlback, 2002). Recently, Zhu *et al* constructed a series of B-domain deletion mutants of fV in an attempt to identify sequences that may influence cofactor activity. These studies identified a region between residues 902 and 1033 of the B-domain that contains an inhibitory sequence that blocks fXa binding to fV (Zhu *et al.*, 2007). These findings confirm that removal of the auto-inhibitory B-domain is a major mechanism for the expression of fVa cofactor activity.

Recently, the isolated C2 domain of fV was found to inhibit all thrombin catalyzed cleavages of fV, whereas the C2 domain of fVIII did not (Suzuki *et al.*, 2006). These results suggest that the C2 domain of fV (a) is a major binding site for thrombin, and (b) binds to thrombin at a site that is distinct from where the C2 domain of fVIII binds.

Stability

Unlike fVIIIa, the A1 and A2 subunits of the fVa heavy chain remain covalently attached. Consequently, regulation of fVa cofactor activity does not occur by the dissociation of the A2 subunit, as described for the regulation of fVIIIa cofactor activity. Instead, regulation of prothrombinase activity is achieved primarily through fVa inactivation by activated protein C (Heeb *et al.*, 1993, van der Neut *et al.*, 2004) and inhibition of fXa by antithrombin (Brufatto *et al.*, 2003).

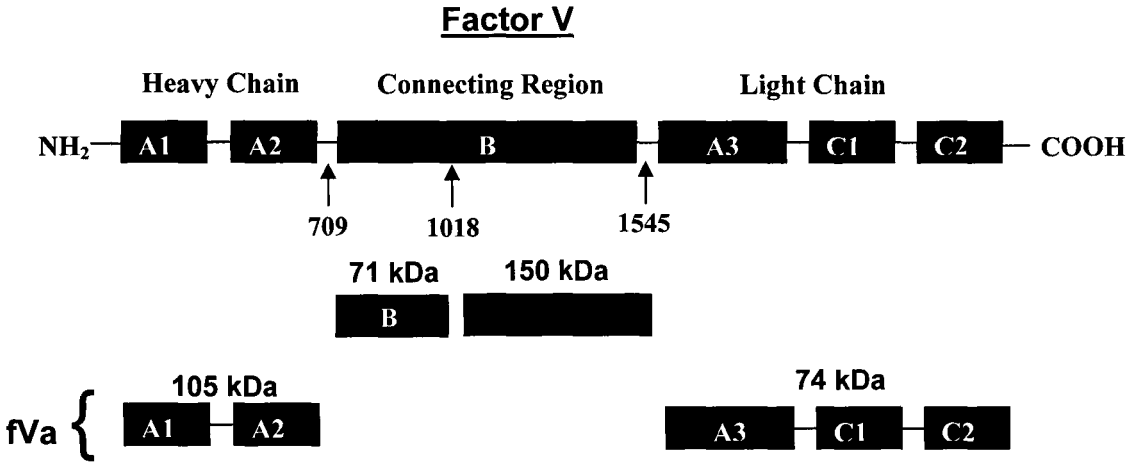

Factor V
Heavy Chain
Connecting Region
Light Chain
NH2
A1
A2
B
A3
C1
C2
COOH
709
1018
1545
71 kDa
150 kDa
B
fVa
105 kDa
A1
A2
74 kDa
A3
C1
C2

Figure 1.3: Models of factor V and Va

The domain structures of fV and fVa are shown with the heavy and light chains and the connecting region identified. The A, B, and C domains that make up the chains are indicated. Sites of cleavage by thrombin are identified by the amino acid residue numbers.

Prothrombinase assembly

Structural models for the prothrombinase complex have been independently proposed by two groups (Autin *et al.*, 2006, Lee *et al.*, 2008). These models have been useful for validating and predicting data from in vitro experiments that question the role of each component of prothrombinase to prothrombin activation.

Role of fVa

To address the role of fVa in prothrombinase, Rosing *et al* performed a kinetic analysis of prothrombin activation with different components of the complex. These studies demonstrated that PS-membranes heighten the affinity of prothrombin for fXa by lowering the K_m by over 2000-fold with only a moderate 4-fold increase in k_{cat} (Rosing *et al.*, 1980). When fVa is present with fXa and PS-membranes, there is an approximately 600-fold decrease in K_m and a 3000-fold increase in k_{cat} compared with the kinetic parameters for prothrombin activation by fXa alone (Nesheim *et al.*, 1979). Taken together, these findings suggest that fVa primarily affects the k_{cat}, whereas PS-membranes increase the affinity of the components for complex assembly (a K_m effect). Thus, it is hypothesized that fVa exerts its role by altering the active site of fXa and that this allosteric effect optimizes enzymatic turnover.

This model has been tested in a number of ways. Using a variant of tick anticoagulant peptide (TAP), a potent and specific inhibitor of fXa, Betz *et al* demonstrated a ~4000-fold higher affinity for fVa-bound fXa compared with fXa alone (Betz *et al.*, 1997). From this it has been proposed that fVa promotes exposure of a prothrombin-binding exosite on fXa (Krishnaswamy and Betz, 1997, Orcutt *et al.*, 2002).

The existence of this fXa exosite is still controversial because it has yet to be directly identified.

In addition to binding fXa, fVa also interacts extensively with prothrombin. Thus, fVa binds prothrombin via three domains: (a) fragment 2 (Krishnaswamy and Walker, 1997), (b) proexosite 1 (Anderson *et al.*, 2000b), and (c) the Gla domain (Blostein *et al.*, 2000). The importance of these interactions to the regulation of prothrombin activation by prothrombinase has not been widely studied, and is a major topic covered in this thesis.

Role of PCPS

Previous studies have suggested that PS-membranes provide a surface onto which the activation complexes assemble, thereby reducing the dimensionality of the reactions and leading to a reduction in the K_m. However, the functional contribution of PS-membranes to prothrombin activation has been the subject of much debate, especially in relation to the pathway of prothrombin activation. Although exposure of anionic phospholipids, such as PS, is required for prothrombinase assembly, recent evidence challenges the concept that a membrane surface is necessary. Majumder *et al* demonstrated effective assembly of fXa and fVa (K_D = 0.6 nM) in the presence of soluble dicapropyl-phosphatidylserine (C6PS) at concentrations well below those needed for micelle formation (Majumder *et al.*, 2005). The rate and pathway of activation were comparable to those obtained in the presence of PS-membranes (Majumder *et al.*, 2005, Rosing *et al.*, 1980). Whether prothrombinase can assemble on soluble PS as well as PS-expressing membranes remains controversial; most experts continue to believe that

assembly occurs mainly on the surface of activated cells or PS-expressing microparticles (Stone and Nelsestuen, 2005).

While *in vitro* experiments are designed to mimic physiologic conditions, synthetic membrane vesicles do not necessarily achieve this goal. The PCPS vesicles that are used in most studies are not the same as cell membranes, such as activated platelets or microparticles. Differences that may affect prothrombinase activity include (a) lipid content, such as phosphatidylethanolamine and cholesterol (Gerads *et al.*, 1990) (b) polysaccharides, such as dextran sulphate (Oshima, 1989), and (c) protein receptors, such as thrombomodulin (Wada *et al.*, 2005). Therefore, results obtained with synthetic membranes *in vitro* cannot necessarily be translated to what occurs with cell-derived membranes *in vivo*. However, PCPS vesicles are a useful surrogate for activated membrane surfaces because they (a) are readily synthesized and stored, (b) unlike cell membranes, are homogeneous in their lipid content, and (c) yield more consistent results.

Because of its critical role in coagulation, many current anticoagulant strategies focus on inhibiting thrombin generation by targeting prothrombinase activity, using direct-fXa inhibitors. This topic will be discussed in further detail in a separate section dealing with anticoagulants.

Prothrombin and prothrombin activation intermediates

Prothrombin, also known as factor II, is a 72 kDa zymogen that circulates in plasma at a concentration of 90 μg/ml (1.4 μM) and has a half-life of 2.5 days (McDuffie *et al.*, 1979). Prothrombin is organized into 4 distinct structural domains (Figure 1.4). The NH_2-terminal fragment 1 contains the Gla-domain and a kringle domain and is covalently linked to fragment 2 via Arg^{155}-Ser^{156} (Rabiet *et al.*, 1986). Fragment 2 consists exclusively of the triple-disulfide loop kringle domain (Suttie and Jackson, 1977). The kringle domains have no known function in coagulation but are homologous to the lysine-binding kringles found in plasminogen and tissue-type plasminogen activator. Fragment 2 is linked to the A chain of thrombin via Arg^{271}-Thr^{272} (Rabiet *et al.*, 1986). The protease domain is composed of the A and B chains of prothrombin, which are covalently attached via Arg^{320}-Ile^{321} (Rabiet *et al.*, 1986). When this bond is cleaved during activation, the A and B chains remain attached by a disulfide bond between Cys^{293}-Cys^{439}. Unlike other coagulation proteases, thrombin does not possess a Gla domain because fragments 1 and 2 are released during the conversion of prothrombin to thrombin.

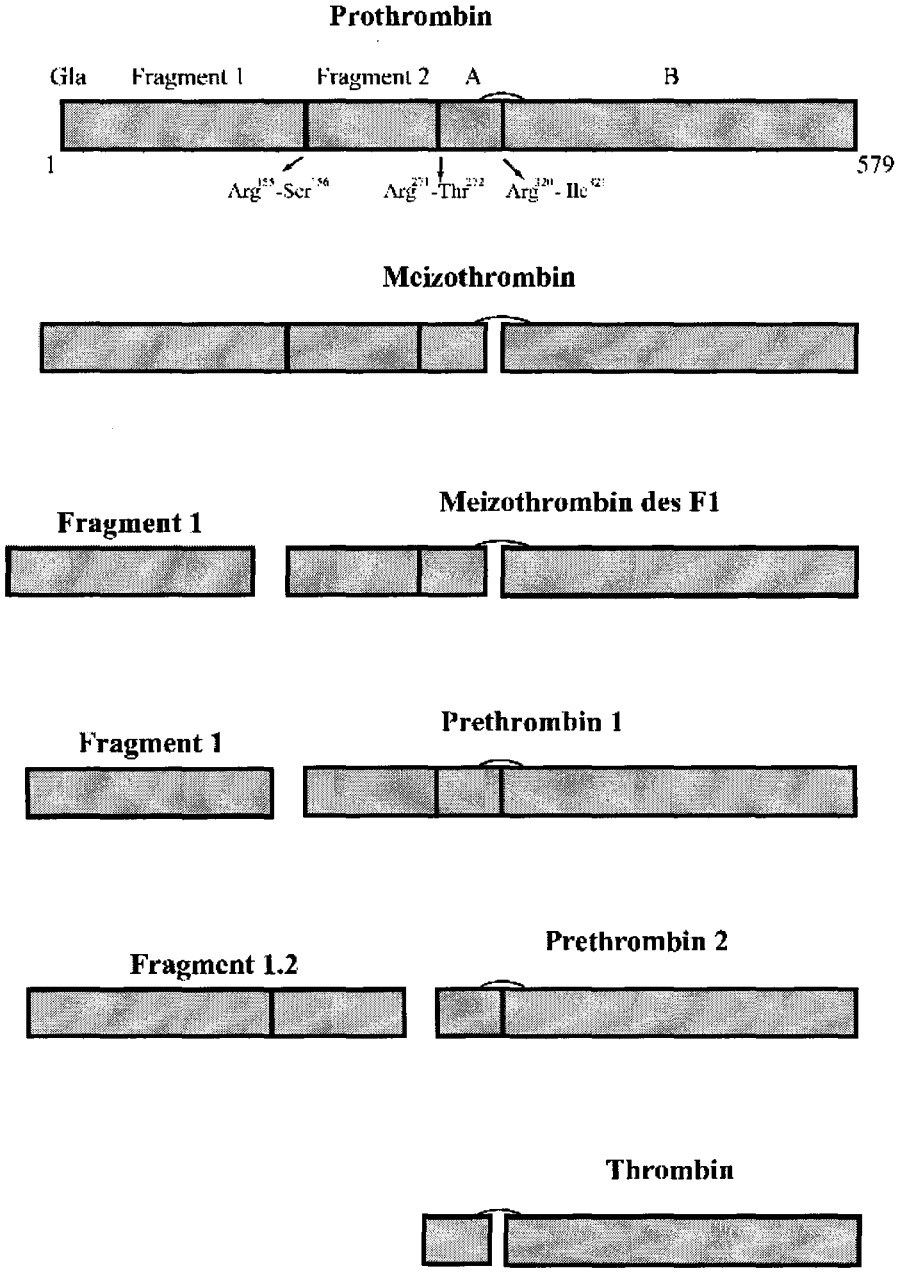

Prothrombin
Gla
Fragment 1
Fragment 2
A
B
1
579
Meizothrombin
Fragment 1
Meizothrombin des F1
Fragment 1
Prethrombin 1
Fragment 1.2
Prethrombin 2
Thrombin

Figure 1.4: Prothrombin activation intermediates

The pathway of prothrombin activation is determined by the order of bond cleavage by fXa. Meizothrombin is generated when fXa within prothrombinase initially cleaves prothrombin at Arg^{320}-Ile^{321}. Prethrombin 2 is formed when fXa alone cleaves prothrombin at Arg^{271}-Thr^{272}, thereby releasing Fragment (F) 1·2. Prothrombin is also cleaved by thrombin at Arg^{155}-Ser^{156}, which releases F1 and generates prethrombin 1. Further proteolysis of prethrombin 1 at Arg^{320}-Ile^{321} by prothrombinase yields meizothrombin des F1. Of the numerous prothrombin intermediates that can be generated in vitro, only prethrombin 2 and meizothrombin are found in the circulation.

Prothrombin activation

Prothrombin is converted to thrombin by cleavage of the Arg^{271}-Thr^{272} and Arg^{320}-Ile^{321} by fXa (Mann *et al.*, 1990). The rate of prothrombin activation is enhanced ~3000-fold when fXa assembles into prothrombinase, as discussed above. When prothrombin binds to prothrombinase, it interacts with fVa, fXa and PS-membranes. Cleavage at Arg^{271} liberates the Gla-domain and both kringle domains from the protease domain, releasing fragment 1.2, the plasma levels of which is often used as an index of *in vivo* prothrombin activation (Boneu *et al.*, 1991). Cleavage at Arg^{320} generates the catalytically functional active site. The creation of the active site occurs via a molecular sexuality mechanism of conformational rearrangement. Upon cleavage at Arg^{320}, the new NH_2 terminus (Ile^{321}) inserts into the Ile-pocket of prothrombin and forms a strong salt bridge with the carboxylate group of Asp^{524} (Bode *et al.*, 1978, Bode and Huber, 1976). Formation of this salt bridge triggers a conformational change within the protease domain that establishes the oxyanion hole and exosite 1 of thrombin (Khan and James, 1998). Both exosite 1 and the oxyanion hole are required for thrombin procoagulant activity, which will be discussed in detail in the section on thrombin.

Staphylocoagulase, an enzyme produced by certain strains of *Staphylococcus aureus*, also can serve as a prothrombin activator. The prothrombin-staphylocoagulase complex crystal structure provided the first structural confirmation of the molecular sexuality mechanism of zymogen activation (Friedrich *et al.*, 2003, Friedrich *et al.*, 2006). Thus, Ile^{1}-Val^{2} of staphylocoagulase occupies the Ile-pocket in a manner similar to Ile^{321}-Val^{322} of prothrombin (Friedrich *et al.*, 2003).

Prothrombin activation intermediates

FXa cleaves prothrombin at two sites and the order of cleavage determines which of the 2 predominant activation pathways is followed. In the presence of fVa and anionic phospholipid, fXa assembles into the prothrombinase complex, which preferentially cleaves Arg^{320}-Ile^{321} rather than Arg^{271}-Thr^{272} and generates the catalytically active meizothrombin as the major intermediate (Orcutt and Krishnaswamy, 2004). The other fXa-mediated pathway of thrombin generation occurs in the absence of phospholipid and fVa and produces prethrombin 2 via preferential cleavage of Arg^{271}-Thr^{272} and release of fragment 1.2 (Orcutt and Krishnaswamy, 2004).

Prothrombin also contains two thrombin sensitive peptide bonds that contribute, although in a minor way, to thrombin generation. Thrombin-catalyzed cleavage of Arg^{155}-Ser^{156} leads to the formation of prethrombin 1 and release of fragment 1 from the NH_2 terminus (Shi *et al.*, 2004). Proteolysis of prethrombin 1 by fXa can yield (a) prethrombin 2 with the release of fragment 2, or (b) meizothrombin des F1 by cleaving at Arg^{320} (Anderson *et al.*, 2003). Furthermore, thrombin also cleaves at Arg^{284}, which yields thrombin des1-13. Despite its truncated A-chain, this non-physiologically relevant thrombin derivative has the same catalytic properties as intact thrombin, (Downing *et al.*, 1975, Lanchantin *et al.*, 1973).

Of the prothrombin derivatives discussed above, meizothrombin, meizothrombin des F1, and thrombin are catalytically active because of cleavage at Arg^{320}, the activation site (Doyle and Haley, 1993). In contrast, prothrombin, prethrombin 1 and prethrombin 2 are inactive because the Arg^{320}-Ile^{321} bond remains intact (Heldebrant *et al.*, 1973).

The proteolytic activity of meizothrombin and meizothrombin des F1 against macromolecular substrates, such as fibrinogen, is less than that of thrombin, although all of the enzymes have similar activity against low molecular weight peptidyl substrates (Doyle and Haley, 1993). Thus, meizothrombin and meizothrombin des F1 possess <10% of the clotting activity of mature thrombin (Shim *et al.*, 2004). Interestingly, meizothrombin has greater activity toward protein C than fibrinogen and, therefore, may play a regulatory role in this anticoagulant pathway (Koike *et al.*, 2003). Furthermore, meizothrombin has been implicated in fV and fVIII activation on the membrane surface (Kroh *et al.*, 2007, Orfeo *et al.*, 2004). These physiological roles of meizothrombin are thought to be aided by its retention of the Gla-domain, which endows it with the capacity to interact with membrane-bound substrates.

Channelling

Although prothrombin activation can proceed through one of the above-mentioned intermediates, there is evidence supporting direct thrombin generation without the accumulation of intermediates (Banerjee *et al.*, 2002, Boskovic *et al.*, 2001, Weinreb *et al.*, 2003). This phenomenon, referred to as channelling, was demonstrated by measuring the maximum rate of thrombin generation during prothrombin activation by prothrombinase, and is suspected to account for 50% of thrombin formation (Boskovic *et al.*, 2001).

Prothrombin entry into prothrombinase

To access fXa within the prothrombinase complex, prothrombin undergoes a series of initial binding events. First, prothrombin is localized to the enzyme complex by

interacting with the PS-membrane surface with a K_D value of 2 μM (Nesheim, 1984). Next, prothrombin binds to the cofactor fVa, which serves as a receptor for both fXa and prothrombin on the surface of platelets (Nesheim, 1984, Tracy *et al.*, 1979). Finally, it is proposed that prothrombin docks on an exosite of fXa, thereby orienting the scissile bonds for proper proteolysis (Orcutt *et al.*, 2002, Wilkens and Krishnaswamy, 2002). fVa is required for the exosite-like properties to be manifested in fXa, suggesting that either (a) fVa serves as a surrogate exosite for fXa, or (b) the interaction of fXa with fVa exposes an exosite on fXa (Buddai *et al.*, 2002, Orcutt *et al.*, 2002, Wilkens and Krishnaswamy, 2002).

Prothrombin interacts with fVa at several distinct sites. Thus, the Gla-domain, fragment 2, and proexosite 1 of the protease domain, have all been shown to be necessary for prothrombin entry into prothrombinase.

Proexosite 1 is the term given to the immature exosite 1 domain on prothrombin. $Hir^{54-65}(SO_3^-)$, the exosite 1-binding COOH-terminus peptide of the thrombin inhibitor hirudin, binds prothrombin and inhibits its activation by the fXa/fVa complex (Anderson *et al.*, 2000a, Anderson *et al.*, 2000b). When fVa was removed from the reaction, $Hir^{54-65}(SO_3^-)$ was no longer able to inhibit prothrombin activation, suggesting that it blocks the fVa-prothrombin interaction. Interestingly, $Hir^{54-65}(SO_3^-)$ was less effective when PCPS vesicles were added because the affinity of prothrombin for fVa within fully assembled prothrombinase is far higher than that of $Hir^{54-65}(SO_3^-)$ for prothrombin (Anderson *et al.*, 2000a, Anderson *et al.*, 2000b, Chen *et al.*, 2003). In more recent studies, bothrojaracin, a snake venom protein that binds thrombin and prothrombin, (Monteiro *et al.*, 2001), and

an acidic peptide derived from the heavy chain of fVa (Bukys *et al.*, 2006a) have also been shown to disrupt fVa binding to proexosite 1 of prothrombin.

In addition to proexosite 1, both fragment 1 and fragment 2 of prothrombin also interact with fVa (Deguchi *et al.*, 1997, Mann, 1994). Interestingly, fragment 2 does not contribute to fVa-dependent prothrombin activation by prothrombinase (Krishnaswamy and Walker, 1997). In contrast, a peptide analogue of residues 1-46 of the NH_2-terminal of prothrombin, which includes the Gla-domain, was shown to inhibit prothrombin activation by prothrombinase in the absence or presence of PCPS vesicles, but not in the absence of fVa (Blostein *et al.*, 2000). The capacity of this Gla-domain peptide to inhibit prothrombin activation was attributed to its ability to bind to fVa (Blostein *et al.*, 2000). Subsequent molecular modelling of prothrombinase suggested that the Gla-domain of prothrombin likely interacts with the C1 domain of the fVa light chain (Autin *et al.*, 2006). It also has been reported that the kingle domain of fragment 1 binds fVa and contributes to prothrombin activation by prothrombinase (Deguchi *et al.*, 1997).

Thus, prothrombin interacts with an extended surface interface of fVa. The result is that fXa effects timely and ordered proteolysis of spatially distinct peptide bonds on prothrombin. However, the mechanism by which fXa gains access to these bonds is controversial.

Mechanism of prothrombin activation

In the absence of cofactors, fXa preferentially cleaves prothrombin first at Arg^{271}, yielding prethrombin 2 , and then at Arg^{320} to generate thrombin (Nesheim and Mann, 1983). However, when assembled within prothrombinase, fXa preferentially cleaves Arg^{320} before Arg^{271}, representing a reversal in cleavage order that yields meizothrombin (Nesheim and Mann, 1983). The exact mechanism through which this occurs remains unclear. To begin to address this issue, Brufatto *et al* developed a series of prothrombin mutants in which the fXa-sensitive Arg residues were replaced with Ala residues (Brufatto and Nesheim, 2003). Kinetic analyses using these mutants demonstrated that in the absence of fVa, the k_{cat} for cleavage at Arg^{271} is 50-fold greater than for Arg^{320}. The addition of fVa enhanced the k_{cat} for cleavage at Arg^{320} but not at Arg^{271}. Thus, in the presence of fVa, fXa activates II_{R271A} and II_{R320A} with equivalent k_{cat} values (Brufatto and Nesheim, 2003). Interestingly, fVa did not affect the K_m for cleavage of the prothrombin mutants. The capacity of fVa to enhance the k_{cat} of prothrombin activation by fXa is consistent with the known effect of fVa on the active site of fXa (Betz *et al.*, 1997, Walker and Krishnaswamy, 1993), and provides a kinetic explanation for the order of bond cleavage with different forms of prothrombinase. Subsequent experiments demonstrated that the interaction between fXa and an acidic region of the heavy chain of fVa explains this kinetic effect (Bukys *et al.*, 2005).

In studies that assessed the change in specificity of fXa cleavage of prothrombin, PCPS vesicles and fVa were always added together. Therefore, the conclusion that fVa regulates the change in fXa specificity does not take into account the potential role of

PCPS vesicles. This is important because a recent study demonstrated that fVa enhances the capacity of fXa to cleave prothrombin, whereas PS-containing membranes determine the prothrombin bond that is cleaved (Weinreb *et al.*, 2003). In support of this concept, soluble C6PS increases the rate of fXa cleavage at Arg^{320} 150-fold, and slightly reduces the rate of fXa cleavage at Arg^{271} (Banerjee *et al.*, 2002, Wu *et al.*, 2002). These findings likely reflect a direct effect of PS on fXa activity because the experiments were conducted with concentrations of C6PS well below those required for micelle formation. C6PS has also been shown to influence the cofactor activity of fVa within prothrombinase by binding to both its light and heavy chain (Zhai *et al.*, 2002). Furthermore, C6PS supports fXa/fVa interactions to a similar extent as a PS-membrane bilayer, and yields rates of prothrombin activation similar to that achieved when prothrombinase assembles on an anionic membrane surface (Majumder *et al.*, 2005). These findings call into question the concept that anionic phospholipid membranes reduce the dimensionality of prothrombin activation by prothrombinase, and suggest that they play a more deliberate role in directing fXa proteolysis of prothrombin.

The reversal of prothrombin bond cleavage when fXa assembles into prothrombinase has been shown to be dependent upon the membrane surface, suggesting that the spatial organization of prothrombin within prothrombinase determines that Arg^{320} is cleaved before Arg^{271}. It is possible that the ordered bond cleavage during prothrombin activation by prothrombinase is the result of different substrate conformations (Orcutt and Krishnaswamy, 2004). This proposal is consistent with the suggestion that prothrombin and meizothrombin adopt different conformations on the

surface of membranes and that the two represent distinct substrates for prothrombinase (Chen and Lentz, 1997). These findings prompted the ratcheting hypothesis of prothrombin activation, whereby initial cleavage at Arg^{320} is required for proper orientation of the Arg^{271} bond relative to the active site of fXa (Bianchini *et al.*, 2005).

Taken together, these data can be organized into a model of prothrombin activation that accounts for the role of each cofactor in determining the pathway of prothrombin activation. Thus, PS-membranes dictate the cleavage of Arg^{320} prior to Arg^{271}. Because of the spatial separation between these bonds, initial cleavage at Arg^{320} orients Arg^{271} toward the active site of fXa. FVa optimizes the active site of fXa and enhances the rate Arg^{320} and Arg^{271} proteolysis to a similar extent.

The process of prothrombin activation provides a level of control over thrombin activity by limiting its generation to sites of vascular injury. This is a critical control mechanism because it permits a rapid and specific response to hemostatic challenges yet limits the generation of systemic thrombin activity, which would be detrimental. Thrombin is a potent procoagulant and unless its activity is regulated, there would be indiscriminate clot formation.

Thrombin

The end product of the coagulation system is the serine protease thrombin (Figure 1.5). Thrombin has numerous functions in maintaining hemostasis. As a procoagulant, thrombin activates platelets, catalyzes the conversion of soluble fibrinogen into insoluble fibrin, and activates an array of clotting factors, including factors V, VIII, XI, and XIII (Mann *et al.*, 1988). Thrombin also functions as an anticoagulant and anti-fibrinolytic enzyme. After binding to thrombomodulin on the surface of endothelial cells (Furie and Furie, 1991), thrombin converts protein C to activated protein C, which then acts as an anticoagulant, and activates thrombin-activatable fibrinolysis inhibitor, which attenuates fibrinolysis (Boffa *et al.*, 1998, Wang *et al.*, 1998). Because of thrombin's multifunctional roles, its regulation is important to maintaining hemostasis.

Active site

Many of the enzymes within the coagulation system are serine proteases, including thrombin. This class of proteases is so named because of the central role that serine plays in peptide bond hydrolysis. The catalytic triad of thrombin consists of His^{57}, Asp^{102}, and Ser^{195}, chymotrypsin numbering (Ct#).

The active site of thrombin is positioned in a concave surface depression that is "guarded" by two protruding loops. The 60- loop consists of residues Leu^{60a}-Glu^{60h}, whereas the γ-loop consists of residues Thr^{144}-Lys^{155} (Ct#). These loops serve to restrict access to the active site. To compensate for this, macromolecular substrates bind additional sites in order to gain access to the active site. These additional sites are termed exosites and

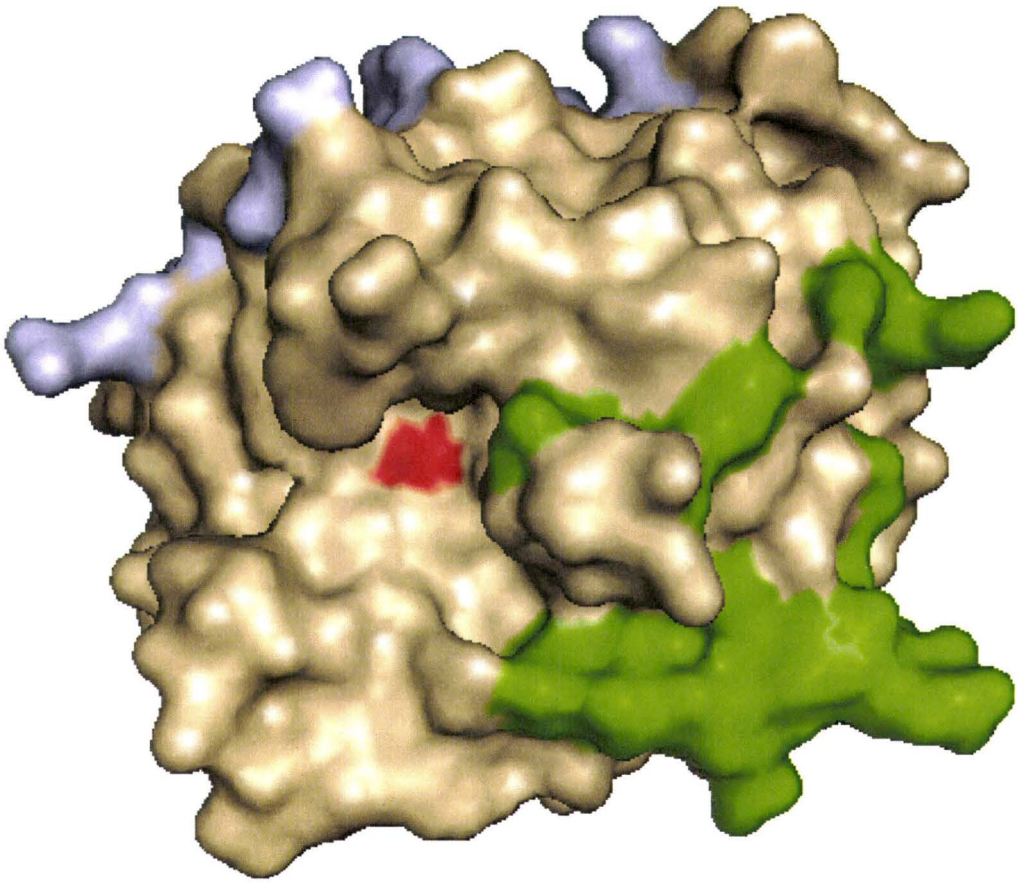


Figure 1.5: Thrombin functional domains

A crystal structure of FPR-thrombin is shown with the active site serine (red), and residues that comprise exosite 1 (green) and exosite 2 (blue) highlighted. The FPRck moiety is omitted for clarity. Exosite 1 consists of basic residues surrounding a hydrophobic cleft, which leads to the active site. Consequently, this domain functions to bind and orient substrates for interaction with the active site. Exosite 2 consists exclusively of arginine and lysine residues, and is highly basic compared with exosite 1. Exosite 2 is noted for its capacity to bind heparin. This image was prepared Pymol with data from the Protein Data Bank (code: 1EB1).

they serve to (a) properly orient the scissile bond for proteolysis, and (b) improve penetration of substrates into the active site.

Exosites

The identification of proteolytic derivatives of thrombin that retained their capacity to cleave small peptidyl substrates but were no longer capable of converting fibrinogen to fibrin provided the first evidence that the activity of thrombin depends on more than just the active site triad (Mohammed *et al.*, 1976). Since then, the specificity of thrombin for macromolecular substrates has been shown to be regulated by exosites, which are docking sites that are distinct from the active site. Exosite 1 is composed of hydrophobic amino acid residues, grouped in a pocket, and surrounded by cationic residues (Adams and Huntington, 2006). This hydrophobic pocket is linked by a surface depression that guides ligands towards the active site (Stubbs and Bode, 1993). As a result, exosite 1 tends to bind ligands that require access to the active site, including fibrinogen (Pechik *et al.*, 2004), hirudin (Chang, 1991), heparin cofactor II (HCII) (Becker *et al.*, 1999), and protease activated receptor 1 (PAR1) (Brass, 2003). Not all exosite 1-binding ligands require access to the active site. For example, thrombin utilizes exosite 1 to bind to thrombomodulin (Huntington and Esmon, 2003) or to the NH_2-terminus of HCII (Becker *et al.*, 1999).

Exosite 2 is composed entirely of arginine and lysine residues and serves to tether thrombin for subsequent interactions. Examples of exosite 2 ligands include glycosaminoglycans, such as heparin (Becker *et al.*, 1999), and the COOH-terminus extension of the variant γ'-chain present on a subpopulation of fibrin(ogen) molecules

(Pospisil *et al.*, 2003). Not only do the exosites bind substrates and cofactors, but they also have been implicated in the regulation of thrombin-mediated activation of fV and fVIII (Myles *et al.*, 2001b, Nogami *et al.*, 2005). Evidence supporting the role of exosites in regulating thrombin activity comes from extensive biochemical assays, mutagenesis studies, and x-ray crystal structures.

Properties of exosite 1

Exosite 1 on prothrombin binds fibrinogen-Sepharose (Kaczmarek *et al.*, 1987) and $Hir^{54-65}(SO_3^-)$ (Anderson *et al.*, 2000a) with much weaker affinity than does thrombin, suggesting that it is immature. During prothrombin activation, exosite 1 functionally matures. Thus, like thrombin, meizothrombin binds $Hir^{54-65}(SO_3^-)$ with a 133-fold higher affinity than prothrombin. In contrast, prethrombin 2 binds $Hir^{54-65}(SO_3^-)$ only 6-fold more tightly than prothrombin (Anderson *et al.*, 2003, Anderson and Bock, 2003). Despite the functional nature of exosite 1 on meizothrombin, the enzyme has negligible fibrinogen clotting activity. Cleavage at Arg^{271}, which releases fragment 2 from the protease domain, has no effect on the affinity for $Hir^{54-65}(SO_3^-)$, but does endow the enzyme with full procoagulant activity (Anderson *et al.*, 2003, Doyle and Mann, 1990). These data demonstrate that procoagulant activity of thrombin requires maturation of the active site and the exosites.

The residues within exosite 1 that are critical for its capacity to bind ligands vary depending on the ligand (reviewed in (Lane *et al.*, 2005)). Tsiang *et al* developed a panel of 77 thrombin mutants where one or several charged residues were replaced with alanine residues (Tsiang *et al.*, 1995). These mutants were used to identify 19 residues that

contribute to the fibrinogen clotting activity of thrombin; 15 of these residues map to the exosite 1 region (Tsiang *et al.*, 1995). The most important residues involved with fibrinogen recognition appear to be Lys^{385}, His^{386}, and Tyr^{391}. These investigators also examined the capacity of the thrombin mutants to bind thrombomodulin and activate protein C. Again, many of the same residues in exosite 1 were identified as critical for this reaction, suggesting that thrombomodulin and fibrinogen dock on largely overlapping sites on exosite 1.

The hydrophobic core is crucial for the specificity of ligand binding to exosite 1. Thus, ligands that bind to exosite 1 of thrombin have a 2-fold higher proportion of hydrophobic residues than ligands that bind exclusively to exosite 2 (Huntington, 2005). Indeed, work by Karshikov *et al* demonstrated that the majority of the binding energy involved in the interaction of hirudin with thrombin is hydrophobic (Karshikov *et al.*, 1992). The charged residues that surround this hydrophobic pocket are mostly involved in aligning complementary hydrophobic residues in a process referred to as "molecular steering" (Myles *et al.*, 2001a). A similar mechanism for thrombin recognition of fibrinogen has also been proposed (Huntington, 2005).

Properties of exosite 2

Whereas exosite 1 is composed of hydrophobic and charged residues, exosite 2 is composed entirely of arginine and lysine residues. As a result, exosite 2 binds ligands that are highly acidic. Such ligands include glycosaminoglycans, Gp1bα, and the γ' extension on the γ'-chain of variant fibrin(ogen). Recent findings have established that exosite 2 plays a supplementary role in regulating thrombin during (a) feedback

activation of fV and fVIII, (b) thrombomodulin-enhanced protein C activation, (c) high affinity binding to fibrin clots, and (d) heparin catalysis of thrombin inhibition by antithrombin. Each of these roles will be explored in greater detail in subsequent sections.

The finding that meizothrombin and thrombin bind $Hir^{54-65}(SO_3^-)$ with similar affinity and exhibit similar capacities to cleave tripeptidyl substrates is interesting in light of the fact that meizothrombin has reduced fibrinogen clotting activity. Fragment 2, which is covalently linked to the protease domain in meizothrombin, binds exosite 2 and renders it inaccessible to ligands (Arni *et al.*, 1993, Liaw *et al.*, 1998). Release of fragment 2 results in the formation of thrombin and the evolution of full procoagulant activity (Anderson *et al.*, 2003). Based on the mutagenesis studies performed by Tsiang *et al*, we know that $Arg^{498}/Arg^{500}/Arg^{503}$ of exosite 2 play a critical role in fibrinogen recognition by thrombin (Tsiang *et al.*, 1995). These data confirm x-ray crystallographic data, which demonstrate that fibrinopeptide A on fibrinogen loops within the active site pocket and makes contact with residues of exosite 2 that line the the active site cleft (Martin *et al.*, 1996). Thus, regions of exosite 2 also play an important role in thrombin clotting activity.

Role of exosite 1 and 2 in thrombin activation of fV and fVIII

As previously discussed, the activation of fV and fVIII by thrombin follows an ordered proteolysis of peptide bonds, resulting in the release of the B-domain and formation of activated cofactors. Thrombin utilizes exosite 1 to initially dock to these substrates, a mechanism that is widely accepted. However, a role for exosite 2 during fV

and fVIII activation has also been identified. Autoantibodies against thrombin isolated from the plasma of a patient who presented with bleeding was directed toward both exosites (Mollica *et al.*, 2006). These antibodies impaired thrombin generation in plasma without binding prothrombin, suggesting that both exosite 1 and 2 are involved in thrombin feedback. Although the role of exosite 2 during cofactor activation appears to be limited, it may be important for regulating the order of cleavages during cofactor activation.

The first evidence of exosite involvement in cofactor activation was described by Esmon and Lollar who showed that RA-thrombin, an exosite 2 mutant of thrombin with mutations at $Arg^{409}/Arg^{413}/Arg^{418}$, activated fVIII more slowly than wild type thrombin (Esmon and Lollar, 1996). Expectedly, $Hir^{54-65}(SO_3^-)$ inhibited the activation of fV and fVIII by thrombin confirming that exosite 1 is critical for both reactions. Since then, Fay *et al* demonstrated that the A2 subunit of fVIII binds to thrombin exosite 2, while the A1 subunit binds to both exosite 1 and 2 (Nogami *et al.*, 2005). Furthermore, the intact heavy chain of fVIII (A1-A2) binds thrombin with higher affinity then either subunit alone, suggesting a synergistic effect of exosite 1 and 2 involvement in thrombin binding to fVIII. Thrombin cleaves fVIII in an ordered fashion and initial cleavage at Arg^{740} enhances the rate of subsequent cleavages at Arg^{1689} and Arg^{372} (Newell and Fay, 2007). Recently Newell *et al* demonstrated that cleavage at Arg^{1698} on the light chain of fVIII influences the rate of cleavage at Arg^{340} (Newell and Fay, 2009). Because of the spatial separation of the cleavage sites on fVIII, and the involvement of exosite 1 and 2 in some, but not all proteolysis steps, the authors proposed that fVIII activation follows a

ratcheting mechanism similar to the one described for prothrombin activation by prothrombinase (Bianchini *et al.*, 2005).

Like the findings with fVIII, both exosite 1 and 2 also appear to be involved in fV activation by thrombin. Thus, Myles *et al* used a mutagenesis approach to show that an extensive interface is involved in thrombin recognition of fV. These data demonstrate that in addition to exosites 1 and 2, the Na^+ binding site and residues surrounding the active site also are important for fV activation by thrombin (Myles *et al.*, 2001b). Furthermore, Bukys *et al* demonstrated that β-thrombin, which lacks exosite 1, is unable to cleave fV at Arg^{1545}, and was significantly less efficient than thrombin at cleaving Arg^{709} (Bukys *et al.*, 2006b). In addition, they demonstrated that membrane-bound meizothrombin activated both fV and fVIII at a higher rate than thrombin (Bukys *et al.*, 2006b). Although the latter findings are not consistent with a role for exosite 2 in fV activation, as proposed by Myles, the membrane surface may enhance the rate of fV activation by meizothrombin adding another layer of complexity.

Sodium binding site and thrombin allostery

Thrombin has been described as an allosteric molecule, meaning that ligands and substrates bind to thrombin and induce conformational changes at both the local level and at distal sites that can influence thrombin function. Thus, allosteric connections have been established to exist between (a) exosite 1 and the active site (Gandhi *et al.*, 2008, Guinto and Di Cera, 1997, Monteiro *et al.*, 1999), (b) exosite 2 and the active site (Han and Tollefsen, 1998, Monteiro *et al.*, 1999), (c) exosite 1 and exosite 2 (Fredenburgh *et al.*, 1997, Petrera *et al.*, 2009), (d) the Na^+ binding site and exosite 1 (Kroh *et al.*, 2007),

and (e) the Na^+ binding site and the active site (Guinto and Di Cera, 1997, Rezaie and Yang, 2005).

Allostery may be an important regulator of thrombin activity. For example, in the presence of Na^+, thrombin is a procoagulant enzyme since it preferentially cleaves fibrinogen over protein C. In the absence of Na^+, thrombin functions as an anticoagulant enzyme because it preferentially activates protein C and has reduced capacity to cleave fibrinogen (Ayala and Di Cera, 1994). In the absence of Na^+, Trp^{547} occludes the active site, whereas in the presence of Na^+, thrombin undergoes a conformational change that translocates Trp^{547} by ~10Å (Pineda *et al.*, 2006). Mutation of Trp^{547} affects protein C activation, but not fibrinogen or PAR1 cleavage, suggesting that occupation of the active site by Trp^{547} promotes protein C activation by thrombin (Arosio *et al.*, 2000). A similar switch in thrombin specificity occurs in the presence of thrombomodulin, which also has been attributed to allosteric rearrangement of Trp^{547} (Cantwell and Di Cera, 2000). Therefore, thrombin is an allosteric molecule, which helps to regulate its activity.

Regulation of Coagulation

There are several different ways in which the coagulation system is regulated. Thus, we have discussed (a) compartmentalization of tissue factor, the initiator of coagulation, (b) ordered assembly of cofactors and enzymes into activation complexes, (c) the specificity of thrombin, and (d) the capacity of thrombin to remain associated with the fibrin clot. In addition to these mechanisms, the coagulation system relies on serine protease inhibitors or serpins, and the anticoagulant protein C pathway to down-regulate hemostasis.

Inhibitors

Serpins are a class of proteins that specifically target and inhibit serine proteases. Because most of the enzymes of the coagulation system are serine proteases, serpins are known to inhibit each step of coagulation.

Serpins inhibit serine proteases by an irreversible "suicide" mechanism that ultimately results in enzyme inactivation (Silverman and Lomas, 2004). Serpins have a protruding reactive center loop (RCL) that contains a protease recognition site tethered between a β-sheet. Upon binding, the active site associates with the scissile bond in the RCL (Johnson *et al.*, 2006). Cleavage of the scissile bond leads to an intermediate covalent ester linkage between inhibitor and enzyme. The complex is then cleared from circulation before significant complex decay can occur (Barker-Carlson *et al.*, 2002).

Antithrombin

The major serpin that regulates the coagulation system is antithrombin. Antithrombin, also called antithrombin III, is a 58 kDa protein that circulates in plasma at a concentration of 5 μM and has a half-life of 3 days (Collen *et al.*, 1977, Conard *et al.*, 1983). Antithrombin inactivates the majority of the enzymes within the coagulation system, including fXIIa, fXIa, fIXa, fXa, thrombin, fVIIa, and kallikrein (Broze, Jr. and Majerus, 1980, Holmer *et al.*, 1981). Mice with reduced levels of antithrombin suffer from spontaneous thrombosis or disseminated intravascular coagulation and complete deficiency is embryonic lethal (Ishiguro *et al.*, 2000).

The x-ray crystal structure of antithrombin demonstrates that the RCL is partially buried (Johnson *et al.*, 2006). In the presence of heparin, however, antithrombin undergoes a conformational change that exposes the RCL to the protease, which enhances the rate of inhibition by 1000-fold (Whisstock *et al.*, 2000). Recently, Izaguirre *et al* demonstrated that a protease-recognition exosite is also exposed on antithrombin in the presence of heparin (Izaguirre *et al.*, 2007).

Heparin cofactor II

Heparin cofactor II, referred to as HCII, is a 66 kDa protein that circulates in plasma at a concentration of 1.4 μM. HCII is a serpin that specifically targets thrombin. HCII deficiency is not lethal and, unlike antithrombin deficiency, it does not cause DIC in genetically deficient mice (He *et al.*, 2002). However this has recently been challenged with the generation of a HCII-// mouse that was not viable (Aihara *et al.*,

2007). As a result, HCII is sometimes regarded as a redundant serpin (van't Veer and Mann, 1997). However, HCII deficiency has recently been shown to accelerate atherogenesis and neointima formation in mice (Vicente *et al.*, 2007) and enhance thrombosis at sites of injury (He *et al.*, 2002).

In addition to its RCL, HCII also possesses an NH_2-terminus acidic tail that unfolds when heparin or dermatan sulphate binds to the body of HCII (Liaw *et al.*, 1999) and binds to exosite 1 (Fortenberry *et al.*, 2004). Deletion of the acidic tail results in a 100-fold decrease in the rate of thrombin inhibition by HCII (Liaw *et al.*, 1999). Recently, the exosite 1-binding tail of HCII was added to a thrombin-inhibiting variant of the serpin α_1-antitrypsin, resulting in a 21-fold enhancement in the rate at which it inhibits thrombin (Sutherland *et al.*, 2006, Sutherland *et al.*, 2007).

α_2-Macroglobulin

In addition to the serpins, α_2-macroglobulin also is an important inhibitor of thrombin. α_2-macroglobulin is a 725 kDa tetramer that circulates in plasma at a concentration of 3 μM and inhibits numerous serine proteases (reviewed in (Sottrup-Jensen, 1989)). The 4 subunits of α_2-macroglobulin are arranged such that they form a cavity, which traps the enzyme (Lonberg-Holm *et al.*, 1987). Unlike the serpin class of inhibitors, the active sites of inhibited enzymes remain intact and can still be detected with small peptidyl substrates that can enter the central cavity of the α_2-macroglobulin tetramer (Hemker *et al.*, 2007).

Protein C anticoagulant pathway

The protein C anticoagulant pathway is another system that reduces thrombin generation. Activated protein C (APC) is generated when thrombomodulin-bound thrombin activates protein C. APC then inhibits thrombin generation by proteolytically inactivating fVa and fVIIIa.

Thrombomodulin

Thrombomodulin is a membrane-bound protein on the surface of endothelial cells that serves as a receptor for thrombin. Thrombin binds to thrombomodulin via exosite 1 with a K_D value of 2.4 nM (Liu *et al.*, 1994). Interestingly, meizothrombin des F1 has also been shown to interact with thrombomodulin with a K_D value comparable to that of thrombin, suggesting that intermediates of prothrombin activation may contribute to protein C activation (Liu *et al.*, 1994). A subpopulation of thrombomodulin molecules contains a chondroitin sulphate moiety that binds to exosite 2 on thrombin, thereby enhancing the affinity for thrombin by 10-fold (Liu *et al.*, 1994, Ye *et al.*, 1993).

When thrombin binds to thrombomodulin, it undergoes an allosteric change that alters the conformation of the active site loops (Koeppe *et al.*, 2005), and enhances the rate of protein C activation by over 1000-fold (Xu *et al.*, 2005). Once bound to thrombomodulin, thrombin has a reduced capacity to convert fibrinogen to fibrin, because (a) exosite 1 is occupied by thrombomodulin, and (b) the active site undergoes an allosteric change (Dang *et al.*, 1995).

Protein C/APC

Protein C is a 62 kDa glycoprotein that circulates in plasma at a concentration of 65 nM. Protein C is activated by thrombin when bound to thrombomodulin. Thus, thrombin cleaves protein C at Arg^{162} to release an activation peptide that results in proper active site formation (Griffin *et al.*, 2007). APC has many physiological roles including (a) anticoagulant (Manithody *et al.*, 2003), (b) anti-inflammatory (Mosnier and Griffin, 2006), (c) anti-apoptotic (Mosnier and Griffin, 2006), and (d) barrier protection (Bae *et al.*, 2007). Because APC is relatively resistant to inhibition by serpins, it has a long plasma half-life (20 min) making it an ideal therapeutic for treating thrombosis and sepsis (Borgel *et al.*, 2007).

The primary role of APC in the coagulation system is to inactivate fVIIIa and fVa, resulting in down regulation of the intrinsic tenase and prothrombinase complexes, respectively (Adams *et al.*, 2004, Malm *et al.*, 2007). The importance of APC is highlighted by fV-Leiden, a common fV mutation (Baker *et al.*, 1994, Bertina *et al.*, 1994). Once activated fV-Leiden is resistant to inactivation by APC. Patients with this mutant form of fV are at increased risk for venous thromboembolism (Kahn *et al.*, 2005, Kearon *et al.*, 2008).

In activation of fVa and fVIIIa

Inactivation of fVIIIa occurs through 2 distinct mechanisms; (a) passive dissociation of the A2 subunit from the A1/A3-C1-C2 dimer, and (b) inactivation by APC. APC inactivates fVIIIa by cleaving at Arg^{336} of the A1 subunit and at Arg^{562} of the A2 subunit (Fay *et al.*, 1991, Varfaj *et al.*, 2006). Cleavage within A1 reduces the

binding affinity between the A1 and A2 subunits, resulting in a reduced k_{cat} and an increased K_m for fX activation by intrinsic tenase. Cleavage within A2 disrupts a critical contact point between fIXa and fVIIIa (Fay *et al.*, 1994). Recent evidence suggests that the cleavage of A1 is the most important for fVIIIa inactivation (Varfaj *et al.*, 2006).

Because fVa is more stable than fVIIIa, inactivation by APC is a critical regulator of its activity. APC inactivates fVa by limited proteolysis of the heavy chain, with Arg^{506} being cleaved first followed by cleavages at Arg^{306} and Arg^{679} (Kalafatis *et al.*, 1994). Cleavage at Arg^{506} causes a 30% reduction in fVa cofactor activity, whereas subsequent cleavage at Arg^{306} results in complete inactivation. Cleavage at Arg^{679} is of lesser importance. The region encompassing residues 311-325 of fVa was identified as a binding site for APC and prothrombin, suggesting that prothrombin and APC interact with similar sites on fVa (Yegneswaran *et al.*, 2007).

Anticoagulants

Cardiovascular disease accounts for greater than 30% of all chronic diseases in the world. Currently, cardiovascular disease is the leading cause of death world-wide. By 2015, the number of annual deaths caused by cardiovascular disease is projected to be 20 million (World Health Organization, 2005). Although, 5% of the NIH budget went toward heart and stroke research, cardiovascular disease remains a significant medical burden. Thrombosis can occur in arteries (e.g., heart attacks or strokes) or in veins (e.g., deep vein thrombosis or pulmonary embolism). Patients who present with thrombosis can be treated with a variety of antithrombotic agents, including antiplatelet agents, anticoagulants or fibrinolytic drugs. Anticoagulants are drugs that inhibit the coagulation system. Many of these agents target thrombin and/or fXa because of the central role they play in clot formation.

Heparins

Heparin is a glycosaminoglycan that consists of a heterogeneous mixture of molecules that range in size from 3 and 50 kDa. Because it is highly sulfated, heparin has one of the highest charge:mass ratios of any biological molecule. Heparin is found within the secretory granules of mast cells (Scully *et al.*, 1986), immune cells which release their contents at sites of vascular injury. The primary role of heparin is not yet resolved, although it has been implicated in protecting sites of injury from bacterial infection (reviewed in (Nader *et al.*, 1999)). Heparin possesses an antithrombin-binding pentasaccharide sequence that mediates its interaction with antithrombin. Approximately

one-third of the molecules of commercial heparin has a pentasaccharide sequence and can bind antithrombin. When these pentasaccharide-containing molecules bind to antithrombin they induce a conformational change, which exposes the RCL and renders it more accessible to fXa. Although this conformational change increases the rate at which fXa is inhibited by antithrombin, it has little effect on the rate of thrombin inhibition (Rezaie, 2006). To accelerate the rate of thrombin inhibition by antithrombin, heparin must also bind to thrombin (Bray *et al.*, 1989). Only heparin molecules that contain a pentasaccharide and at least 5 additional saccharide units are long enough to simultaneously bind to antithrombin and thrombin. Shorter chains will catalyze fXa inhibition by antithrombin but not thrombin inhibition.

An example of a shorter heparin molecule is fondaparinux, a synthetic analogue of the antithrombin-binding pentasaccharide (Choay *et al.*, 1983). Fondaparinux increases the rate of fXa inhibition by antithrombin. In contrast, because it is too short to bridge antithrombin to thrombin, fondaparinux has no effect on the rate of thrombin inhibition. Fondaprinux also is too short to bind to platelet factor 4. Because heparin-induced thrombocytopenia (HIT), a serious allergic reaction to heparin, is caused by antibodies against heparin/platelet factor 4, this complication does not occur with fondaparinux (Lassen *et al.*, 2002).

The most common side effect of heparin is bleeding. Advantages of heparin in this setting include its short half-life and the availability of an antidote. Thus, protamine sulphate, a cationic protein derived from salmon sperm, binds to heparin and neutralizes its anticoagulant activity. The interaction of heparin with protamine sulphate is chain

length dependent; longer chains bind more tightly than shorter chains. Thus, fondaparinux is too short to bind to protamine sulphate. Consequently, in contrast to heparin, fondaparinux lacks an antidote. Therefore, there remains a need for novel anticoagulants that target specific clotting enzymes and have an antidote.

Hirudin

When thrombin binds to fibrin it is not readily inhibited by the heparin-antithrombin complex (Weitz *et al.*, 1990). Because clot bound thrombin retains catalytic activity, it can trigger thrombus growth. Therefore, inhibitors that not only inhibit free thrombin, but also inhibit fibrin-bound thrombin may have advantages over heparin. Hirudin is such an agent.

Hirudin is a thrombin inhibitor originally isolated from the salivary glands of leeches. This 6900 Da protein binds specifically to thrombin, with its acidic COOH-terminal tail binding to exosite 1 and its globular NH_2-domain interacting with the active site (Rydel *et al.*, 1991). Although hirudin is not a covalent inhibitor, its high affinity for thrombin (K_D value of 20 fM) makes the interaction essentially irreversible (Stone and Hofsteenge, 1986). Because of its specificity and high affinity, hirudin has advantages over heparin as an anticoagulant because it does not interfere with other proteins of the coagulation system. At present, hirudin is used to treat patients with HIT who cannot receive heparin (White, 2005). However, hirudin has a narrow therapeutic window, which necessitates constant monitoring to reduce the risk of bleeding (Mehta *et al.*, 2002). Furthermore, there is no antidote for hirudin.

Derivatives of hirudin have been developed in an attempt to overcome these limitations. Bivalirudin is a recombinant 20 amino acid analogue of hirudin that reversibly inhibits thrombin because once bound, it is cleaved by thrombin. In patients undergoing balloon angioplasty, bivalirudin is at least as effective as heparin, but is associated with less bleeding (Antman *et al.*, 2002). Based on these observations, bivalirudin is licensed as an alternative to heparin in such patients (Maroo and Lincoff, 2004).

Synthetic inhibitors of coagulation

Fondaparinux is one example of a synthetic inhibitor that targets a specific enzyme in the coagulation pathway (Mehta *et al.*, 2005, Wiebe *et al.*, 2003). Other examples include dabigatran etexilate, an oral thrombin inhibitor (Wienen *et al.*, 2007), and rivaroxaban, an oral fXa inhibitor (Gerotziafas *et al.*, 2007). Recent clinical trials have demonstrated that dabigatran etexilate and rivaroxaban are non-inferior to enoxaparin, a low-molecular-weight heparin, for prevention of venous thromboembolism in patients undergoing elective hip or knee replacement surgery (Eriksson *et al.*, 2006, Wolowacz *et al.*, 2009). Based on these findings, dabigatran etexilate and rivaroxaban are licensed for this indication in many countries including Canada. Ongoing clinical trials are evaluating their utility in other disorders where they are being compared with vitamin K antagonists, such as warfarin (Mueck *et al.*, 2008, Wolowacz *et al.*, 2009). Because vitamin K agonists have a slow onset of action, a narrow therapeutic window and numerous food and drug interactions, frequent monitoring is needed (recently reviewed in (Eikelboom and Weitz, 2007)). In contrast, rivaroxaban and dabigatran

etexilate have a rapid onset of action. Food has no effect and drug-drug interactions are uncommon. These agents produce such a predictable level of anticoagulation that they can be given in fixed doses without routine coagulation monitoring rendering them promising alternatives to warfarin for long term indications.

Although oral agents are useful for long term treatment, they are less attractive for short-term use. Furthermore, there are no antidotes for rivaroxaban or dabigatran etexilate, which makes them problematic for patients at high risk for bleeding. As a result, there is still a need for novel parenteral anticoagulants that have a rapid onset of action and can be readily reversed with specific and safe antidotes. One such class of agents is the anticoagulant aptamers.

Aptamers

Aptamers, derived from the latin word '*aptus*' (meaning "to fit"), are single stranded oligonucleotides that bind a specific target (Djordjevic, 2007). The targets can range from proteins (Griffin *et al.*, 1993b) to saccharides (Su *et al.*, 2007) as well as small organic molecules (Noeske *et al.*, 2006) and metal particles (Smirnov and Shafer, 2000). Aptamers are generated by passing a large library of random oligonucleotides over an immobilized target to select for sequences that bind. These lead aptamers are then amplified using the polymerase chain reaction to generate an enriched pool of oligonucleotides. This process is then repeated under increasingly stringent conditions to select for oligonucleotide sequences with high affinity for the target. This methodology is referred to as Systematic Evolution of Ligands by Exponential enrichment or SELEX

(Klug and Famulok, 1994). Using SELEX, aptamers have been developed as drugs to treat macular degeneration (Fraunfelder, 2005), HIV (Nimjee *et al.*, 2005b), and thrombosis (Chan *et al.*, 2008b, DeAnda, Jr. *et al.*, 1994).

Properties of aptamers

Aptamers fold into a secondary structure that forms a binding pocket specific for its target (reviewed in (Nimjee *et al.*, 2005b)). The aptamer-protein interface can be stabilized by a variety of interactions including salt bridges, hydrogen bonding, and van der Waals forces (Hermann and Patel, 2000). In addition, the 3-dimensional structure of aptamers are complementary to the topology of the protein surface, which enhances the specificity of the aptamer for its target (Hermann and Patel, 2000). Thus, aptamers are well-suited to be developed as anticoagulants.

In addition to the tight binding and high specificity for its target, aptamers have several properties that make them ideal anticoagulants. First, aptamers are non-immunogenic, as reported in a recent clinical trial for Macugen, the anti-VEGF aptamer (Eyetech Study Group, 2003). Second, although the plasma half-life of aptamers is short, they can be modified so as to prolong their half-life. Factors that influence aptamer stability in the circulation include renal clearance, 3'-exonuclease degradation, and the intrinsic instability of RNA. Strategies that can modify these processes include (a) adding polyethylene glycol (Chan *et al.*, 2008b) or streptavidin (Dougan *et al.*, 2000), modifications that reduce renal clearance, (b) development of circularized aptamers (Di Giusto and King, 2004) or addition of a 3'-cap, such as biotin (Dougan *et al.*, 2000) to protect against exonuclease degradation, and (c) use of DNA aptamers or modified

ribonucleotides in place of RNA (Dougan *et al.*, 2000). Third, complementary antidotes can be designed, which neutralize the anticoagulant properties of the aptamer (Rusconi *et al.*, 2002). Because aptamers rely on precise intramolecular interactions to maintain their three dimensional structure, a complementary oligonucleotide that binds the aptamer will denature this structure and prevent the aptamers from binding its target.

A major disadvantage of aptamers as anticoagulants is that they lack oral bioavailability. Because of this limitation, aptamer-based anticoagulants are best suited for short-term indications where rapid reversal is an asset. Examples include cardiopulmonary bypass surgery and dialysis (Nimjee *et al.*, 2006).

Anticoagulant aptamers

To-date, several anticoagulant aptamers have been developed. RB006 is an RNA-aptamer that binds to fIX/fIXa (Rusconi *et al.*, 2002) and blocks fIX activation by extrinsic tenase and fX activation by intrinsic tenase (Gopinath *et al.*, 2006). RB006, and its antidote, RB007, have completed phase 1 evaluation in healthy volunteers and the aptamer/antidote pair also has been studied in patients with stable coronary artery disease (Chan *et al.*, 2008a). RB006 produces rapid, predictable and dose-dependent anticoagulation that is immediately reversed upon administration of RB007.

An aptamer directed against fVIIa is a potent inhibitor of the extrinsic pathway of coagulation. This nuclease-resistant RNA aptamer binds fVII and fVIIa with high affinity (K_D value of 10 nM) and inhibits fX activation by the tissue factor/fVIIa complex in a dose-dependent manner (Rusconi *et al.*, 2000). Because it binds fVII with high

affinity, this aptamer is likely to be a potent anticoagulant. However, this aptamer has yet to be evaluated in humans.

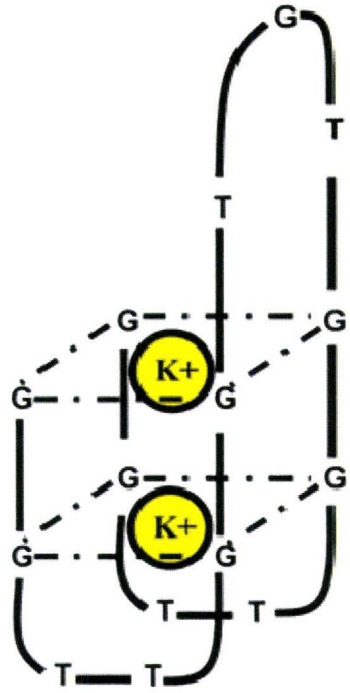
Aptamers have also been developed to influence the role of hematopoietic cells in thrombosis. For example, RNA aptamers that bind P-selectin (Jenison *et al.*, 1998), L-selectin (Watson *et al.*, 2000), or von Willebrand factor (Oney *et al.*, 2007) have been developed. Of these, the anti-vWF aptamer is the furthest along in clinical development (Gilbert *et al.*, 2007), and will next be tested in the setting of acute coronary syndromes.

Among the most widely studied aptamers are those that bind thrombin. These include HD1, a 15-oligonucleotide DNA aptamer that binds exosite 1, HD22, a 29-oligonucleotide DNA aptamer, which binds exosite 2.

HD1

HD1 is one of the most widely studied aptamers. HD1 was developed by Gibbs *et al* and was designed to bind thrombin exosite 1 and inhibit its capacity to clot fibrinogen and activate platelets (Griffin *et al.*, 1993b) (Figure 1.6) . The NMR structure of HD1 shows that it folds into a compact chair-like structure with anti-parallel strand orientation (Kelly *et al.*, 1996). This structure is stabilized, in part, by 2 intramolecular G-quartets. Recent evidence suggests that HD1 may form a dimer in solution (Fialova *et al.*, 2006). G-quartets can be stabilized by cations, such as K^+, positioned in the middle of each G-quartet. These cations reduce the repulsive forces that are generated from intramolecular folding of the negatively charged oligonucleotide (Wilcox *et al.*, 2008). In addition to metal cations, the secondary structure of HD1 is stabilized by thrombin (Baldrich and O'Sullivan, 2005). Although heating and rapid cooling induces G-quartet

A.



B.

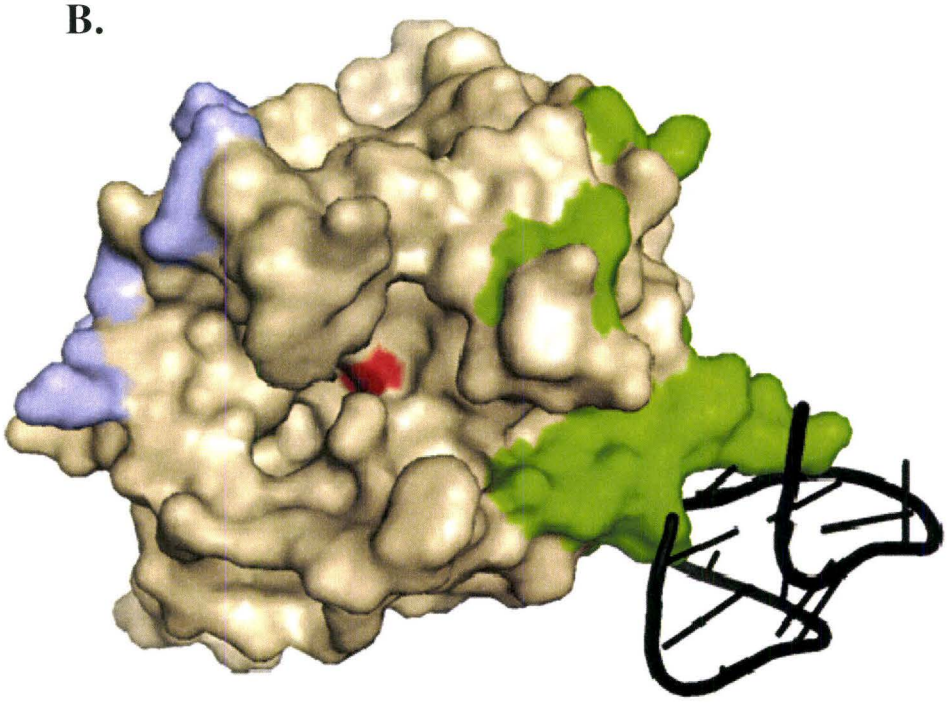


Figure 1.6: HD1 and its interaction with thrombin

A, HD1 is a DNA aptamer consisting of 15 nucleotides that folds into a chair-shaped secondary structure stabilized by 2 G-quartets. The repulsion forces of this secondary structure can be neutralized by potassium (K^+) ions, which localize to the center of each G-quartet and stabilize HD1. The solid lines depict covalent bonds, whereas the dashed lines represent hydrogen bonds holding the Hoogsteen base-paired guanine bases together within each G-quartet. *B*, HD1 (black) bound to exosite 1 (green) of thrombin is depicted, with exosite 2 shown in blue and the active site serine indicated in red. This image was prepared using Pymol with data obtained from Protein Data Bank (code: 1HUT).

formation in aptamers, recent work by Nagatoishi *et al* demonstrated that in the presence of thrombin, HD1 spontaneously forms a G-quartet (Nagatoishi *et al.*, 2007). Although Li^+ (Williamson *et al.*, 1989) and high concentrations of Na^+ disrupt G-quartets (Hianik *et al.*, 2007), the clinical utility of this information is limited.

The X-ray crystal structure of the HD1-thrombin complex demonstrates that the central T-G-T loop of HD1 interacts with thrombin exosite 1, whereas the 3' and the 5' terminal guanines are directed toward the surface of thrombin, but do not make direct contact with exosite 1 (Padmanabhan *et al.*, 1993). Scanning alanine-mutagenesis of thrombin exosite 1 reveals that surface arginine residues within exosite 1 are the most important contacts for HD1 (Tsiang *et al.*, 1995).

Because it inhibits thrombin activity, HD1 was tested as an anticoagulant in several different animal models. In a cardiopulmonary bypass model in dogs, a 0.5 mg/kg·min infusion of HD1 provided effective anticoagulation (DeAnda, Jr. *et al.*, 1994). In this model, HD1 had a circulation half-life of about 2 min and the clotting times returned to normal 5 min after stopping the aptamer infusion. Subsequent studies in monkey plasma demonstrated that HD1 is susceptible to 3'-exonuclease degradation (Lee *et al.*, 1995). Bio-distribution studies reveal the accumulation of HD1 in the kidneys and liver of rats, consistent with active clearance mechanisms (Reyderman and Stavchansky, 1998). Because HD1 has potential as an anticoagulant, further investigation into its mechanisms of action is warranted.

HD22

HD22 competes with heparin for binding to exosite 2 on thrombin. Crosslinking experiments reveal that HD22 makes significant contacts with Val^{573}-Gly^{578} of thrombin near exosite 2 (Tasset *et al.*, 1997). In clotting assays, HD22 produces only a 2-fold prolongation of thrombin clotting time. In contrast, in functional assays, HD22 causes a 45-fold reduction in the heparin-catalyzed rate of thrombin inhibition by antithrombin and a 3-fold reduction in the heparin-catalyzed rate of thrombin inhibition by HCII (Jeter *et al.*, 2004). Because exosite 2 is not directly involved in thrombin's interaction with bulk fibrin, HD22 was tested for its capacity to detect clot-bound thrombin (Dougan *et al.*, 2003). Despite successful detection of fibrin-bound thrombin *in vitro*, ^{125}I-HD22 was unable to reliably detect clot-bound thrombin in a rabbit jugular vein injury model. As a result, HD22 is best used as a ligand to study the role of exosite 2 in regulating thrombin activity (Mosesson, 2007, Segers *et al.*, 2007).

Coagulation assays

In order to study coagulation in the laboratory or in patients, global tests that assess the integrity of the extrinsic and intrinsic pathways of coagulation are useful. Such tests are used to (a) identify acquired or hereditary deficiencies of clotting factors, (b) monitoring anticoagulants, and (c) comparing the potency of various anticoagulants. Although coagulation assays typically use clot formation as their end-point, newer techniques monitor thrombin generation on a continuous basis.

Clot-based assays

The integrity of the coagulation system can be tested by measuring the clotting times of re-calcified citrated plasma after initiation of coagulation by agents that trigger the extrinsic or intrinsic system (Bates and Weitz, 2005).

The prothrombin time (PT) assesses the extrinsic and common pathway. Clotting is initiated by addition of calcium and relipidated tissue factor or thromboplastin. Deficiencies in fVII, fX, fV, prothrombin, or fibrinogen can cause a prolongation of the PT. In addition, anticoagulants such as warfarin or rivaroxaban also can be monitored using the PT.

The activated partial thromboplastin time (aPTT) assesses the intrinsic and common pathways. The test involves preincubation of plasma with a contact activator, such as silica or kaolin, and phospholipid prior to calcium addition. Deficiencies in fXII, prekallikrein, high molecular weight kininogen, fXI, fIX, fVIII, fX, fV, prothrombin, or fibrinogen can prolong the aPTT. This test can be used to screen patients for haemophilia or to monitor heparin or hirudin.

Although clot-based assays are widely used, they provide only limited information about the dynamics of thrombin generation because clotting occurs with as little as 2 nM thrombin, which represents only 2-5% of total thrombin production (Brummel *et al.*, 2002). To overcome this problem, thrombin generation assays have been developed to monitor the dynamics of thrombin production in plasma. Although these assays are mostly used for research purposes, recent studies suggest that they might provide information about the severity of hereditary bleeding disorders (Trossaert *et al.*,

2008), or the susceptibility of patients to acute coronary syndromes (Brummel-Ziedins *et al.*, 2008).

Thrombin generation assay

The thrombin generation assay (TGA) measures thrombin activity over the entire interval where thrombin is active. This covers the initial period where clotting occurs at low nM levels of thrombin to the late phase where activation has ceased and thrombin is consumed by inhibitors. The TGA is initiated by addition of tissue factor and Ca^{2+} to citrated plasma. The assay depends on a fluorogenic substrate that reacts specifically with thrombin. The substrate z-Gly-Gly-Arg-AMC (7-amino-4-methoxy coumarin) has a higher K_m and a lower k_{cat} relative to conventional thrombin substrates, such as Tosyl-Phe-Pro-Arg-p-Nitroanilide (Devreese *et al.*, 2007). This substrate allows thrombin activity to be monitored over extended periods of time (hours) without being entirely consumed. For a number of reasons, each experimental condition must be calibrated with a known concentration of thrombin. The calibrator corrects for fluorogenic substrate consumption, quenching of the fluorescent signal, as well as residual thrombin chromogenic activity from the thrombin-α2-macroglobulin complex (DE Smedt *et al.*, 2008, Hemker *et al.*, 2007).

Stages of thrombin generation

The thrombin generation profile follows a specific biphasic pattern, which includes (a) a lag phase, where thrombin activity is undetectable, (b) a propagation phase, where the concentration of thrombin increases rapidly, and (c) a termination phase, where

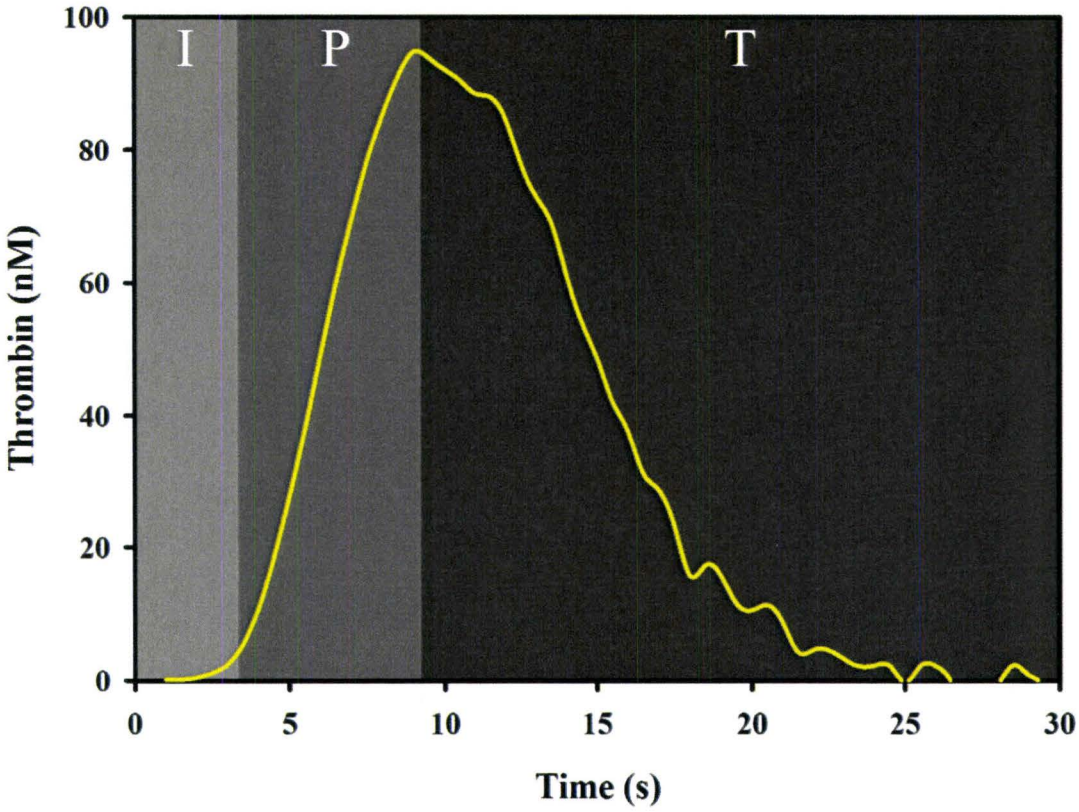

I
P
T
Thrombin (nM)
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (s)

Figure 1.7: The phases of thrombin generation

The thrombin activity in plasma during a typical thrombin generation assay is depicted. Thrombin generation can be divided into 3 distinct phases. The initiation phase (I) is defined as the time between initiation of thrombin generation and the generation of 2 nM active thrombin. The propagation phase (P) is marked by rapid thrombin generation effected by the assembly of the intrinsic tenase and prothrombinase complexes. Once the concentration of thrombin reaches a maximum, the termination phase (T) begins.

the concentration of thrombin decreases rapidly (Figure 1.7). Variations in the timing and magnitude of the thrombin signal during each of these phases are reflective of procoagulant or anticoagulant forces that influence thrombin generation.

Initiation phase

The initiation or lag phase occurs early in the thrombin generation, where the concentration of thrombin is close to zero. During this time, the tissue factor/fVIIa complex assembles and activates fX and fIX. FXa produces only small amounts of thrombin because fVa is lacking. This thrombin then activates fV and fVIII, which promotes assembly of the intrinsic tenase and prothrombinase complexes, leading to a burst of thrombin production in the propagation phase (reviewed in (Mann *et al.*, 2003b)).

Initial work by Butenas *et al* described the sequence of events during the initiation phase by using a reconstituted coagulation proteome and a series of fluorogenic substrates that could detect thrombin, fXa, and fVIIa at concentrations in the fM-pM range (Butenas *et al.*, 1997). This landmark study showed that during the initiation phase (a) the fVa heavy chain is rapidly produced but generation of the light chain is delayed, (b) thrombin is the only relevant activator of fV because hirudin blocked its activation, (c) fXa is only generated by the TF/fVIIa complex because fXa generation in the absence of fVIII was the same as that observed in its presence, and (d) fXa alone cannot convert prothrombin into thrombin, suggesting that the procofactor fV plays a role in prothrombin activation in the initial stages of thrombin generation (Butenas *et al.*, 1997). Because the initiation phase is driven by TF/fVIIa, tissue factor pathway inhibitor (TFPI) is the most relevant inhibitor during the initial stages of thrombin generation. However,

once fVIIIa is formed and the intrinsic tenase complex is assembled, TF/fVIIa plays a minimal role in thrombin generation (van't Veer and Mann, 1997). As a result, the rate at which TFPI inhibits TF/fVIIa creates a threshold barrier for thrombin generation because it limits the transition from initiation to propagation to instances where TF is in abundance. The importance of TFPI to coagulation is noted by the fact that $TFPI^{-/-}$ mice die during embryonic development (Huang *et al.*, 1997).

Propagation phase

The propagation phase, which is also called the consolidation phase, occurs after initiation and is marked by rapid thrombin formation. Rapid thrombin generation requires assembly of the prothrombinase complex. Because fV is fully activated during the initiation phase, the limiting component of the prothrombinase complex is fXa (Butenas *et al.*, 1997). The increased demand for fXa is provided by the intrinsic tenase complex, which activates fX more efficiently than extrinsic tenase. Greater fXa production leads to assembly of prothrombinase and a subsequent burst of thrombin.

Clot formation occurs at the beginning of the propagation phase when approximately 2 nM thrombin has been generated (Brummel *et al.*, 2002). Thus, the majority of thrombin is generated after clot formation. The presence of fibrin causes a prolongation of the lag time, a higher peak thrombin concentration and lower levels of α_2-macroglobulin-thrombin complexes (Hemker *et al.*, 2003, Hemker *et al.*, 2005). These data suggest that fibrin-bound thrombin is resistant to inhibition, consistent with previous reports (Becker *et al.*, 1999). Recently, the rate of thrombin formation during the initiation phase was shown to be temperature dependent, whereas it was not

temperature dependent after clot formation (Hemker *et al.*, 2005). Thus, thrombin generation during the initiation phase is controlled by the chemical reaction rates, whereas during the propagation phase it is regulated by rates of diffusion. These data demonstrate that fibrin regulates thrombin generation during the propagation phase.

Termination phase

Antithrombin, HCII, and α_2-macroglobulin continuously inhibit thrombin. However, during the propagation phase, thrombin generation exceeds inhibition. The depletion of prothrombin leads to a reduction in the rate of thrombin generation. When the rates of thrombin generation and inhibition are equivalent, the thrombin concentration reaches a peak (van't Veer and Mann, 1997). The rate of thrombin inhibition then exceeds the rate of formation, called the termination phase.

Limitations of the CAT assay

Despite the interest in the CAT assay in basic and clinical research, the test has certain inherent limitations. The most significant limitation of the TGA is the competitive inhibition of thrombin by the fluorogenic substrate (Butenas and Mann, 2007). The influence of substrate concentration influences the lag time and the peak thrombin (Hemker *et al.*, 2006b). Thus, the fluorogenic substrate may compete with thrombin feedback activation of fV and fVIII, and with serpins for the active site of thrombin. An alternate approach is to measure the levels of thrombin-antithrombin complexes or fragment 1.2 in plasma. However, the levels of these biomarkers are affected by the quality of the blood collection and the assays are tedious and time-consuming (Brummel-Ziedins *et al.*, 2005).

Chapter 2: Hypotheses and Aims

Hemostasis, or maintenance of the flow and stability of the circulatory system, is dependent on the generation and regulation of thrombin. If thrombin generation at sites of injury is insufficient, bleeding occurs. In contrast, if an excess of thrombin is generated, thrombosis occurs.

Anticoagulants dampen thrombin generation or activity. This can be achieved by (a) inhibiting prothrombin activation by prothrombinase, and/or (b) directly inhibiting thrombin. We hypothesized that an agent that binds to exosite 1 on thrombin and the corresponding domain on prothrombin would not only inhibit thrombin activity, but also inhibit prothrombin activation by prothrombinase. To test this hypothesis we examined whether HD1, an aptamer known to bind exosite 1 on thrombin, also binds prothrombin and, if so, whether its activity would be greater than that of an agent that only binds to thrombin. In addition, building on the observation that the complementary aptamer RB007 neutralizes the anticoagulant activity of RB006, a factor IXa-directed aptamer, we hypothesized that a complementary aptamer could be developed that would neutralize the activity of HD1. To test this hypothesis, we synthesized and characterized antiHD1, a complementary aptamer against HD1.

Objectives

1. To determine the specificity of HD1 and HD22 for thrombin and prothrombin
2. To compare the effects of HD1 and $Hir^{54\text{-}65}(SO_3^-)$ on thrombin generation
3. To synthesize antiHD1 and determine its capacity to neutralize the anticoagulant activity of HD1

Chapter 3: HD1, a thrombin-directed aptamer, binds exosite 1 on prothrombin with high affinity and inhibits its activation by prothrombinase

Foreword

This manuscript has been published in the Journal of Biological Chemistry (Vol.281, NO.49, pp.37477-37485, December 8, 2006). The authors are: Colin A. Kretz, Alan R. Stafford, James C. Fredenburgh, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz. According to the policies of the American Society of Biochemistry and Molecular Biology, no formal permission is required to reproduce this manuscript for non-commercial purposes.

All of the experiments contained within this manuscript were performed by me. Furthermore, this manuscript was drafted by me. Chapter 3 is a direct representation of the above referenced manuscript.

Summary

Incorporation of prothrombin into the prothrombinase complex is essential for rapid thrombin generation at sites of vascular injury. Prothrombin binds directly to anionic phospholipid membrane surfaces where it interacts with the enzyme, factor Xa, and its cofactor, factor Va. We demonstrate that HD1, a thrombin-directed aptamer, binds prothrombin and thrombin with similar affinities (K_D values of 86 nM and 34 nM, respectively), and attenuates prothrombin activation by prothrombinase by over 90% without altering the activation pathway. HD1-mediated inhibition of prothrombin activation by prothrombinase is factor Va-dependent because (a) the inhibitory activity of HD1 is lost if factor Va is omitted from the prothrombinase complex, and (b) prothrombin binding to immobilized HD1 is reduced by factor Va. These data suggest that HD1 competes with factor Va for prothrombin binding. Kinetic analyses reveal that HD1 produces a 2-fold reduction in the k_{cat} for prothrombin activation by prothrombinase and a 6-fold increase in the K_m, highlighting the contribution of the factor Va-prothrombin interaction to prothrombin activation. As a high affinity, prothrombin exosite 1-directed ligand, HD1 inhibits prothrombin activation more efficiently than $Hir^{54-65}(SO_3^-)$. These findings suggest that exosite 1 on prothrombin exists as a proexosite only for ligands whose primary target is thrombin rather than prothrombin.

Introduction

Thrombin is the most versatile component of the hemostatic system, mediating procoagulant, anticoagulant, and anti-fibrinolytic pathways (Di Cera, 2003). The diverse activities of thrombin are regulated, at least in part, by electropositive exosites flanking its active site (Lane *et al.*, 2005). Exosite 1 binds ligands that interact with the active site of thrombin; including fibrinogen, heparin cofactor II, and protease-activated receptor, the major thrombin receptor on cells (Lane *et al.*, 2005). In contrast, exosite 2, which binds ligands such as heparin (De Cristofaro *et al.*, 1998, Fortenberry *et al.*, 2004) and platelet glycoprotein Ibα (Adam *et al.*, 2003, De Candia *et al.*, 2001, De Cristofaro *et al.*, 2000), serves to tether thrombin for subsequent interactions with substrates or inhibitors.

Prothrombin, the precursor of thrombin, lacks an active site and has immature or inaccessible exosites (Anderson *et al.*, 2000a, Kaczmarek *et al.*, 1987, Ni *et al.*, 1993). Because exosite 1 on prothrombin exhibits reduced affinity for certain ligands, it has been designated proexosite 1 (Anderson *et al.*, 2000a). This proexosite gains functional activity during prothrombin conversion to thrombin, as evidenced by fluorescent ligand binding studies (Anderson and Bock, 2003). Thus, Anderson *et al.* reported that fluorescein-hirudin$^{54\text{-}65}(SO_3^-)$ [f-Hir$^{54\text{-}65}(SO_3^-)$][1], the exosite 1-binding COOH-terminus of hirudin, binds thrombin with an affinity 130-fold higher than that for prothrombin (K_D values of 25 nM and 3.2 μM, respectively) (Anderson and Bock, 2003). Prothrombin activation intermediates display intermediate affinities for f-Hir$^{54\text{-}65}(SO_3^-)$ that increase with the extent of activation (Anderson *et al.*, 2003, Anderson and Bock, 2003).

Diminished affinity of other thrombin ligands for proexosite 1 on prothrombin also has been observed (Fischer *et al.*, 1998, Wu *et al.*, 1994).

In contrast to the progressive maturation of proexosite 1, exosite 2 displays more abrupt development. Exosite 2 is not accessible until fragment 2 (F2) is released from prothrombin. Thus, prethrombin 2 (pre2) and thrombin have similar affinities for heparin, whereas meizothrombin (mIIa) and meizothrombin des F1 [mIIa(-F1)], which retain the F2 domain, do not bind heparin (Schoen and Lindhout, 1987).

Understanding the functional maturation of the exosites on thrombin has increased in importance with emerging evidence that the exosites serve not only as binding domains, but also as allosteric regulators of thrombin activity (Fredenburgh *et al.*, 1997, Mengwasser *et al.*, 2005, Rezaie and Yang, 2003). Numerous studies reveal that ligand binding to either exosite can modify the activity of thrombin. Thus, peptide (Hortin and Trimpe, 1991, Mengwasser *et al.*, 2005), glycosaminoglycan (Cosmi *et al.*, 2003, Pike *et al.*, 2005), and nucleotide (Griffin *et al.*, 1993b) ligands have all been shown to modulate thrombin. Thrombin-binding DNA aptamers represent a unique class of ligand. These engineered oligonucleotides, which fold into characteristic secondary structures, form binding pockets for specific ligands (Mao and Gmeiner, 2005, Schultze *et al.*, 1994). In the case of thrombin, aptamer HD1 binds selectively to exosite 1 (Bock *et al.*, 1992), whereas aptamer HD22 binds to exosite 2 (Dougan *et al.*, 2003). Because of their high affinity and selective binding, HD1 and HD22 serve as useful tools to probe the structure-function relationship of the exosites on thrombin. The crystal structure of the thrombin-HD1 complex has been defined (Padmanabhan *et al.*, 1993), as has the

structure of the thrombin-Hir$^{54\text{-}65}$(SO_3^-) complex (Vijayalakshmi *et al.*, 1994). These structures suggest that the two exosite 1-directed ligands bind to overlapping, but discrete, subdomains. Thus, Hir$^{54\text{-}65}$(SO_3^-) largely interacts with the hydrophobic cleft of exosite 1, whereas HD1 binds to charged residues surrounding this cleft. Given their distinct binding sites on thrombin, it is possible that HD1 and Hir$^{54\text{-}65}$(SO_3^-) do not exhibit identical interactions with prothrombin and its intermediates. To explore this possibility, we used HD1 and Hir$^{54\text{-}65}$(SO_3^-) to examine the functional maturation of exosite 1 and HD22, the exosite 2-binding DNA aptamer, to report exosite 2 maturation.

Materials

Reagents:

Human prothrombin, thrombin, and factor Xa, were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Factor Va and dansylarginine N-(3-ethyl-1,5-pentanediyl) amide (DAPA) were from Haematologic Technologies, Inc (Essex Junction, VT). D-Phe-Pro-Arg chloromethyl ketone (FPRck) and 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone (dEGR) were obtained from Calbiochem (San Diego, CA). Fluorescein isothiocyanate (FITC) was from Sigma (Oakville, Ontario, Canada). HD1 (5'-GGTTGGTGTGGTTGG-3'), HD22 (5'-AGTCCGTGGTGGTAGGGCAGGT TGGGGTGACT-3'), the exosite 1 and 2-directed DNA aptamers, respectively, and HD23 (5'-AGTCCGTAAAGCAGGTTAAAATGACT-3'), a scrambled oligonucleotide sequence of HD22, and their 3' FITC- or biotin-labelled counterparts, were synthesized

by the Molecular Biology and Biotechnology Institute at McMaster University (Hamilton, ON, Canada). Before use, all aptamers were subjected to renaturation by heating to 95°C for 5 min followed by cooling on ice for 10 min (Griffin *et al.*, 1993b). Ecarin, a snake venom protein derived from *Echis carinatus*, was from Pentapharm (Basel, Switzerland). DEAE-Sepharose, PD-10 Sephadex, G-10 Sephadex, Mono-Q Sepharose, and SP-C50 Sephadex were obtained from GE Healthcare (Dorval, Quebec, Canada). Recombinant tick anticoagulant peptide, a factor Xa-directed inhibitor, was a generous gift from Dr. G. Vlasuk, Corvas International, Inc. (San Diego, CA). Hirudin and its Tyr^{63}-sulfated COOH-terminal peptide, $Hir^{54-65}(SO_3^-)$, were from Bachem (King of Prussia, PA). Chromozym Thrombin (Chz-Th) was from Roche (Indianapolis, IN), whereas S2765 and S2238 were from Chromogenix (Milano, Italy).

L-α-phosphatidyl-L-serine (PS) from bovine brain and L-α-phosphatidyl-choline (PC) type III-E from egg yolk were from Avanti Polar Lipids Inc. (Alabaster, AL) and Sigma, respectively. PCPS vesicles were synthesized using a modification of previously published methods (Barenholz *et al.*, 1977, Bloom *et al.*, 1979). A phosphate assay was used to determine the concentration of PCPS vesicles (Ames, 1966). Vesicles were stored at -80°C in 10 % sucrose.

Labelled proteins:

To label $Hir^{54-65}(SO^-_3)$ with FITC, 0.11 mg of peptide was dissolved in 250 µl 0.2 M Na_2HCO_3 buffer, pH 9.0, and 20 µl FITC (25.7 µM in DMSO) was added to a final concentration of 2 µM. After wrapping the mixture in aluminum foil and mixing the

sample end-over-end for 90 min at 23°C, 20 µl of 1 M NH_4Cl was added to stop the reaction. The sample was then applied to a 10 ml G10 Sephadex column that was pre-treated with 5 mg/ml ovalbumin and washed with 20 mM Tris-HCl, 0.15 mM NaCl, pH 7.4 (TBS). 0.5-ml fractions were collected and the fluorescent fraction, which was identified by monitoring the effluent with a UV light, was recovered in a single tube. Absorbance of the fractions was determined at 492 nm and protein concentrations were calculated based on $\varepsilon = 6.8x\ 10^4\ M^{-1}cm^{-1}$ (Mercola *et al.*, 1972), assuming 1:1 incorporation of FITC into $Hir^{54-65}(SO_3^-)$.

Methods

Preparation of prothrombin activation intermediates: All prothrombin activation intermediates were prepared using modifications of published methods (Church et al., 1991, Mann, 1976). The progress of each reaction was monitored using SDS-PAGE analysis (Laemmli, 1970) on 4-15% acrylamide Ready Gels (Bio-Rad, Hercules, CA) under reducing and non-reducing conditions.

Pre1 was prepared by incubating prothrombin (1 mg/ml) with 200 nM thrombin in 17 mM imidazole-HCl, 144 mM NaCl, pH 7.4 for 2 h at 37 °C. The reaction was terminated by addition of 500 nM FPR-ck and the absence of residual thrombin activity was confirmed using Chz-Th. The sample was then subjected to chromatography on a 5 ml DEAE Sepharose column.

Pre2 was prepared by digesting pre1 with factor Xa. Briefly, 1 mg/ml pre1 in 25 % trisodium citrate buffer (pH 8.8) was incubated with 50 nM factor Xa for 45 min at

37°C. The reaction was terminated by addition of 1 μM FPR-ck and 1 μM dEGR and inhibition of the residual thrombin or factor Xa activity was confirmed with Chz-Th or S2765, thrombin- and factor Xa-directed substrates, respectively. The mixture was then subjected to chromatography on a SP-C50 Sephadex column.

mIIa and mIIa(-F1) were generated by treating prothrombin or pre1, respectively, with Ecarin. Reactions were conducted in the presence of FPR-ck to prevent autocatalytic cleavage. Prothrombin or pre1 (1 mg/ml in TBS) was incubated with 250 μg/ml Ecarin diluted in 10 mM HEPES, 150 mM NaCl, pH 7.0 containing 25 mM $CaCl_2$ in the presence of 20 μM FPR-ck for 3 h at 37°C. Samples were then subjected to anion exchange chromatography on a Q-Sepharose column.

Protein concentrations were determined using the following extinction coefficients: $\varepsilon = 1.64$, $\varepsilon = 1.95$, $\varepsilon = 1.44$, and $\varepsilon = 1.64$ $ml \cdot mg^{-1} \cdot cm^{-1}$ for pre1, pre2, mIIa, and mIIa(-F1), respectively (Rabiet *et al.*, 1986). All proteins were concentrated using an Amicon Centriprep YM-10 (Beverly, MA). The integrity of each of the prothrombin intemediates was assessed by SDS-PAGE (Appendix 3.1) and aliquots were stored at -80°C.

Binding studies:

Functional assessment of exosites 1 and 2 was performed using fluorescein-labeled HD1 (f-HD1) and HD22 (f-HD22), respectively. f-HD1 or f-HD22 (30 nM), diluted in TBS containing 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$ and 0.1 % polyethylene glycol (aptamer buffer), was added to 10 x 4 mm quartz cuvettes maintained

at 23°C with a circulating water bath and stirred using a micro stir bar. Fluorescence was monitored at an emission wavelength of 535 (cut-off filter set at 520 nm) and slit width of 8 nm, with excitation wavelength at 492 nm and a slit width of 6 nm, using a Perkin Elmer LS 50B luminescence spectrophotometer (Wellesley, MA). After allowing the baseline fluorescence (Io) to stabilize, aptamers were titrated with 2-20 µl aliquots of solutions containing 10 µM of prothrombin, prothrombin intermediates, or thrombin allowing the fluorescence signal to stabilize between each titrant addition. To prevent fluorophore dilution, the titrant contained 30 nM of fluorescent aptamer. Once the fluorescence signal reached a plateau, intensity values (I) were obtained from time drive profiles.

After plotting I/I_o as a function of the protein titrant concentration, data were fit by nonlinear regression using Table Curve (Jandel Scientific, San Rafael, CA) to the equation:

$$\frac{\alpha}{2}\left(1+\frac{K_d+P}{A_o}-\sqrt{\left(1+\frac{K_d+P}{A_o}\right)^2-4\cdot\frac{P}{A_o}}\right)$$

where P is the concentration of the titrated protein, A_o is the concentration of aptamer, α is the maximal fluorescence change, and K_D is the dissociation constant.

Competition experiments were performed to examine the capacity of $Hir^{54\text{-}65}(SO_3^-)$ to displace f-HD1 from thrombin or prothrombin. f-HD1 (60 nM), in 900 µl aptamer buffer, was added to the cuvette and fluorescence was monitored. Prothrombin or thrombin was then added to final concentrations of 220 nM or 110 nM, respectively.

After the fluorescence signal stabilized, samples were titrated with 10-25 µl aliquots of 30 µM $Hir^{54-65}(SO_3^-)$. For all titrations, titrant solutions contained 60 nM f-HD1 to prevent fluorophore dilution. Reciprocal titrations were performed in a similar fashion where 1.1 µM prothrombin or 110 nM thrombin was added to 30 nM f-$Hir^{54-65}(SO_3^-)$, and the samples were then titrated with aliquots of 10 µM HD1 containing 30 nM f-$Hir^{54-65}(SO_3^-)$.

Surface Plasmon Resonance (SPR):

SPR was used as an independent method to examine the interaction of HD1 with prothrombin. Biotinylated HD1 (b-HD1) was immobilized on a SA sensor chip, which is coated with streptavidin. To increase streptavidin reactivity, the flow cells were first washed 3 times with 1 M NaCl, 50 mM NaOH at a flow rate of 10 µl/min using a BIAcore 1000 (Piscataway, NJ). b-HD1 (250 nM) was then passed through the flow cells at 5 µl/min for 10 min, after which the cells were washed with BIAcore aptamer buffer at 10 µl/min for 2 min. To examine whether factor Va competes with b-HD1 for prothrombin binding, a sample containing 300 nM prothrombin was injected into a b-HD1-coated flow cell in the absence or presence of 1000 nM factor Va and Req values were recorded under 'kinetic' or 'quickinject' modes. To correct for non-specific DNA-protein binding, Req values obtained when samples were passed over a b-HD23 flow cell were subtracted. Binding of prothrombin to HD1 in the presence of factor Va was then expressed as a percentage of that measured in its absence.

Prothrombin activation experiments:

A discontinuous assay system was used to examine the effect of HD1 or $Hir^{54-65}(SO_3^-)$ on prothrombin activation by prothrombinase using a modification of a previously published method (Anderson et al., 2001). A 10x stock solution of prothrombinase, consisting of 60 μM PCPS, 2.5 nM factor Xa, and 6 nM factor Va, diluted in aptamer buffer was preincubated for 10 min at 23 °C. 1 μM prothrombin was incubated with HD1 or $Hir^{54-65}(SO_3^-)$, in concentrations ranging from 0 to 2.5 μM, for 5 min in a series of wells in a 96 well plate (90 μl volume). To start the reactions, 10 μl of the prothrombinase stock solution was added to each prothrombin-containing well. At intervals up to 10 min, individual reactions were terminated by addition of 4.5 μl of a solution containing 5.5 μM tick anticoagulant peptide and 200 mM EDTA. Generated thrombin was quantified by measuring hydrolysis of 600 μM Chz-Th at 405 nm for 10 min using a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA). Rates of substrate cleavage (mOD/min), as determined by instrument software, were used to calculate thrombin concentration based on the specific activity of thrombin cleavage of Chz-Th, as determined in a separate experiment. By plotting thrombin concentration versus time, rates of prothrombin activation were determined. In some experiments, the effect of HD1 on prothrombin activation by factor Xa was measured in the absence of factor Va or PCPS. For these studies, 1 μM prothrombin was activated with either 50 nM factor Xa and 6 μM PCPS, 0.25 nM factor Xa and 0.3 nM factor Va, or with 2 nM factor Xa and 20 nM factor Va. As a control, the effect of HD1 on Ecarin-mediated

prothrombin activation also was examined. For these studies, 1 μM prothrombin was activated with 100 μg/ml Ecarin in the absence or presence of 10 μM HD1 and thrombin generation was measured as described above.

To examine the effect of HD1 on the kinetics of prothrombin activation by prothrombinase, prothrombin (in concentrations ranging from 0 to 8 μM) was incubated with prothrombinase (0.25 nM factor Xa, 0.6 nM factor Va, and 5 μM PCPS) in the absence or presence of 25 μM HD1 for varying intervals up to 60 sec. Initial rates of thrombin production (nM/s) were plotted versus prothrombin concentration (nM) and fit by nonlinear regression to the Michaelis-Menten equation:

$$V = (Vmax) X (S)/(Km + S)$$

where K_m is the Michaelis-Menten constant, and V_{max} is the maximum rate of prothrombin activation (nM IIa/s). k_{cat} was calculated by dividing V_{max} by the factor Xa concentration.

SDS-PAGE analysis of prothrombin activation:

To examine the effect of HD1 on the prothrombin activation pathway, activation intermediates generated in the absence or presence of HD1 were assessed by SDS-PAGE. Prothrombin (14 μM) was incubated with 70 μM DAPA in the absence or presence of 50 μM HD1. Reactions were initiated by addition of the preassembled prothrombinase complex, consisting of 2 nM factor Xa, 20 nM factor Va, and 6 μM PCPS vesicles (final concentrations) diluted in aptamer buffer. At intervals, 5 μl-aliquots were removed into

sample buffer, boiled for 2 min, and subjected to SDS-PAGE analysis under reducing and non-reducing conditions.

Statistical methods:

Unless otherwise indicated, experiments were performed at least 3 times. Results are presented as the mean ± standard error of the mean.

Results

Competitive binding of exosite 1 ligands to thrombin and prothrombin:

Inspection of the crystal structures of thrombin in complex with $Hir^{54-65}(SO_3^-)$ or with HD1 suggests that these exosite 1-directed ligands interact with distinct, but partially overlapping, domains on thrombin (Padmanabhan *et al.*, 1993, Vijayalakshmi *et al.*, 1994). Whether these ligands interact with the same domains on prothrombin as they do on thrombin is currently unknown. To begin to address this, we first examined the capacity of $Hir^{54-65}(SO_3^-)$ to displace f-HD1 from prothrombin or thrombin. As illustrated in Figure 3.1A, the fluorescence intensity increases by 17 ± 1.1 % when 110 nM thrombin is added to a cuvette containing 30 nM f-HD1. This increase in fluorescence intensity is negated when unlabeled HD1 is added (data not shown), consistent with reversible binding. The fluorescence intensity value also returns to baseline when the f-HD1-thrombin complex is titrated with $Hir^{54-65}(SO_3^-)$, suggesting that the $Hir^{54-65}(SO_3^-)$ binding site on thrombin overlaps with that of HD1 (Figure 3.1A). In

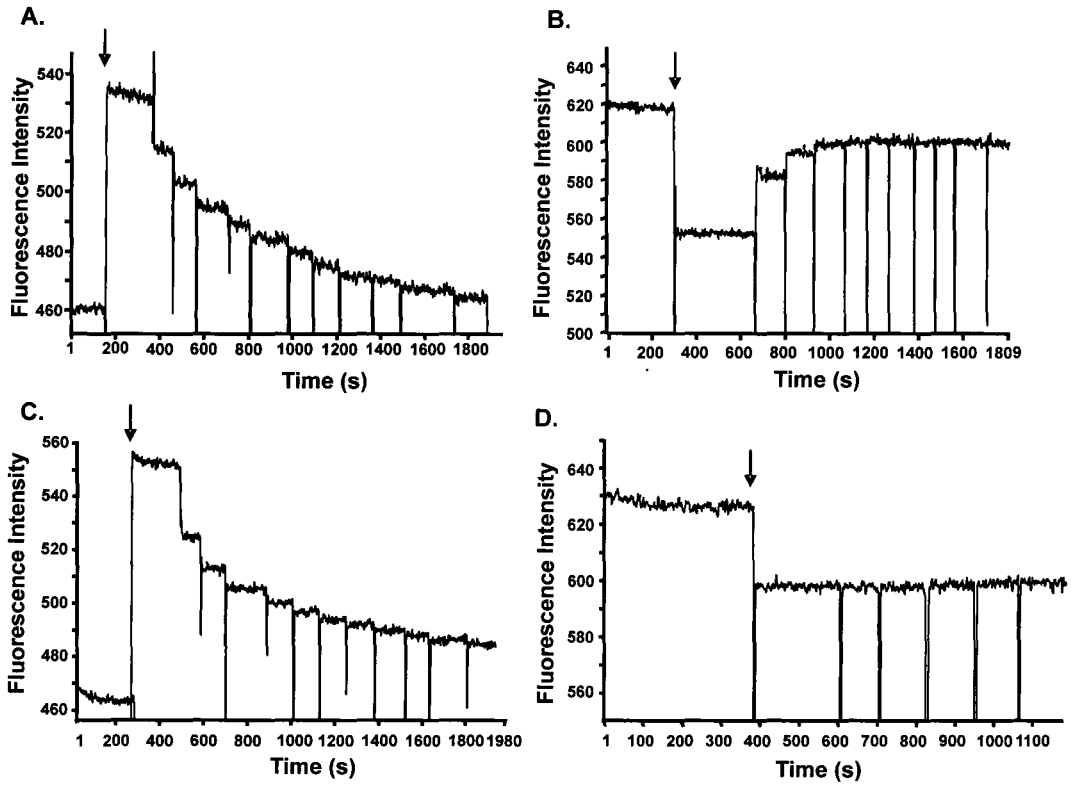

A.
Fluorescence Intensity
540
520
500
480
460
1 200 400 600 800 1000 1200 1400 1600 1800
Time (s)
B.
Fluorescence Intensity
640
620
600
580
560
540
520
500
1 200 400 600 800 1000 1200 1400 1600 1809
Time (s)
C.
Fluorescence Intensity
560
540
520
500
480
460
1 200 400 600 800 1000 1200 1400 1600 1800 1980
Time (s)
D.
Fluorescence Intensity
640
620
600
580
560
1 100 200 300 400 500 600 700 800 900 1000 1100
Time (s)

Figure 3.1: Competition between HD1 and $Hir^{54-65}(SO_3^-)$ for binding exosite 1 on thrombin and prothrombin

Competitive binding studies were conducted to assess binding differences between HD1 and the canonical exosite 1 ligand, $Hir^{54-65}(SO_3^-)$. A cuvette containing 30 nM f-HD1 was allowed to equilibrate and fluorescence was monitored at excitation and emission wavelengths of 492 and 535 nm, respectively. At the time indicated by the arrow, 110 nM thrombin (*A*) or 220 nM prothrombin (C) was added to the cuvette and the mixture was then titrated with a fluorophore-balanced solution of 10 μM $Hir^{54-65}(SO_3^-)$ up to final concentrations of 1.2 and 7 μM, respectively. In reverse titrations, the complex of 30 nM f-$Hir^{54-65}(SO_3^-)$ with 110 nM thrombin (B) or 1.1 μM prothrombin (D) was titrated with a balanced solution of 10 μM HD1 up to final HD1 concentrations of 1 μM.

the reciprocal experiments, addition of thrombin to f-Hir$^{54-65}$(SO_3^-) results in a 10 ± 2.8 % reduction in fluorescence intensity (Figure 3.1B). When the f-Hir$^{54-65}$(SO_3^-)-thrombin complex is titrated with HD1, the fluorescence intensity increases, but does not return to baseline. These findings suggest that the HD1 binding site on thrombin only partially overlaps with the Hir$^{54-65}$(SO_3^-) binding site.

Studies were then repeated using prothrombin in place of thrombin. As illustrated in Figure 3.1C, addition of prothrombin to f-HD1 results in a 16 ± 0.5 % increase in fluorescence intensity. Upon titration with Hir$^{54-65}$(SO_3^-), the fluorescence intensity decreases but does not reach baseline levels. In the reverse experiment, fluorescence intensity decreases by 5 ± 0.4 % when prothrombin is added to f-Hir$^{54-65}$(SO_3^-). There is no change in fluorescence intensity when the f-Hir$^{54-65}$(SO_3^-)-prothrombin complex is titrated with HD1, suggesting that the HD1 binding site on prothrombin does not overlap with the f-Hir$^{54-65}$(SO_3^-) binding site. Addition of unlabelled Hir$^{54-65}$(SO_3^-) to the cuvette resulted in fluorescence returning to baseline confirming reversibility (data not shown). These data suggest that prothrombin binds both exosite 1 ligands and that the Hir$^{54-65}$(SO_3^-) binding site on prothrombin only partially overlaps with the HD1 binding site.

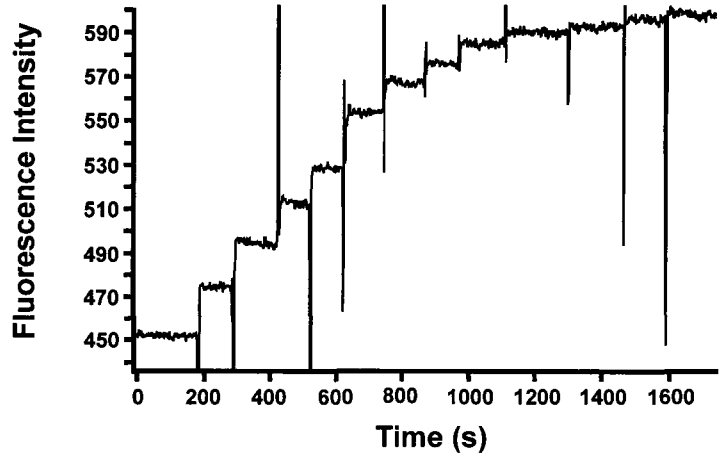
Binding of aptamers to prothrombin derivatives:

Because the change in fluorescence intensity that occurs when f-HD1 complexes with prothrombin is similar in magnitude to that which occurs when it binds thrombin, we measured the affinity of f-HD1 for prothrombin, prothrombin intermediates, and thrombin. The fluorescence intensity of 30 nM f-HD1 was monitored before and after titration with prothrombin, prothrombin intermediates, or thrombin, and the relative

changes in intensity signal were plotted versus protein concentration. The addition of prothrombin to f-HD1 results in a maximal 33 ± 1.7 % increase in fluorescence intensity and yields a saturable curve with a K_D value of 86 ± 8.4 nM (Figure 3.2). Thus, the affinity of f-HD1 for prothrombin is much higher than that of $Hir^{54-65}(SO_3^-)$ (below) and is comparable to the K_D value of 40 nM reported for domain 2 of staphylocoagulase (Panizzi *et al.*, 2006). Binding experiments were subsequently performed to measure the affinity of f-HD1 for prothrombin derivatives. As outlined in Table 3.1, f-HD1 binds mIIa, mIIa(-F1), pre1, and pre2 with K_D values of 35 ± 5.5, 45 ± 0.3, 86 ± 1.0, and 66 ± 16 nM, respectively. F-HD1 binds thrombin with a K_D value of 34 ± 4.8 nM. Thus, f-HD1 binds prothrombin and all its activation intermediates with an affinity similar to that for thrombin. These findings suggest that the HD1 binding site on prothrombin undergoes little structural change during prothrombin conversion to thrombin.

Studies were then done using f-$Hir^{54-65}(SO_3^-)$ in place of HD1. Consistent with previous reports (Anderson *et al.*, 2003, Anderson and Bock, 2003), $Hir^{54-65}(SO_3^-)$ binds prothrombin with an affinity 44-fold lower than that for thrombin (K_D values of 3000 ± 1400 nM and 68 ± 5.2 nM, respectively; Table 3.1). These data suggest that, unlike the HD1 binding site on prothrombin, the f-$Hir^{54-65}(SO_3^-)$ binding site on prothrombin undergoes changes during prothrombin conversion to thrombin that heighten its affinity for the ligand.

A.


Fluorescence Intensity
590
570
550
530
510
490
470
450
0
200
400
600
800
1000
1200
1400
1600
Time (s)

B.

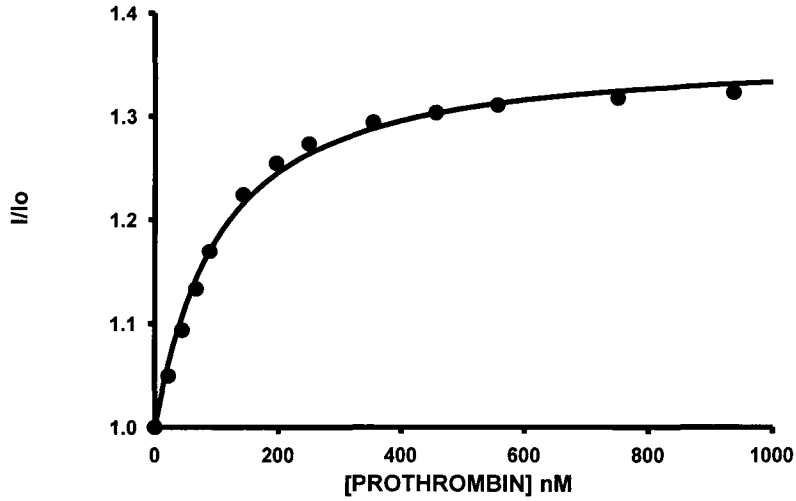

I/Io
1.4
1.3
1.2
1.1
1.0
0
200
400
600
800
1000
[PROTHROMBIN] nM

Figure 3.2: Interaction of f-HD1 with prothrombin

The fluorescence of 30 nM f-HD1 was monitored as it was titrated with aliquots of 10 μM prothrombin (A). Fluorescence intensity values (I) determined after each addition of prothrombin were divided by the initial fluorescence intensity (Io). I/Io values were then plotted as a function of prothrombin concentration (B), and the data were fit by nonlinear regression analysis (line) to obtain the K_D value.

Previous structural (Arni *et al.*, 1994) and functional (Cote *et al.*, 1997, Han *et al.*, 1997) data indicate that F2 binds exosite 2. Consequently, exosite 2 should only be accessible on thrombin and pre2, derivatives lacking F2. To explore this concept, we measured the affinity of f-HD22, an exosite 2-directed DNA aptamer, for prothrombin, prothrombin intermediates, and thrombin. As expected, f-HD22 binds only to pre2 and thrombin, with K_D values of 42 ± 6.8 and 29 ± 3.1 nM, respectively (Table 3.1). Thus, these data confirm the concept that exosite 2 is only accessible on prothrombin derivatives lacking F2.

Effect of HD1 on prothrombin activation by prothrombinase:

Exosite 1 on prothrombin contributes to prothrombin activation by prothrombinase by mediating, at least in part, the interaction of prothrombin with factor Va (Anderson *et al.*, 2000b). Because HD1 binds exosite 1 of prothrombin with high affinity, we set out to determine whether HD1 attenuates prothrombin activation by competing with factor Va for prothrombin binding. The rate of prothrombin activation by complete prothrombinase was measured in the absence or presence of HD1. HD1 attenuates prothrombin activation by prothrombinase in a dose-dependent and saturable fashion (Figure 3.3), inhibiting the reaction by 93 ± 1.0 % with an IC_{50} value of 134 ± 60 nM, a value comparable to the K_D of f-HD1 for prothrombin.

Prothrombin derivative	f-HD1	f-Hir$^{54-65}$(SO_3^-)	f-HD22
	K_D (nM)		
Prothrombin	86 ± 8.4	3000 ± 1400	No Binding
Meizothrombin	35 ± 5.5	nd*	No Binding
Meizothrombin des F1	45 ± 0.3	nd	No Binding
Prethrombin 1	86 ± 1.0	nd	No Binding
Prethrombin 2	66.1 ± 15.8	nd	42 ± 6.8
Thrombin	34 ± 4.8	68 ± 5.2	29 ± 3.1

Table 3.1: K_D values of f-HD1, f-Hir$^{54-65}$(SO_3^-), and f-HD22 for prothrombin, prothrombin activation intermediates and thrombin: K_D values were determined by titrating f-HD1, f-Hir$^{54-65}$(SO_3^-), or f-HD22 with prothrombin, the various prothrombin activation intermediates, or thrombin as described in the legend to Fig. 2. Where no change in fluorescence was observed, the result was interpreted as no binding. Data represent the mean ± standard error of three separate experiments.

*nd-indicates that values were not determined.

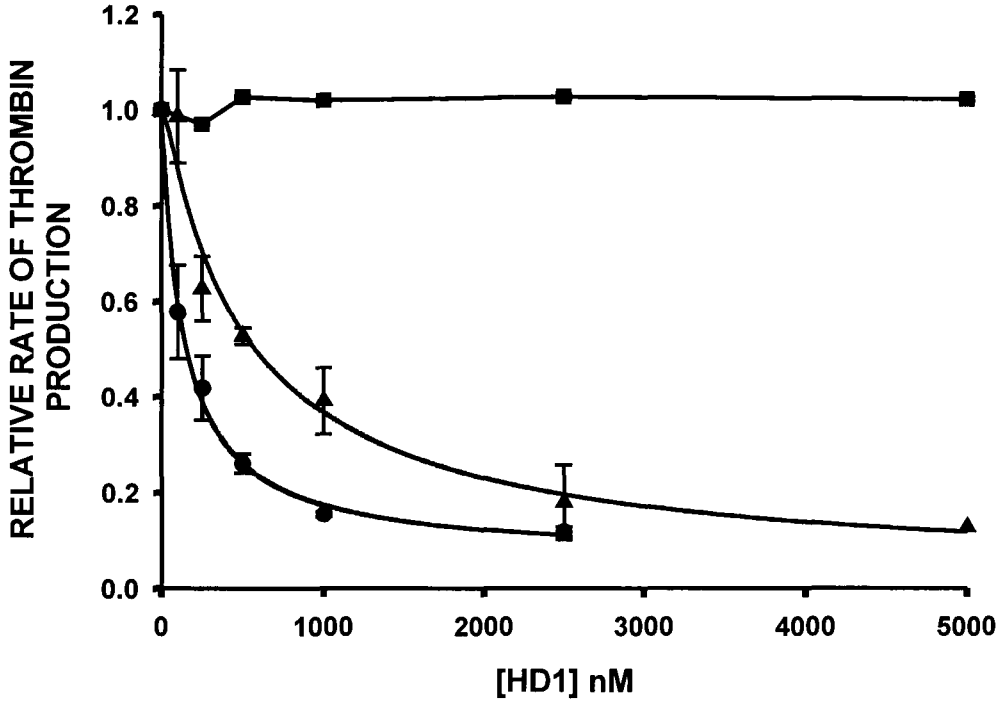

RELATIVE RATE OF THROMBIN PRODUCTION
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
5000
[HD1] nM

Figure 3.3: Effect of HD1 on the rate of prothrombin activation by prothrombinase

The initial rate of activation of 1 μM prothrombin by 0.25 nM fXa, 0.3 nM factor Va, and 6 μM PCPS was measured in the presence of increasing concentrations of HD1 (●). Similar titrations of HD1 were repeated in the absence of PCPS (▲), or in the absence of fVa (■). Thrombin generation was determined by measuring hydrolysis of Chz-Th. Data were plotted as the rate of prothrombin activation measured in the presence of HD1 relative to that determined in its absence. Each point represents the mean of three experiments while the bars reflect the standard error. Data were fit by nonlinear regression analysis (lines).

To begin to define the mechanism by which HD1 inhibits this reaction, prothrombin was activated by factor Xa in the presence or absence of individual components of the prothrombinase complex (Figure 3.3). When PCPS vesicles were omitted from the prothrombinase complex, HD1 inhibited prothrombin activation by over 85 % with factor Va concentrations of either 0.3 or 20 nM yielding IC_{50} values of 505 ± 97 and 472 ± 366 nM, respectively. In contrast, HD1 had no effect on prothrombin activation when factor Va was omitted from the prothrombinase complex. These results suggest that the inhibitory effect of HD1 on prothrombin activation is factor Va-dependent, consistent with other known exosite 1 ligands (Anderson *et al.*, 2000b, Chen *et al.*, 2003, Monteiro and Zingali, 2002). In support of this concept, HD1 had no effect on prothrombin activation by Ecarin (data not shown), a factor Va-independent activator of prothrombin.

mIIa is the predominant intermediate formed during prothrombin activation (Banerjee *et al.*, 2002, Krishnaswamy *et al.*, 1987). mIIa generation is PCPS- and factor Va-dependent, whereas pre2 is the predominant intermediate that is generated in the absence of cofactors. To determine whether HD1 alters the extent of mIIa generation, 14 μM prothrombin was activated by prothrombinase in the absence or presence of 50 μM HD1 and aliquots of the reaction mixture were subjected to SDS-PAGE analysis under reducing (Figure 3.4) or non-reducing (data not shown) conditions to distinguish mIIa from prothrombin. In the absence of HD1, mIIa is the major intermediate and prothrombin activation is complete by 90 min (Figure 3.4). When HD1 is added, prothrombin activation also proceeds through mIIa, but prothrombin activation remains

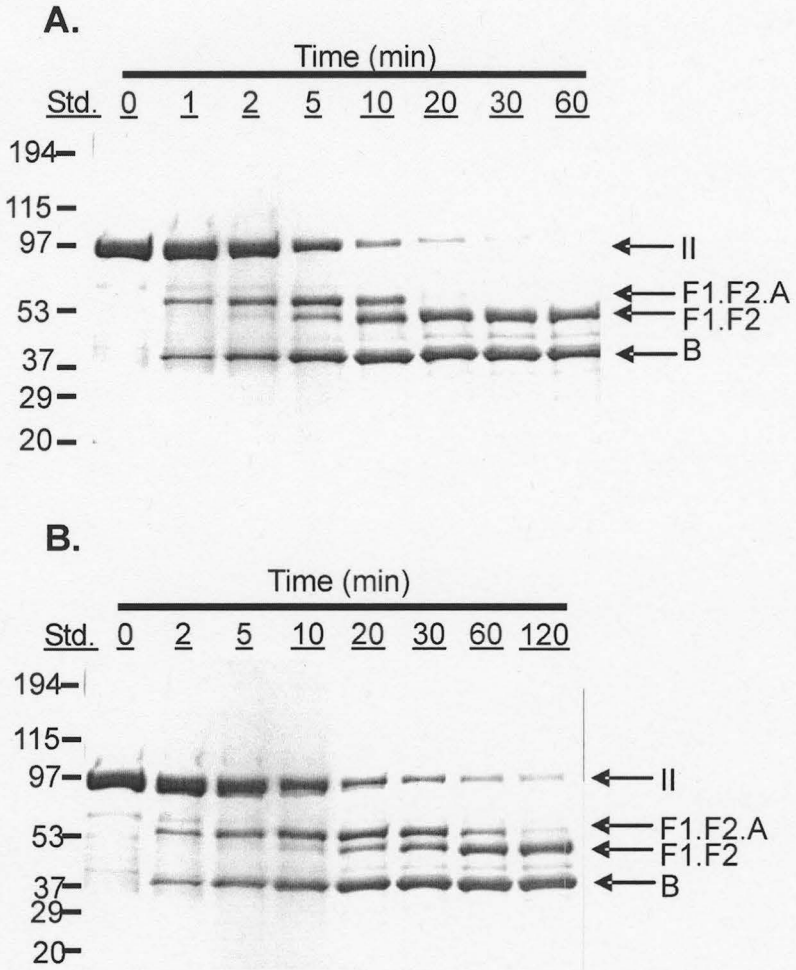

A.
Time (min)
Std. 0 1 2 5 10 20 30 60
194
115
97
53
37
29
20
II
F1.F2.A
F1.F2
B
B.
Time (min)
Std. 0 2 5 10 20 30 60 120
194
115
97
53
37
29
20
II
F1.F2.A
F1.F2
B

Figure 3.4: SDS-PAGE analysis of prothrombin activation by prothrombinase in the absence (A) or presence (B) of HD1

Prothrombin (14 μM) was activated by 2 nM factor Xa, 20 nM factor Va, and 6 μM PCPS in aptamer buffer containing 70 μM DAPA in the absence or presence of 50 μM HD1. At the times indicated, 5 μl aliquots were removed and subjected to SDS-PAGE analysis under reducing conditions. Molecular weight markers are identified on the left and prothrombin-derived bands are identified on the right.

incomplete at 240 min. Delayed conversion of the F1.2.A fragment to F1.2 in the presence of HD1 reflects the inhibitory effect of HD1 on thrombin. Thus, HD1 attenuates prothrombin activation by prothrombinase, but appears to have minimal effects on the prothrombin activation pathway.

In terms of the kinetic mechanism of prothrombin activation, current thinking is that factor Va increases the k_{cat} for factor Xa cleavage of prothrombin, whereas PCPS lowers the K_m of the reaction (Kalafatis, 2005, Rosing *et al.*, 1980). Because HD1 attenuates prothrombin activation by prothrombinase in a factor Va-dependent fashion, we examined the effect of HD1 on the kinetic parameters of activation. Prothrombin, in concentrations ranging from 0 to 8 μM, was activated with prothrombinase in the absence or presence of 25 μM HD1, and the rate of thrombin generation was quantified (Figure 3.5). In the absence of HD1, the K_m and k_{cat} values are 255 ± 97 nM and 12.2 ± 1.4 s^{-1}, respectively; values comparable to the previously reported K_m of 120 nM and k_{cat} of 32.4 s^{-1} (Bukys *et al.*, 2005). With 25 μM HD1, the reaction proceeds at a slower rate, yielding a K_m of 1480 ± 223 nM and a k_{cat} of 6.2 ± 0.7 s^{-1}. Thus, the catalytic efficiency is 12-fold lower in the presence of HD1 than it is in its absence, reflecting a 6-fold increase in K_m and 2-fold reduction in k_{cat}. These findings explain why HD1 produces over 90 % inhibition of prothrombin activation by prothrombinase (Figure 3.3).

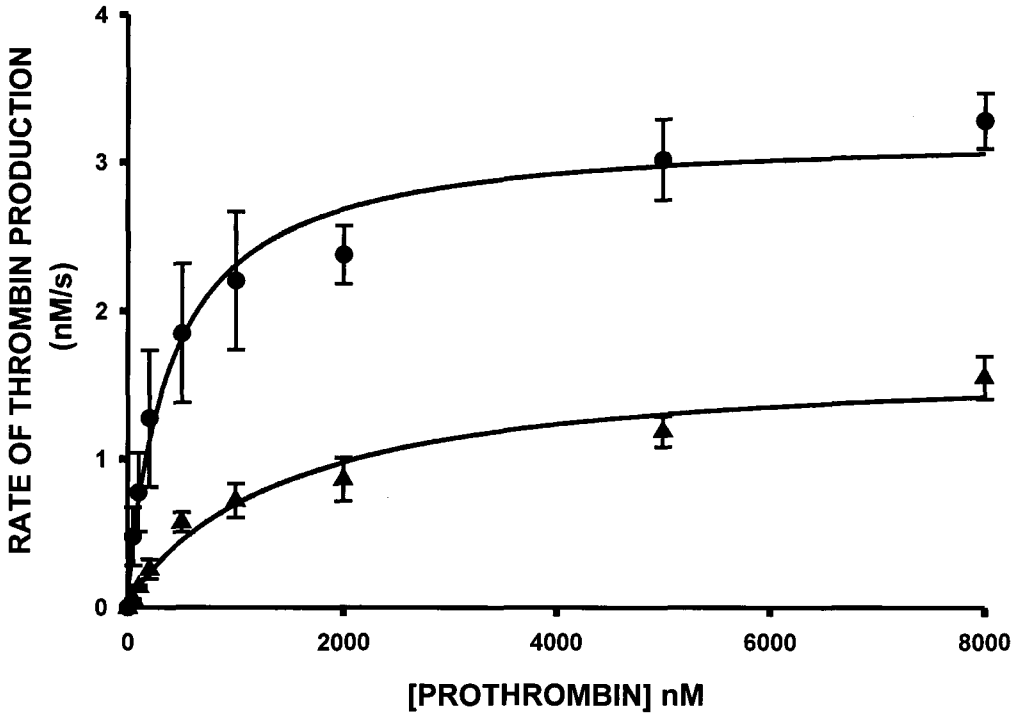

RATE OF THROMBIN PRODUCTION
(nM/s)
4
3
2
1
0
0
2000
4000
6000
8000
[PROTHROMBIN] nM

Figure 3.5: Kinetic analysis of HD1 inhibition of prothrombin activation by prothrombinase

Initial rates of prothrombin activation by 0.25 nM factor Xa, 0.6 nM factor Va, and 5 μM PCPS vesicles are plotted as a function of total prothrombin concentration in the absence (●) or presence (▲) of 25 μM HD1. Rates of prothrombin activation were determined by measuring hydrolysis of Chz-Th using a discontinuous assay as described under "Methods". The lines represent nonlinear regression analysis of the Michaelis Menten equation performed on the average relative rate of thrombin generation. Each point represents the mean of three separate experiments, while the bars reflect the standard error.

Effect of HD1 on the prothrombin-factor Va interaction:

To determine whether HD1 competes with factor Va for prothrombin binding, a competition experiment was conducted using SPR. In this experiment, prothrombin binding to immobilized b-HD1 was examined in the absence or presence of 1 μM factor Va. When factor Va is present, prothrombin binding to immobilized b-HD1 is reduced by 56 ± 2.2 % (Appendix 3.2). These findings support the concept that HD1 disrupts the factor Va-prothrombin interaction.

Discussion

Efficient prothrombin activation by prothrombinase is essential for generating a burst of thrombin at sites of vascular injury (Mann *et al.*, 2003a). For efficient activation to occur, prothrombin must incorporate into the prothrombinase complex. Assembly is accomplished not only by prothrombin Gla-domain-dependent interaction with negatively charged phospholipids (Banerjee *et al.*, 2002), but also by prothrombin binding to factor Va (Krishnaswamy *et al.*, 1987). Current thinking is that exosite 1 on prothrombin mediates, at least in part, its interaction with factor Va (Anderson *et al.*, 2000b, Chen *et al.*, 2003). However, exosite 1 is proposed to exist as an immature proexosite on prothrombin based on the observation that $Hir^{54-65}(SO_3^-)$ binds prothrombin with an affinity much lower than that for thrombin and weakly attenuates prothrombin activation (Anderson *et al.*, 2000a, Chen *et al.*, 2003). Our data with HD1 refine the concept of proexosite 1 on prothrombin and provide new information on exosite maturation during

prothrombin conversion to thrombin. We demonstrate that, unlike $Hir^{54-65}(SO_3^-)$, HD1 (a) binds prothrombin and thrombin with similar high affinity, (b) potently inhibits prothrombin activation by prothrombinase in a factor Va-dependent fashion, and (c) competes with factor Va for prothrombin binding. Therefore, HD1 provides unique insight into the structure and function of exosite 1 on prothrombin. The different inhibitory profiles of HD1 and $Hir^{54-65}(SO_3^-)$ can partly be explained by disparity in their binding interactions with prothrombin and thrombin.

Comparison of $Hir^{54-65}(SO_3^-)$ and HD1 binding to prothrombin and thrombin:

Examination of the crystal structures of the $Hir^{54-65}(SO_3^-)$- and HD1-thrombin complexes suggests that the two exosite 1-directed ligands bind to distinct, but contiguous regions of exosite 1 (Padmanabhan *et al.*, 1993, Vijayalakshmi *et al.*, 1994). Competition experiments support this concept because $Hir^{54-65}(SO_3^-)$ competes with f-HD1 for binding to both prothrombin and thrombin. These findings can be explained in two ways. First, the capacity of $Hir^{54-65}(SO_3^-)$ and HD1 to compete for binding could reflect extended conformational changes associated with ligand binding. Thus, exosite 1 residues are disordered in their native state. Once $Hir^{54-65}(SO_3^-)$ binds, however, these residues become highly ordered (Malkowski *et al.*, 1997, Vijayalakshmi *et al.*, 1994) and this conformational rearrangement may displace HD1 from its nearby binding site. A second potential explanation is that $Hir^{54-65}(SO_3^-)$ directly competes for the charged residues that bind HD1. Consistent with a steric mechanism, HD1 incompletely displaces $Hir^{54-65}(SO_3^-)$ from prothrombin or thrombin. Thus, HD1 displays a small thrombin-

binding footprint with five residues identified as important for binding (Tsiang *et al.*, 1995). In contrast, $Hir^{54-65}(SO_3^-)$, which makes extended contacts with thrombin (Vijayalakshmi *et al.*, 1994), has a considerably larger footprint. The fact that HD1 only partially competes with $Hir^{54-65}(SO_3^-)$ suggests that the footprints of the two ligands overlap, but the residues mediating the interaction of HD1 with thrombin or prothrombin represent only a portion of those that mediate $Hir^{54-65}(SO_3^-)$ binding. Thus, our competition studies highlight differences in the structure of exosite 1 of prothrombin. The distinct interactions of HD1 and $Hir^{54-65}(SO_3^-)$ with prothrombin prompted investigation of the functional maturation of exosite 1 in prothrombin.

Exosite 1 on prothrombin:

In contrast to $Hir^{54-65}(SO_3^-)$, HD1 binds prothrombin and prothrombin intermediates with affinities similar to that for thrombin. These findings suggest that the charged residues that constitute the HD1 binding site in exosite 1 undergo minimal conformational rearrangement during prothrombin conversion to thrombin. In contrast, the $Hir^{54-65}(SO_3^-)$ binding site undergoes significant conformational maturation during prothrombin activation. Cleavage of the Arg^{320}-Ile^{321} bond likely contributes to maturation of the exosite 1 hydrophobic cleft because the affinity of $Hir^{54-65}(SO_3^-)$ for the meizo-derivatives of prothrombin is much higher than those for prothrombin, pre1, and pre2 (Anderson *et al.*, 2003, Anderson and Bock, 2003).

Our data suggest that the concept of a proexosite on prothrombin needs to be refined. Although it may be a proexosite in terms of its affinity for thrombin substrates, such as fibrinogen, it does not serve as a proexosite for HD1. It also should not be

considered a proexosite in the context of factor Va, whose primary role is to bind prothrombin rather than thrombin. In fact, our data, as well as those of others (Anderson *et al.*, 2000a, Anderson *et al.*, 2000b, Chen *et al.*, 2003, Yegneswaran *et al.*, 2003), indicate that exosite 1 is an important contributor to the prothrombin/factor Va interaction.

Prothrombin interaction with factor Va:

We show that HD1 inhibits prothrombin activation by prothrombinase in a factor Va-dependent fashion, and that factor Va competes with prothrombin for HD1 binding. Taken together, these data highlight the importance of exosite 1 in the prothrombin/factor Va interaction, a concept supported by previous work with other exosite 1-directed ligands, such as $Hir^{54-65}(SO_3^-)$ (Anderson *et al.*, 2000b, Chen *et al.*, 2003) or Bothrojaracin, a prothrombin-binding protein isolated from the venom of *Bothrops jararaca* (Monteiro and Zingali, 2002). Although HD1 attenuates prothrombin activation, it does not totally block it because exosite 1 is not the sole binding site for factor Va. Thus, residues 473-487 of the B chain (Yegneswaran *et al.*, 2004), F2 (Krishnaswamy and Walker, 1997), and the Gla-domain (Blostein *et al.*, 2000) and kringle 1 of F1 (Deguchi *et al.*, 1997) also contribute to the prothrombin/factor Va interaction. Despite the extensive contacts that prothrombin makes with factor Va, however, the potent inhibitory effect of HD1 on prothrombin activation by prothrombinase suggests that exosite 1 on prothrombin plays an important part in the prothrombin/factor Va interaction.

The relative contribution of factor Va and PS-membranes to direct factor Xa activity toward prothrombin is uncertain. Initial studies suggested that factor Va determines which prothrombin intermediate is generated by factor Xa-mediated cleavage (Krishnaswamy *et al.*, 1987, Rosing *et al.*, 1986). More recent work challenges this concept, suggesting instead that PS dictates the activation pathway, whereas factor Va enhances the rate of thrombin production regardless of the pathway (Banerjee *et al.*, 2002, Krishnaswamy *et al.*, 1987, Weinreb *et al.*, 2003, Wu *et al.*, 2002). Our observation that HD1 reduces the rate of prothrombin activation by prothrombinase, without affecting the activation pathway through which prothrombin is converted to thrombin, suggests that exosite 1 on prothrombin is a major contact for its entry into the prothrombinase complex but does not contribute to directing factor Xa cleavage of prothrombin.

Role of exosite 1 on prothrombin in its activation by prothrombinase:

HD1 is one of several exosite 1-directed ligands that has been shown to inhibit prothrombin activation by prothrombinase. $Hir^{54\text{-}65}(SO_3^-)$ and Bothrojaracin inhibit prothrombin activation by the factor Xa/factor Va complex, but not by complete prothrombinase (Monteiro and Zingali, 2002, Zingali *et al.*, 2001). Similarly, DYDYQ, a pentapeptide analog of a portion of factor Va, binds prothrombin with a K_D value of 850 nM and inhibits prothrombinase activity with an IC_{50} value of 1.6 μM (Beck *et al.*, 2004). Although all of these ligands bind exosite 1 on prothrombin, none inhibits prothrombin activation by prothrombinase as potently as HD1.

At saturating concentrations, HD1 inhibits prothrombin activation by prothrombinase by over 90 %. Kinetic analysis indicates that HD1 affects the K_m of the reaction more than the k_{cat}. This observation is consistent with the concept that exosite 1 on prothrombin is a major docking site for factor Va and is important for prothrombin incorporation into the prothrombinase complex. The continued enhancement of prothrombin activation observed in the presence of HD1 may reflect factor Va interactions with factor Xa and/or prothrombin that are independent of exosite 1 (Krishnaswamy and Walker, 1997). Because of its high affinity for prothrombin, HD1 provides a unique tool to examine the contribution of the prothrombin/factor Va interaction to efficient prothrombin activation by prothrombinase.

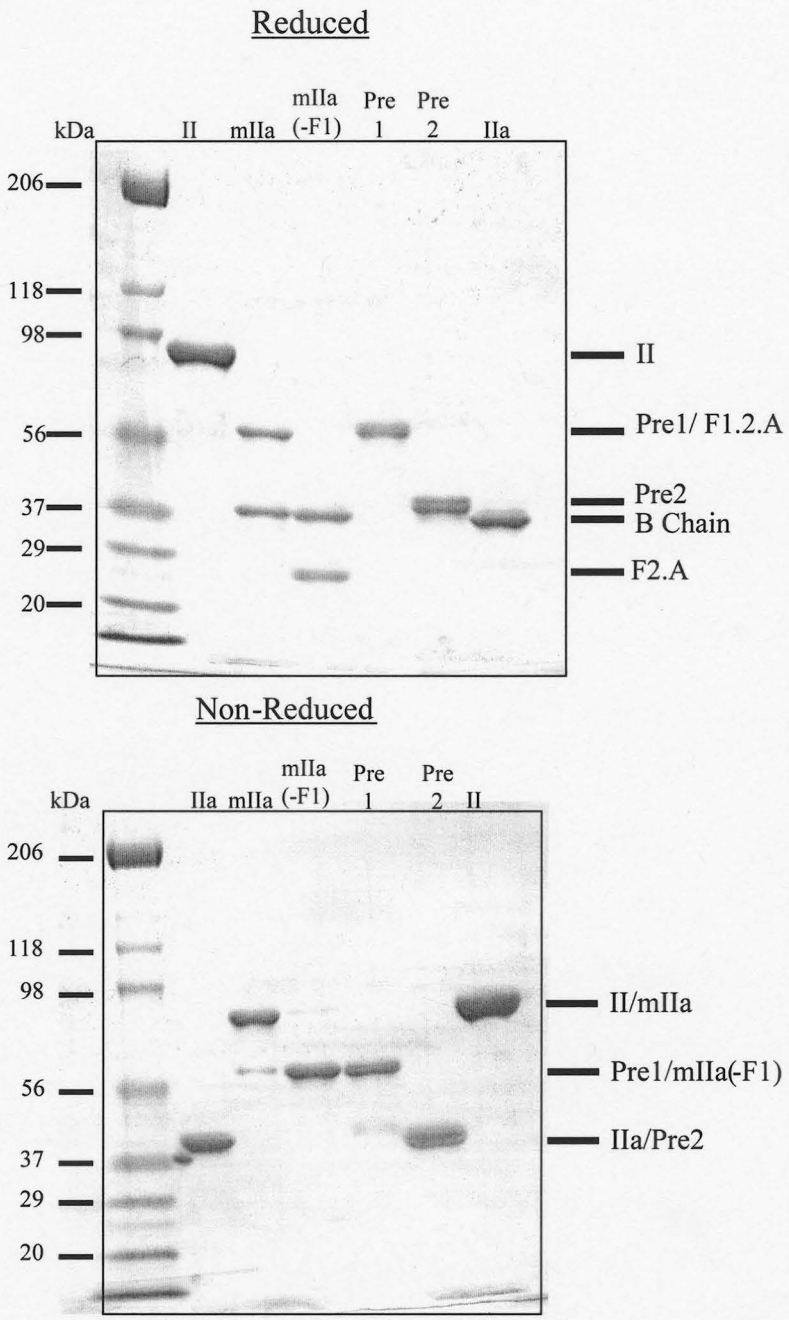
Relevance:

Our data suggest that the notion that prothrombin possesses a proexosite 1 needs to be refined. Whereas $Hir^{54-65}(SO_3^-)$ and fibrinogen bind prothrombin more weakly than thrombin, this is not the case with HD1. Likewise thrombomodulin may also bind prothrombin more tightly than $Hir^{54-65}(SO_3^-)$ or fibrinogen because thrombomodulin binds mIIa and mIIa(-F1) with high affinity (Cote *et al.*, 1997), and is a more potent inhibitor of prothrombin activation by prothrombinase than $Hir^{54-65}(SO_3^-)$ (Chen *et al.*, 2003). Therefore, exosite 1 on prothrombin is fully capacitated for binding ligands, such as factor Va, that have a direct prothrombin-dependent function. In contrast, this domain serves as a proexosite for thrombin-directed ligands or substrates.

HD1 was initially developed as an anticoagulant because of its capacity to directly inhibit thrombin clotting activity (Griffin *et al.*, 1993b, Tsiang *et al.*, 1995). Our data

suggest that HD1 has dual anticoagulant activity. Thus, in addition to blocking thrombin activity, HD1 also attenuates thrombin generation by inhibiting prothrombin activation by prothrombinase. Positioned at the junction between the intrinsic and extrinsic pathways of coagulation, prothrombinase is an attractive target for new anticoagulants. By attenuating thrombin generation, the intent is to preserve sufficient thrombin activity to permit hemostasis. Such an approach may produce less bleeding than one that targets thrombin directly. Our data, suggest that an aptamer that preferentially binds prothrombin over thrombin would specifically target prothrombinase by blocking the factor Va/prothrombin interaction.

Appendix 3.1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kDa
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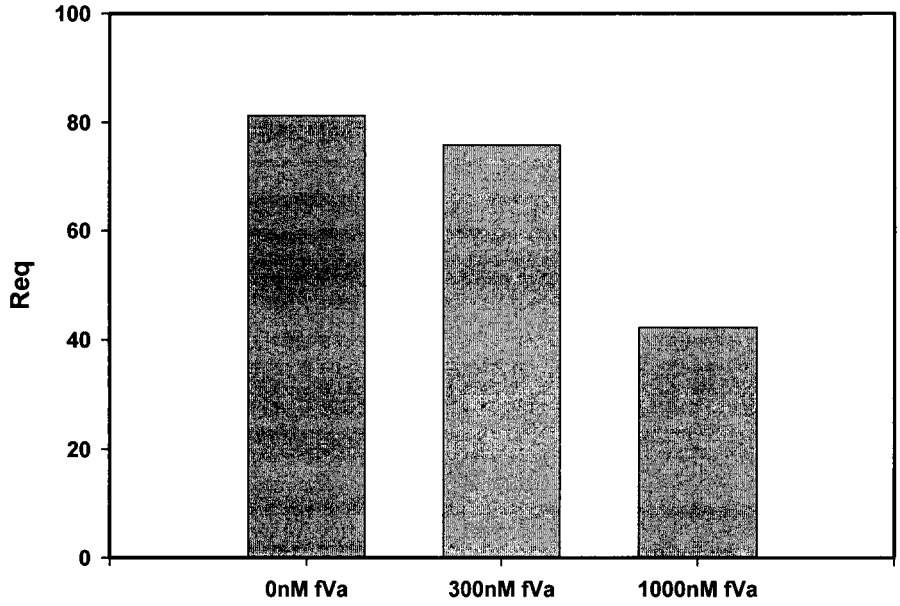
mIIa
mIIa (-F1)
Pre 1
Pre 2
IIa
206
118
98
56
37
29
20
II
Pre1/ F1.2.A
Pre2
B Chain
F2.A
Non-Reduced
kDa
IIa
mIIa
mIIa (-F1)
Pre 1
Pre 2
II
206
118
98
56
37
29
20
II/mIIa
Pre1/mIIa(-F1)
IIa/Pre2

Appendix 3.1: Purity of prothrombin activation intermediates

Following the purification of the prothrombin activation intermediates, SDS-PAGE analysis was performed on each preparation to determine their purity and integrity. 3 μg of each protein sample was added to 10 μL of sample buffer and placed in a boiling water bath for 3 min. Samples were then loaded onto a 4-15% acrylamide Tris-HCl gel and electrophoresed for 1 h. The bands were visualized by Fast Stain. The Non-reduced gel shows the purity of the different preparations. The mIIa preparation shows some degree of breakdown to mIIa(-F1), however these levels are not significant enough to affect our results. The mIIa(-F1) preparation has some mIIa that should not interfere with our results. Both pre1 and pre2 appear to be very pure preparations. The reduced gel indicates that the mIIa and mIIa(-F1) preparations are properly formed and that the pre1 and pre2 maintain their Arg^{320}-Ile-^{321} bond.

Appendix 3.2



Appendix 3.2: Displacement of Prothrombin from immobilized b-HD1 by fVa

A BIAcore instrument was used for SPR experiments to assess the competition of prothrombin from biotin-streptavidin immobilized HD1 by fVa. A streptavidin sensor chip was conjugated with b-HD1 following initial pre-conditioning washes with 1 M NaCl 50 mM NaOH. Samples with 300 nM prothrombin and increasing concentrations of fVa were injected into the flow cell under kinetic mode at a flow rate of 25 μL/min for 2 min. The response units associated with bulk transport was controlled for by flowing identical samples over a flow cell conjugated with the scrambled oligonucleotide sequence, b-HD23. The difference in prothrombin binding to b-HD1 in the presence of fVa was reported as equilibrium response units.

Chapter 4: HD1, a thrombin- and prothrombin-binding DNA aptamer, inhibits thrombin generation by attenuating prothrombin activation and thrombin feedback reactions

Foreword

This manuscript has been accepted for publication in Thrombosis and Hemostasis, and is to appear in the October 2009 issue. The authors are: Colin A. Kretz, Karl K Cuddy, Alan R. Stafford, James C. Fredenburgh, Robin Roberts, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz.

Unless noted, all of the experiments contained within this manuscript were performed by me. Furthermore, this manuscript was drafted by me. Thrombin generation assays were conducted in collaboration with Dr. Catherine P. Hayward. Nola Fuller, acknowledged in this paper, trained me in the use of the Calibrated Automated Thrombography (CAT) instrument for the thrombin generation experiments. Professor Robin Roberts conducted the 3-way ANOVA analysis of the thrombin generation data used to compare the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on CAT parameters. Karl Cuddy performed the SDS-PAGE analysis of factor VIII activation by thrombin (Figure 4.2).

Summary

HD1, a DNA aptamer, binds exosite 1 on thrombin and blocks its clotting activity. Because HD1 also binds prothrombin and inhibits its activation by prothrombinase, we hypothesized that HD1 would be a more potent inhibitor of coagulation than other exosite 1-directed ligands, such as $Hir^{54-65}(SO_3^-)$. Supporting this concept, the effect of HD1 on the prothrombin time and activated partial thromboplastin time was 2-fold greater than that of $Hir^{54-65}(SO_3^-)$ even though both agents inhibited thrombin-mediated factor (f) V and fVIII activation to a similar extent. In thrombin generation assays, HD1 (a) delayed the lag time, (b) reduced peak thrombin concentration, and (c) decreased endogenous thrombin potential to a greater extent than $Hir^{54-65}(SO_3^-)$. To eliminate thrombin feedback, studies were repeated in fV- and/or fVIII-deficient plasma supplemented with fVa and/or fVIIIa. Only HD1 prolonged the lag time in fV- and fVIII-deficient plasma supplemented with fVa and fVIIIa. In contrast, HD1 and $Hir^{54-65}(SO_3^-)$ inhibited the lag time in fVIII-deficient plasma supplemented with fVIIIa and in normal plasma. The more potent anticoagulant properties of HD1, therefore, reflect its capacity to attenuate fV activation by thrombin and inhibit prothrombinase assembly. These findings identify prothrombin as a potential target for new anticoagulants.

Introduction

The hemostatic system responds to injury by generating a clot at the site of damage to stop the loss of blood. Blood coagulation is initiated by tissue factor (TF) exposed at the site of vascular injury (Versteeg and Ruf, 2007). TF initiates a series of reactions that culminate in the generation of thrombin, which converts fibrinogen to fibrin, a primary component of the clot. Thrombin also amplifies its own generation by activating factors (f) VIII and V; critical cofactors for upstream activation complexes. In the presence of fVIIIa and anionic phospholipid membranes, fIXa assembles into the intrinsic tenase complex, thereby enhancing the rate of fXa generation by $2x10^5$-fold over that effected by fIXa alone (van Dieijen *et al.*, 1981). Similarly, fVa and anionic phospholipid membranes combine with fXa to produce the prothrombinase complex, which enhances prothrombin activation by $3x10^5$-fold compared with fXa alone (Nesheim *et al.*, 1979, van Rijn *et al.*, 1984). Thus, assembly of these activation complexes is critical for generating a burst of thrombin at the site of vascular injury.

Thrombin generation in plasma can be divided into 3 phases (reviewed in (Hemker *et al.*, 2006a)). The initiation phase begins when TF combines with fVIIa to produce fXa, which generates minute amounts of thrombin. This is followed by the propagation phase, which occurs when the intrinsic tenase and prothrombinase complexes assemble, and is characterized by a period of rapid thrombin generation. The termination phase, which begins after the concentration of thrombin reaches a peak, is marked by a decrease in the concentration of thrombin. This decrease occurs because the rate of thrombin generation is slower than the rate of its inhibition by antithrombin and heparin cofactor II (HCII).

Thrombin generation is complete at the end of the termination phase when thrombin activity is no longer detected.

Thrombin interacts with its substrates via exosite 1, a ligand-binding domain distinct from its active site. Exosite 1 is composed of hydrophobic amino acid residues, grouped in a pocket and surrounded by cationic residues (Adams and Huntington, 2006). This hydrophobic pocket is linked to the active site by a surface depression that helps guide macromolecular substrates for proteolysis (Stubbs and Bode, 1993). Thus, the docking of macromolecular substrates, such as fVIII and fV, to exosite 1 of thrombin is essential for their efficient conversion into activated cofactors (Bukys *et al.*, 2006b, Nogami *et al.*, 2005).

Thrombin can be inhibited directly by agents that target the active site and/or exosite 1, or indirectly by attenuating prothrombin conversion to thrombin. HD1 and $Hir^{54-65}(SO_3^-)$ bind exosite 1 on thrombin and block thrombin-mediated conversion of fibrinogen to fibrin (Griffin *et al.*, 1993b, Naski *et al.*, 1990). HD1 is a thrombin-specific DNA aptamer composed of 15 nucleotides (Griffin *et al.*, 1993b), whereas $Hir^{54-65}(SO_3^-)$ is a dodecapeptide analog of the COOH-terminus of hirudin, a potent leech-derived inhibitor of thrombin (DiMaio *et al.*, 1990, Thurieau *et al.*, 1994). In buffer systems, we recently demonstrated that in addition to inhibiting thrombin activity, HD1 also attenuates thrombin generation by inhibiting prothrombin activation by prothrombinase (Kretz *et al.*, 2006). This reflects the capacity of HD1, but not $Hir^{54-65}(SO_3^-)$, to bind exosite 1 on prothrombin and prevent its interaction with fVa. Because HD1 inhibits prothrombin activation as well as thrombin activity, whereas $Hir^{54-65}(SO_3)$ only inhibits

thrombin activity, we hypothesized that HD1 would be a more potent inhibitor of coagulation than $Hir^{54-65}(SO_3^-)$. To explore this hypothesis, we compared the effects of HD1 and $Hir^{54-65}(SO_3^-)$ in (a) plasma-based clotting assays, (b) thrombin generation assays, and (c) the thrombin-mediated feedback reactions that contribute to thrombin generation.

Materials and Methods

Reagents:

Human prothrombin, thrombin, factor Xa, and factor IXa were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Human plasma-derived fV was a generous gift from Dr. Michael Nesheim (Queen's University, Kingston, ON), whereas fVa was from Haematologic Technologies Inc. (Essex Junction, VT). HD1 (5'-GGTTGGTGTGGTTGG-3') was synthesized by the Molecular Biology and Biotechnology Institute at McMaster University (Hamilton, ON). Before use, HD1 was subjected to renaturation by heating to 95°C for 5 min followed by cooling on ice for 10 min (Griffin *et al.*, 1993b). Hirudin and its Tyr^{63}-sulfated COOH-terminal peptide, $Hir^{54-65}(SO_3^-)$, were from Bachem Bioscience, Inc. (King of Prussia, PA). The activated partial thromboplastin time (aPTT) and prothrombin time (PT) were performed using HemosIL APTT-SP and Recombiplastin (relipidated recombinant tissue factor), respectively, from Instrumentation Laboratory (Lexington, MA). FVIII, in the form of Kogenate (a recombinant antihemophilic factor), was from Bayer, Inc. (Toronto, ON). FVIIIa was

generated by reacting 0.5 μM fVIII with 50 nM thrombin for 30 s in 20 mM Tris-HCl, 140 mM NaCl, pH 7.4, containing 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 0.1% PEG (aptamer buffer). The reaction was terminated with 50 nM hirudin, and the resultant fVIIIa was used immediately. D-Phe-Pro-Arg chloromethyl ketone (FPRck) was obtained from EMD Chemicals, Inc. (Mississauga, ON). Human HCII, antithrombin, and plasmas deficient in fV, fVIII, or fV and fVIII were obtained from Affinity Biologicals, Inc. (Ancaster, ON). Batroxobin, a snake venom enzyme from *Bothrops atrox*, Prionex, and Pefafluor TG were all from Pentapharm Inc. (Basel, Switzerland). Chromozym Thrombin (Chz-Th) was from Roche Diagnostics (Laval, QC), whereas S2765 and S2238 were from Chromogenix (Milano, Italy). PCPS vesicles were synthesized using a modification of previously published methods (Barenholz *et al.*, 1977, Bloom *et al.*, 1979).

Plasma pool:

A plasma pool, prepared from blood collected from informed healthy donors into 3.8% sodium citrate, was aliquoted and stored at -80°C.

Preparation of defibrinated plasma:

To generate defibrinated plasma, 0.25 units/ml Batroxobin was added to either control or factor-depleted plasma containing plastic inoculation loops (Fisher Scientific, Toronto, ON). After incubation for 30 min at 37°C, the inoculation loops with attached fibrin were removed and the defibrinated plasma was stored in aliquots at -80°C.

Plasma clotting assays:

We first compared the effect of HD1 and $Hir^{54-65}(SO_3^-)$ on the PT, aPTT, thrombin time and fXa clotting time. To 100 µl aliquots of plasma in wells of a 96-well plate was added 20 µM HD1 or $Hir^{54-65}(SO_3^-)$. After a 5 min incubation, 200 µl Recombiplastin was added for determination of the PT. For aPTT determination, 100 µl PTT reagent was added and the mixture was incubated for 5 min at 37°C prior to addition of 100 µl $CaCl_2$ reagent. Thrombin and fXa clotting times were determined in 100 µl aliquots of control plasma or plasma deficient in fV and fVIII (fVfVIIIdp) supplemented with 30 nM fVa, 0.3 nM fVIIIa and 5 µM PCPS. Clotting was initiated by adding 15 mM $CaCl_2$ and 10 nM thrombin or fXa. In all cases, turbidity was monitored at 340 nm for 10 min at 37°C using a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA) and the clotting time was calculated as the time to half maximal absorbance.

SDS-PAGE analysis of thrombin-mediated activation of fV or fVIII:

The effect of HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin-mediated activation of fV or fVIII was assessed using SDS-PAGE (Laemmli, 1970). After incubating 10 nM thrombin with 5 µM HD1 or $Hir^{54-65}(SO_3^-)$ for 5 min in aptamer buffer containing 0.05% Prionex, reactions were initiated by adding 0.6 µM fV or 3 µM fVIII. At intervals, 5 µl aliquots were removed and subjected to SDS-PAGE under reducing conditions on 4-15% polyacrylamide gradient gels (Ready Gels, Bio-Rad, Hercules, CA). Fixed gels displaying fVIII or fV activation were stained with Fast Stain (Zoion Biotech, Worchester, MA) or Sypro Ruby (Bio-Rad), respectively, and imaged using a Typhoon 9410 (Amersham Biosciences, Piscataway, NJ).

Thrombin generation:

The Calibrated Automated Thrombinography (CAT) assay (Hemker and Beguin, 1995) was used to compare the effect of 0-25 μM HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin generation in (a) control plasma, (b) defibrinated control plasma, or (c) defibrinated plasma deficient in fV (fVdp), fVIII (fVIIIdp), or fV and fVIII (fVfVIIIdp). Briefly, 100 μl of plasma was added to round-bottom wells of a 96-well plate together with 30 μM PCPS and 0.5 pM TF from 1:2 diluted PPP Low reagent (Thrombinoscope, Maastricht, The Netherlands). To fVdp was added 10 nM fVa (fVdp/+fVa), whereas 1 nM fVIIIa was added to the fVIIIdp (fVIIIdp/+fVIIIa). For experiments involving fVfVIIIdp, 10 nM fVa and 1 nM fVIIIa were added (fVfVIIIdp/+fVa+fVIIIa) and thrombin generation was initiated by addition of 5 nM fIXa. The thrombin generation reactions were initiated by adding FluCa, a mixture of $CaCl_2$ and fluorescent substrate (Thrombinoscope). Appropriate reference wells containing thrombin calibrator were prepared for each condition and HD1 or $Hir^{54-65}(SO_3^-)$ and fVa or fVIIIa were added where appropriate. Plates were read on a Labsystems Fluoroskan Ascent FL plate reader (MTX Lab Systems, Inc, Vienna, VA) for 2 h at 30 s intervals at excitation and emission wavelengths of 360 nm and 460 nm, respectively, and the CAT assay software (Thrombinoscope) was used to calculate the lag time, peak thrombin concentration, and area under the curve or endogenous thrombin potential (ETP). With fVIIIdp and fV/fVIIIdp, the lag time was manually determined as the time to reach 10 nM thrombin.

Effect of HD1 and Hir$^{54-65}$(SO_3^-) on the rates of thrombin inhibition by antithrombin or HCII:

The rate of inhibition of 20 nM thrombin by 1 μM antithrombin or HCII was measured at 23°C in the absence or presence of 2.5 μM HD1 or Hir$^{54-65}$(SO_3^-) using a discontinuous assay under pseudo-first order conditions. Second order inhibition rate constants (k_2) were determined according to published methods (Becker *et al.*, 1997).

Statistical methods:

Unless otherwise indicated, experiments were performed at least 3 times. Results are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences in the effects of HD1 and Hir$^{54-65}$(SO_3^-) on clotting times was examined using Student's t-tests (InStat 3 GraphPad Software, Inc., La Jolla, CA), whereas their inhibitory effects on thrombin generation were explored via two-way analysis of variance (Kelinbaum *et al.*, 1988). The latter technique yields a test of the difference in mean (averaged over concentration range) between HD1 and Hir$^{54-65}$(SO_3^-), the so-called main effect, and the difference in the concentration response between HD1 and Hir$^{54-65}$(SO_3^-), the ligand-concentration effect. Thrombin generation also was compared in a pair-wise fashion between control plasma and the various factor-deficient/activated factor-supplemented plasmas. The deficient plasmas represent an additional experimental "condition" and necessitated exploration via three-way analysis of variance (Kelinbaum *et al.*, 1988). The relevant tests were the relationship between plasma condition with ligand (the extent to which the difference between HD1 and Hir^{54-}

$^{65}(SO_3^-)$ depends on plasma condition), and with the ligand-concentration effect (the extent to which the difference in concentration response between HD1 and $Hir^{54-65}(SO_3^-)$ varies with plasma condition). For all analyses, a p value ≤ 0.05 was considered statistically significant.

Results

Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on clotting times in plasma:

Because of its higher affinity for prothrombin, we hypothesized that HD1 would be a more potent inhibitor of coagulation than $Hir^{54-65}(SO_3^-)$. To test this, we first compared the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on the aPTT and PT, which measure the integrity of the intrinsic and extrinsic pathways of coagulation, respectively. At equimolar concentrations, HD1 and $Hir^{54-65}(SO_3^-)$ prolonged the aPTT 3- and 2-fold, respectively, and the PT 7- and 4-fold, respectively (Table 4.1). Thus, HD1 is significantly ($p<0.001$) more potent than $Hir^{54-65}(SO_3^-)$ in these global clotting assays. To determine whether this difference reflects the capacity of HD1 to inhibit prothrombin activation by prothrombinase, we next compared the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on the thrombin and fXa clotting times. Whereas HD1 and $Hir^{54-65}(SO_3^-)$ had similar effects on the thrombin clotting time (Table 4.1), consistent with their similar capacity to inhibit thrombin clotting activity, HD1 had a significantly ($p<0.05$) greater effect on the fXa clotting time than $Hir^{54-65}(SO_3^-)$, consistent with the capacity of HD1 to inhibit prothrombin activation.

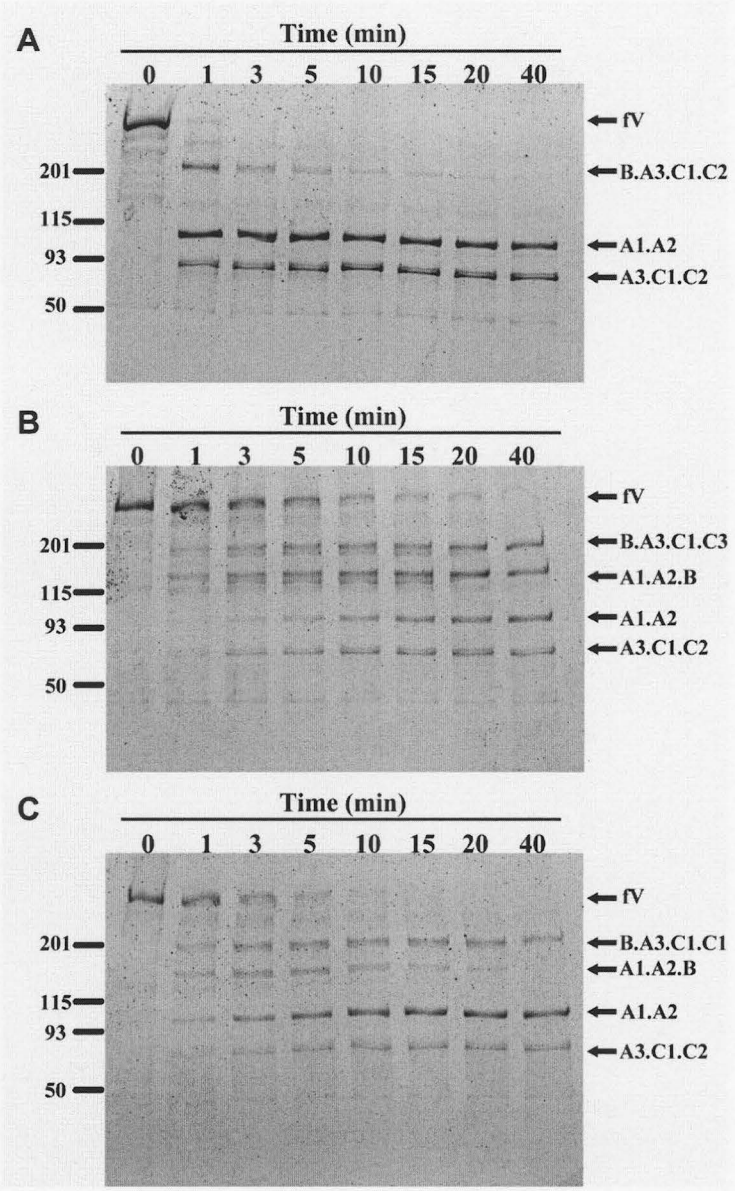
Assay	Normal			fVfVIIIdp/+fVa+fVIIIa		
	Baseline	HD1	Hir$^{54-65}$(SO_3^-)	Baseline	HD1	Hir$^{54-65}$(SO_3^-)
aPTT	40 ± 2	130 ± 2	74 ± 1	-	-	-
PT	11 ± 1	73 ± 1	39 ± 1	-	-	-
fXa time	90 ± 4	177 ± 7	156 ± 3	126 ± 6	350 ± 11	209 ± 3
Thrombin Time	27 ± 2	62 ± 4	74 ± 10	49 ± 11	94 ± 3	87 ± 7

Table 4.1: Effect of HD1 or Hir$^{54-65}$(SO_3^-) on clotting times in normal plasma or plasma depleted of fV and fVIII and supplemented with fVa and fVIIIa

Clotting assays were performed in the absence or presence of 20 μM HD1 or Hir$^{54-65}$(SO_3^-). The data represent the mean ± SEM of 3 separate experiments, each done in duplicate. Both inhibitors significantly prolong all clotting parameters beyond that of baseline ($p < 0.05$). Compared with Hir$^{54-65}$(SO_3^-), HD1 produces a significantly greater prolongation of all of the clotting times except for the thrombin time ($p < 0.05$).

Effect of HD1 or Hir$^{54-65}$(SO_3^-) on fVIII or fV activation by thrombin:

Activation of fV and fVIII is essential for the rapid thrombin generation that is elicited in global coagulation assays, such as the PT or aPTT. To explore potential differences in the effects of HD1 and Hir$^{54-65}$(SO_3^-) on this feedback step, we used SDS-PAGE to compare their capacity to inhibit fV and fVIII activation by thrombin. In the absence of inhibitor, the A1-A2 heavy chain and the A3-C1-C2 light chain of fVa were fully formed after 40 min (Figure 4.1 A). With 5 μM HD1 or Hir$^{54-65}$(SO_3^-), the B-A3-C1-C2 and A1-A2-B activation intermediates accumulated and formation of A3-C1-C2 was delayed, consistent with attenuated cleavage at Arg^{1018}, Arg^{709}, and Arg^{1535}, respectively (Figure 4.1 B and C). The effect of HD1 or Hir$^{54-65}$(SO_3^-) on thrombin-mediated activation of fVIII was assessed in the same fashion. In the absence of inhibitors, the A1-A2 heavy chain appeared first, followed by slower formation of the A1 and A2 chains and the A3-C1-C2 light chain (Figure 4.2 A). With 5 μM HD1 or Hir$^{54-65}$(SO_3^-), formation of the A1, A2, and A3-C1-C2 chains was delayed, consistent with attenuated cleavage at Arg^{740}, Arg^{340}, and Arg^{1689} (Figure 4.2 B and C). These results demonstrate that HD1 and Hir$^{54-65}$(SO_3^-) inhibit fV and fVIII activation by thrombin to a similar extent. Therefore, the more potent inhibitory effect of HD1 in plasma clotting assays is unlikely to reflect greater inhibition of thrombin-mediated feedback activation of fV or fVIII by HD1 relative to Hir$^{54-65}$(SO_3^-).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50
fV
B.A3.C1.C2
A1.A2
A3.C1.C2
B
Time (min)
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201
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93
50
fV
B.A3.C1.C3
A1.A2.B
A1.A2
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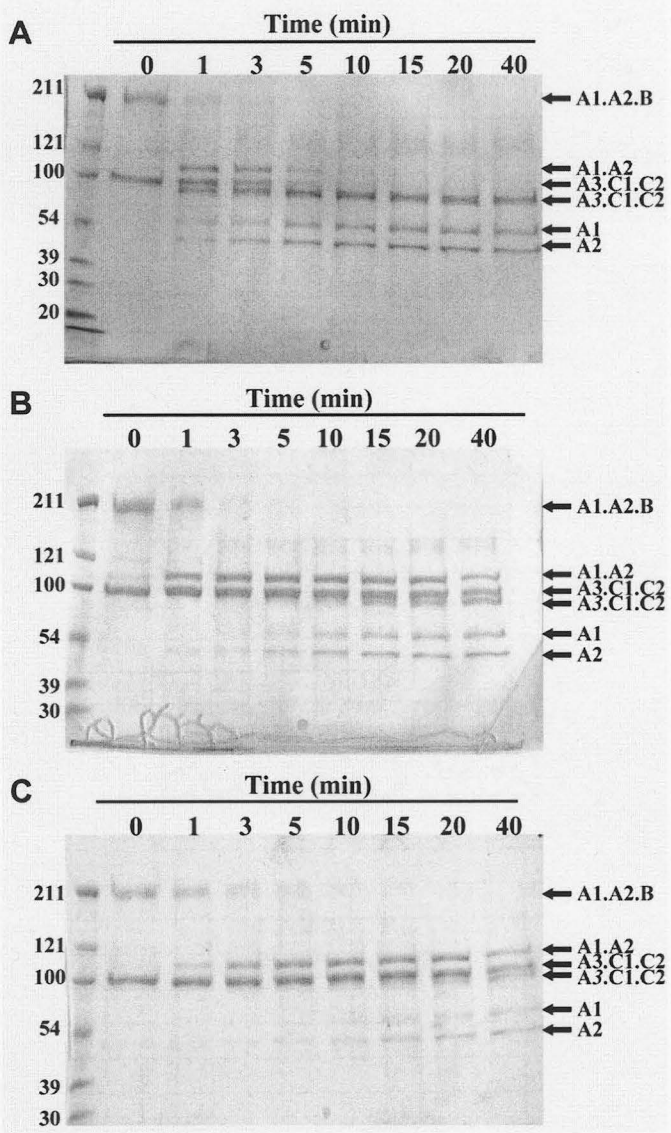
C
Time (min)
0 1 3 5 10 15 20 40
201
115
93
50
fV
B.A3.C1.C1
A1.A2.B
A1.A2
A3.C1.C2

Figure 4.1: SDS-PAGE analysis of fV activation by thrombin in the absence or presence of $Hir^{54-65}(SO_3^-)$ or HD1

Factor (f) V (0.6 μM) was activated by 10 nM thrombin in the absence (A) or presence of 5 μM HD1 (B) or $Hir^{54-65}(SO_3^-)$ (C). At the times indicated, aliquots were removed and subjected to SDS-PAGE analysis under reducing conditions. Gels were stained with Sypro Ruby and bands were visualized by fluorescence. Molecular weight markers are identified on the *left*, whereas fV-derived bands are identified by their domain composition on the *right*.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Time (min)
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121
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54
39
30
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A1.A2.B
A1.A2
A3.C1.C2
A3.C1.C2
A1
A2
B
Time (min)
0 1 3 5 10 15 20 40
211
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A1.A2.B
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A3.C1.C2
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Time (min)
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211
121
100
54
39
30
A1.A2.B
A1.A2
A3.C1.C2
A3.C1.C2
A1
A2

Figure 4.2: SDS-PAGE analysis of fVIII activation by thrombin in the absence or presence of $Hir^{54-65}(SO_3^-)$ or HD1

Factor (f) VIII (3 μM) was activated by 10 nM thrombin in the absence (A) or presence of 5 μM HD1 (B) or $Hir^{54-65}(SO_3^-)$ (C). At the times indicated, aliquots were removed and subjected to SDS-PAGE analysis under reducing conditions. Gels were stained with Fast Stain. Molecular weight markers are identified on the *left*, whereas fVIII-derived bands are identified by their domain composition on the *right*.

Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on clotting times in fVfVIIIdp:

Having shown that HD1 and $Hir^{54-65}(SO_3^-)$ inhibit thrombin-mediated activation of fVIII and fV to a similar extent, we next tested their capacity to block plasma clotting when the feedback reactions were bypassed in an effort to simplify the reactions that contribute to clot formation. Thus, the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on thrombin and fXa clotting times were measured in fVfVIIIdp supplemented with 3 nM fVa, 0.3 nM fVIIIa, and 5 μM PCPS. The thrombin clotting time of the deficient plasma was 41 ± 7 s and remained minimally affected by the addition of fVa and fVIIIa (49 ± 11 s). Addition of either 2.5 μM HD1 or $Hir^{54-65}(SO_3^-)$ produced a similar 2-fold prolongation of the thrombin clotting time (Table 4.1). This result is consistent with the findings in normal plasma and reflects the comparable affinities of HD1 and $Hir^{54-65}(SO_3^-)$ for thrombin. When fXa was added to fVfVIIIdp, no clotting was observed (data not shown). With the addition of fVa and fVIIIa, the fXa clotting time was 126 ± 6 s. Whereas 2.5 μM HD1 prolonged the fXa clotting time ~3-fold, 2.5 μM $Hir^{54-65}(SO_3^-)$ only prolonged it ~1.5-fold, a significantly lower value ($p < 0.001$) (Table 4.1). These results are consistent with the capacities of HD1 and $Hir^{54-65}(SO_3^-)$ to inhibit coagulation in normal plasma, but suggest that HD1 and $Hir^{54-65}(SO_3^-)$ inhibit thrombin generation to different extents.

Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin generation in control plasma:

HD1 prolongs plasma clotting times to a greater extent than $Hir^{54-65}(SO_3^-)$ despite their equivalent capacity to inhibit thrombin feedback reactions. Therefore, we used the CAT assay to better understand how each inhibitor attenuates thrombin generation in a dynamic system. Thus, defibrinated plasma was incubated with increasing concentrations of HD1 or $Hir^{54-65}(SO_3^-)$ before initiating thrombin generation with $CaCl_2$ and 0.5 pM TF. In the absence of HD1 or $Hir^{54-65}(SO_3^-)$, thrombin activity was detected after a 4.2 ± 0.2 min lag time, culminating in a 95.1 ± 13.4 nM peak thrombin concentration that was achieved at 9.8 ± 0.8 min (Table 4.2). Subsequently, thrombin concentration decreased until ~25 min, at which point thrombin activity was no longer detectable. In the presence of increasing concentrations of HD1, thrombin generation decreased in a dose-dependent fashion (Figure 4.3 A), as evidenced by a prolongation in the lag time (Figure 4.3 C) and a reduction in the peak thrombin concentration and ETP (Figure 4.3 D, E). At 25 μM HD1, thrombin generation was completely inhibited because the ETP approached baseline. Quantitatively, $Hir^{54-65}(SO_3^-)$ was a weaker inhibitor of thrombin generation than HD1. Thus, the dose-dependent prolongation of the lag time reached a maximum of 13.5 min at 25 μM $Hir^{54-65}(SO_3^-)$, which was 5-fold lower than the respective value for 25 μM HD1. In contrast to HD1, higher concentrations of $Hir^{54-65}(SO_3^-)$ were unable to reduce the peak thrombin concentration below the control value. Thus, at 25 μM $Hir^{54-65}(SO_3^-)$, the peak thrombin concentration was 170.1 ± 2.1 nM, representing a 44% increase from control.

Plasma	Lag (min)	Peak (nM)	ETP (nM min)	Time to Peak (min)
PPP	4.5 ± 0.2	75 ± 3.3	1079 ± 37.5	10.5 ± 0.2
Normal	4.2 ± 0.2	95.1 ± 13.4	923 ± 30	9.8 ± 0.8
fVdp/+fVa	3.2 ± 0.2[a]	185.2 ± 6.1[a]	1140 ± 9[a]	7.2 ± 0.2[a]
fVIIIdp/+fVIIIa	3.0 ± 0.2[a]	102.4 ± 3.5	1177 ± 4[a]	11.8 ± 0.2
fVfVIIIdp/+fVa+fVIIIa	1.5 ± 0.2[a]	335.5 ± 5.4[a]	1182 ± 6[a]	4.0 ± 0.2[a]

Table 4.2: Effect of plasma conditions on thrombin generation parameters

The CAT assay parameters were determined in normal or cofactor depleted plasma. All plasmas were defibrinated except PPP, where fibrinogen was not removed. Values represent mean ± SEM. The parameters for PPP are not significantly different from those for normal plasma, indicating that fibrinogen removal has minimal effects on the thrombin generation parameters.

[a] denotes values that are significantly different from the corresponding value in normal plasma ($p < 0.05$).

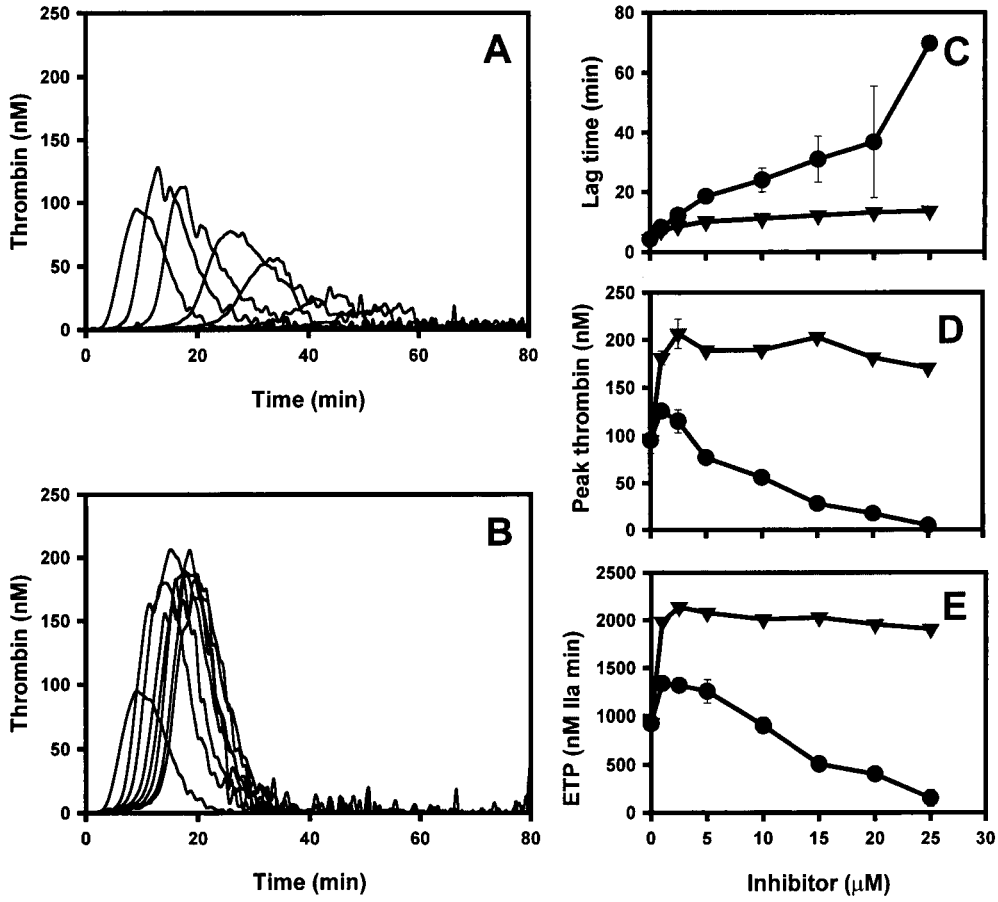

A
B
C
D
E
Thrombin (nM)
Time (min)
Lag time (min)
Peak thrombin (nM)
ETP (nM IIa min)
Inhibitor (μM)

Figure 4.3: Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin generation in defibrinated plasma

The effect of increasing concentrations (0-25 μM) of (A) HD1 or (B) $Hir^{54-65}(SO_3)$ on thrombin generation in defibrinated normal plasma was determined using the CAT assay. TF (0.5 pM) was added to the plasma before initiating thrombin generation with $CaCl_2$. The thrombin concentrations were calculated and plotted versus time. Data in panels A and B represent the mean of 3 separate experiments, and the profiles that represent increasing concentrations of HD1 or $Hir^{54-65}(SO_3^-)$ are shown from left to right. The effect of increasing concentrations of HD1 (●) or $Hir^{54-65}(SO_3^-)$ (▼) on lag time (C), peak thrombin concentration (D) and ETP (E) are plotted. The data in panels C-E represent the mean ± SEM of two separate experiments, each performed in duplicate.

Formal comparisons of the thrombin dose responses shown in Figures 4.3 C, D, and E indicate highly statistically significant ($P<0.001$) differences in (a) the averaged HD1 and $Hir^{54-65}(SO_3^-)$ effect (main effect) and (b) the slopes of the concentration-response curves (ligand-concentration effect) for each parameter of thrombin generation. Similar data were obtained in control plasma that was not defibrinated (Appendix 4.1), suggesting that the differences in the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on thrombin generation are fibrinogen-independent. Therefore, although HD1 and $Hir^{54-65}(SO_3^-)$ bind exosite 1 on thrombin with similar affinities and inhibit thrombin activation of fV and fVIII to a similar extent, HD1 is more potent at prolonging the lag time and decreasing the peak thrombin concentration in normal plasma.

Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin generation in cofactor-deficient plasma:

We next monitored thrombin generation in defibrinated fVdp/+fVa to bypass the fV feedback activation step. No thrombin activity was detected in defibrinated fVdp without added fVa (data not shown). When defibrinated fVdp was supplemented with 10 nM fVa, thrombin activity was detected after a brief 3.2 ± 0.2 min lag time, culminating in a peak thrombin concentration of 185.2 ± 6.1 nM (Table 2). Neither HD1 nor $Hir^{54-65}(SO_3^-)$ had an effect on the lag time (Fig. 4.4C, $p=0.77$). The peak thrombin concentration was significantly reduced with increasing concentrations of HD1, but not with $Hir^{54-65}(SO_3^-)$ (Fig. 4.4D, $p<0.001$). Thus, with 25 μM HD1, peak thrombin concentration was 99.7 ± 5.8 nM; a 50% reduction from control. In contrast,

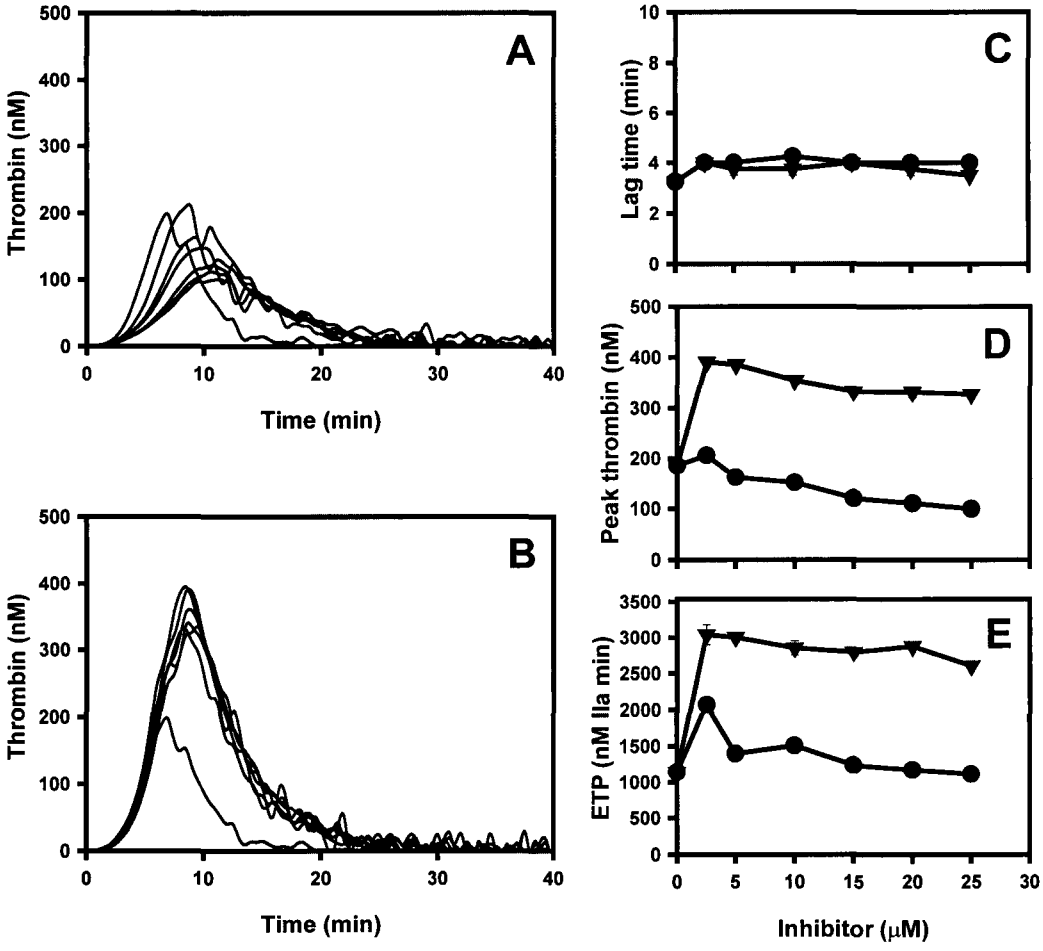

A
Thrombin (nM)
Time (min)
B
Thrombin (nM)
Time (min)
C
Lag time (min)
D
Peak thrombin (nM)
E
ETP (nM IIa min)
Inhibitor (μM)

Figure 4.4: Effect of HD1 or Hir$^{54-65}$(SO_3^-) on thrombin generation in fV-deficient, defibrinated plasma

The effect of increasing concentrations (0-25 μM) of (A) HD1 or (B) Hir$^{54-65}$(SO_3) on thrombin generation in fV-depleted plasma containing 10 nM fVa was determined using the CAT assay. TF (0.5 pM) was added to the plasma before initiating thrombin generation with $CaCl_2$. The thrombin concentrations were calculated and plotted versus time. Data in panels A and B represent the mean of 3 separate experiments, and the profiles that represent the effect of increasing concentrations of HD1 or Hir$^{54-65}$(SO_3^-) are shown from left to right. The effect of increasing concentrations of HD1 (●) or Hir$^{54-65}$(SO_3^-) (▼) on lag time (C), peak thrombin concentration (D) and ETP (E) are plotted. The data in panels C-E represent the mean ± SEM of two separate experiments, each performed in duplicate.

$Hir^{54-65}(SO_3^-)$ caused an increase in the peak thrombin concentration. Thus, with 2.5 μM $Hir^{54-65}(SO_3^-)$, the peak thrombin increased to 390.4 ± 8.6 nM, a 2-fold higher value than control. Peak thrombin concentration decreased with higher concentrations of $Hir^{54-65}(SO_3^-)$ but remained elevated with respect to that measured in both control plasma and in plasma with corresponding concentrations of HD1. At 25 μM $Hir^{54-65}(SO_3^-)$, the peak thrombin was 327.3 ± 2.1 nM, which is still ~2-fold higher than the control (Figure 4D). Higher concentrations of HD1 reduced the ETP to a significantly greater extent than $Hir^{54-65}(SO_3^-)$ (Figure 4E, $p<0.001$). HD1 also was more potent than $Hir^{54-65}(SO_3^-)$ at prolonging the time to peak thrombin (data not shown). Therefore, HD1 potently inhibits thrombin generation in defibrinated fVdp/+fVa, whereas $Hir^{54-65}(SO_3^-)$ failed to effect a significant reduction. This result may reflect the ability of HD1, but not $Hir^{54-65}(SO_3^-)$, to inhibit prothrombinase.

To study the effect of HD1 and $Hir^{54-65}(SO_3^-)$ on fV activation in plasma, we measured thrombin generation in defibrinated fVIIIdp/+fVIIIa. Although the thrombin generation profile in defibrinated fVIIIdp was unaffected by the addition of 1 nM of fVIIIa (data not shown), to maintain consistency, fVIIIa was added in subsequent experiments. In the absence of inhibitors, the lag time was 3 min (Table 4.2). The peak thrombin concentration was 102.4 ± 3.5 nM, a value 10% higher than that in control plasma, but 45% lower than that in fVdp/+fVa. With increasing concentrations of HD1 or $Hir^{54-65}(SO_3^-)$, a slightly elevated but constant thrombin activity (2-8 nM) was detected prior to the propagation phase (in the first 2-3 min) compared with that observed in control plasma (Figure 4.5 A and B). Consequently, the lag time in defibrinated

fVIIIdp/+fVIIIa was manually determined as the time to reach 10 nM thrombin. $Hir^{54-65}(SO_3^-)$ caused a dose-dependent prolongation of the lag time, which levelled at 6 min in the presence of 25 μM $Hir^{54-65}(SO_3^-)$. Although HD1 also prolonged the lag time in a dose-dependent fashion, this effect was not saturable, similar to the results in normal plasma ($p=0.76$). Interestingly, in the range of 1-5 μM, HD1 and $Hir^{54-65}(SO_3^-)$ prolonged the lag time to a similar extent (Figure 4.5 C). However, at concentrations higher than 5 μM, HD1 prolonged the lag time to a greater extent than $Hir^{54-65}(SO_3)$. Although both HD1 and $Hir^{54-65}(SO_3^-)$ caused an initial increase in peak thrombin concentration, further addition of HD1 caused a more pronounced decrease than $Hir^{54-65}(SO_3^-)$ (Figure 4.5 D, $p<0.001$). Thus, at 25 μM HD1, the peak thrombin concentration decreased to 14.0 ± 2.6 nM, compared with 148.9 ± 0.8 nM for 25 μM $Hir^{54-65}(SO_3^-)$. In addition, HD1 was more potent than $Hir^{54-65}(SO_3^-)$ at inhibiting the ETP in fVIIIdp/+fVIIIa (Figure 4.5 E, $p<0.001$). These results demonstrate that the ability of $Hir^{54-65}(SO_3^-)$ to prolong the lag time of thrombin generation is due to its capacity to inhibit thrombin feedback activation of fV, and not fVIII.

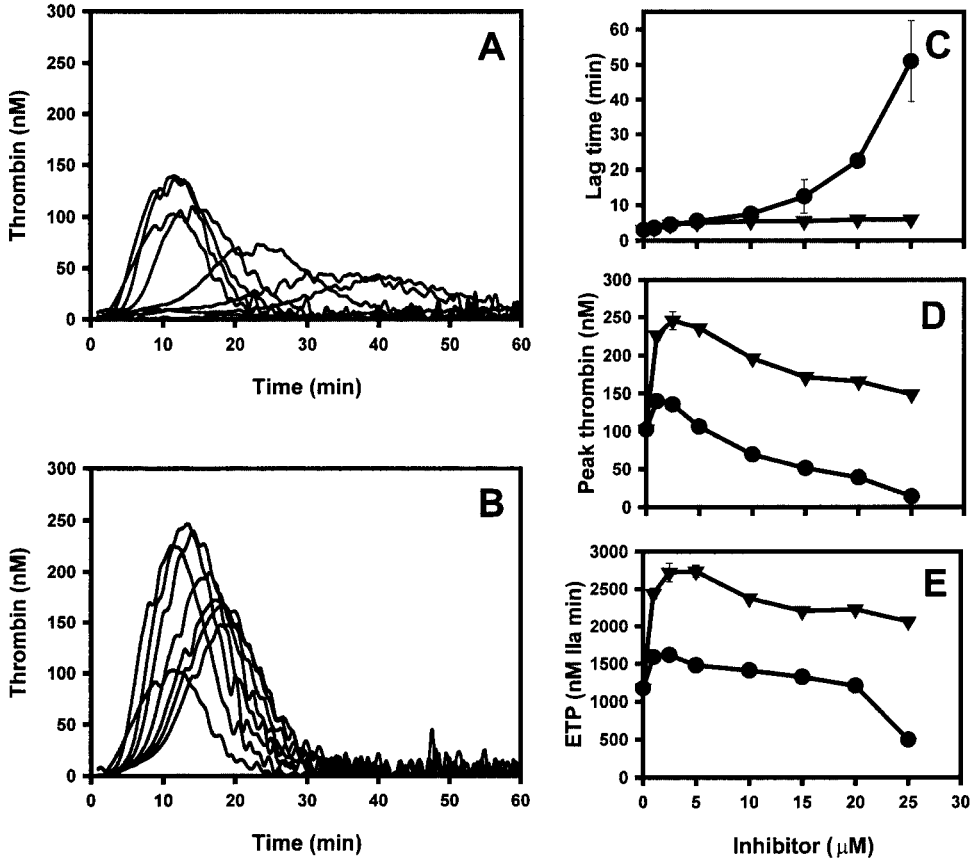

A
B
C
D
E
Thrombin (nM)
Time (min)
Lag time (min)
Peak thrombin (nM)
ETP (nM IIa min)
Inhibitor (μM)

Figure 4.5: Effect of HD1 or $Hir^{54-65}(SO_3^-)$ on thrombin generation in fVIII-deficient defibrinated plasma

The effect of increasing concentrations (0-25 μM) of (A) HD1 or (B) $Hir^{54-65}(SO_3)$ on thrombin generation in fVIII-deficient plasma containing 1 nM fVIIIa was determined using the CAT assay. TF (0.5 pM) was added to the plasma before initiating thrombin generation with $CaCl_2$. The thrombin concentrations were calculated and plotted versus time. Data in panels A and B represent the mean of 3 separate experiments, and the profiles that represent the effect of increasing concentrations of HD1 or $Hir^{54-65}(SO_3^-)$ are shown from left to right. The effect of increasing concentrations of HD1 (●) or $Hir^{54-65}(SO_3^-)$ (▼) on lag time (C), peak thrombin concentration (D) and ETP (E) are plotted. The data in panels C-E represent the mean ± SEM of two separate experiments, each performed in duplicate.

It is clear from these thrombin generation experiments that inhibition of fV activation contributes to the capacity of HD1 and $Hir^{54-65}(SO_3^-)$ to inhibit thrombin generation. However, this does not explain the differences between these inhibitors because HD1 and $Hir^{54-65}(SO_3^-)$ inhibit fV and fVIII activation by thrombin to a similar extent. To determine whether differences between HD1 and $Hir^{54-65}(SO_3^-)$ can be explained by their different capacities to inhibit prothrombin activation by prothrombinase, we compared the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on thrombin generation in defibrinated fVfVIIIdp/+fVa+fVIIIa. In the absence of HD1 or $Hir^{54-65}(SO_3^-)$, the peak thrombin concentration was 335.5 ± 5.4 nM (Table 4.2). The lag time was 1.5 ± 0.2 min, a value significantly shorter than that in defibrinated fVdp/+fVa ($p < 0.05$), which highlights the contribution of fVIII activation to thrombin generation (Table 4.2). The ETP was 1182.5 ± 5.5 μM·min (Table 4.2), a value similar to that determined in defibrinated fVdp/+fVa. Lag time increased with higher concentrations of both HD1 and $Hir^{54-65}(SO_3)$, but the effect was stronger with HD1 (Figure 4.6 C, $p<0.001$). HD1 also was more potent than $Hir^{54-65}(SO_3^-)$ at reducing the peak thrombin concentration (Figure 4.6 D, $p<0.001$), whereas neither HD1 nor $Hir^{54-65}(SO_3^-)$ significantly reduced the ETP (Figure 4.6 E). These results demonstrate that (a) in the absence of feedback reactions, HD1 is more potent than $Hir^{54-65}(SO_3)$ at blocking thrombin generation, and (b) HD1 inhibition of prothrombinase causes a reduction in the peak thrombin concentration as well as a prolongation of the lag time.

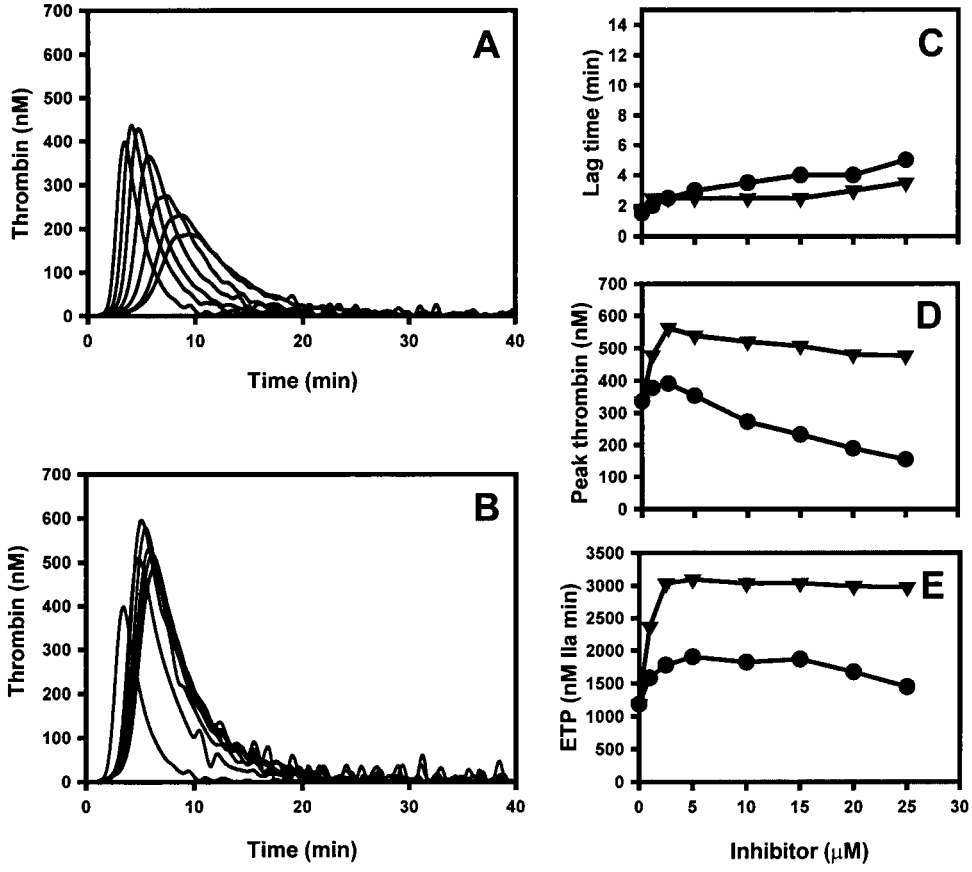

A
Thrombin (nM)
Time (min)
B
Thrombin (nM)
Time (min)
C
Lag time (min)
D
Peak thrombin (nM)
E
ETP (nM IIa min)
Inhibitor (μM)

Figure 4.6: Effect of HD1 or Hir$^{54-65}$(SO_3^-) on thrombin generation in fV/fVIII-deficient defibrinated plasma

The effect of increasing concentrations (0-25 μM) of (A) HD1 or (B) Hir$^{54-65}$(SO_3^-) on thrombin generation in fV- and fVIII-depleted plasma containing 10 nM fVa and 1 nM fVIIIa was determined using the CAT assay. FIXa (5 pM) was added prior to initiating thrombin generation with $CaCl_2$. Thrombin concentrations were calculated and plotted versus time. Data in panels A and B represent the mean of 3 separate experiments, and the profiles that represent the effect of increasing concentrations of HD1 or Hir$^{54-65}$(SO_3^-) are shown from left to right. The effect of increasing concentrations of HD1 (●) or Hir$^{54-65}$(SO_3^-) (▼) on lag time (C), peak thrombin concentration (D) and ETP (E) are plotted. The data in panels C-E represent the mean ± SEM of two separate experiments, each performed in duplicate.

Effect of HD1 and $Hir^{54-65}(SO_3^-)$ on thrombin inhibition by antithrombin or HCII:

Despite their effects on the peak thrombin, HD1 and $Hir^{54-65}(SO_3^-)$ did not cause the expected proportional reduction in the ETP in doubly depleted plasma. This finding, coupled with the increase in peak thrombin concentration and ETP observed with lower concentrations of HD1 or $Hir^{54-65}(SO_3^-)$, suggests that these ligands influence the termination phase. To explore this possibility, we examined the effect of HD1 and $Hir^{54-65}(SO_3^-)$ on the rates of thrombin inhibition by antithrombin or HCII. In the absence of HD1 or $Hir^{54-65}(SO_3^-)$, the k_2 values for thrombin inhibition by antithrombin or HCII were $3.8 \pm 0.35 \times 10^3$ and $5.30 \pm 0.8 \times 10^2$ $M^{-1}s^{-1}$, respectively; values consistent with previous reports (Rezaie, 1996). At 2.5 μM, both HD1 and $Hir^{54-65}(SO_3^-)$ significantly ($p < 0.005$) reduced the rate of thrombin inhibition by antithrombin (to $1.5 \pm 0.2 \times 10^3$ and $1.2 \pm 0.4 \times 10^3$ $M^{-1}s^1$, respectively) and HCII (to $2.58 \pm 0.5 \times 10^2$ and $3.13 \pm 0.1 \times 10^2$ $M^{-1}s^{-1}$, respectively). Therefore, HD1 and $Hir^{54-65}(SO_3^-)$ reduce the rates of thrombin inhibition by antithrombin or HCII to a similar extent.

Discussion

Previously, we demonstrated that HD1 not only inhibits thrombin activity, but also attenuates prothrombin activation by prothrombinase, whereas $Hir^{54-65}(SO_3^-)$ only inhibits thrombin activity (Kretz *et al.*, 2006). Because of its dual mechanism of inhibition, we hypothesized that HD1 would be a more potent inhibitor of coagulation than $Hir^{54-65}(SO_3^-)$. In support of this concept, we observed that at equimolar concentrations, HD1 (a) prolongs the aPTT and PT to a greater extent than $Hir^{54-65}(SO_3)$ in control plasma, and (b) prolongs the fXa clotting time to a greater extent than $Hir^{54-65}(SO_3^-)$ in both control and fVfVIIIdp reconstituted with fVa and fVIIIa. In contrast, HD1 and $Hir^{54-65}(SO_3^-)$ have similar effects on the thrombin clotting time, reflecting the fact that by binding exosite 1 on thrombin with similar affinities (Kretz *et al.*, 2006), they attenuate thrombin-mediated conversion of fibrinogen to fibrin to the same extent (Meh *et al.*, 1996, Pospisil *et al.*, 2003). These findings, though consistent with our previous findings in buffer systems (Kretz *et al.*, 2006, Pospisil *et al.*, 2003), do not provide quantitative evidence of the differences between $Hir^{54-65}(SO_3^-)$ and HD1 in plasma.

CAT is a useful tool for studying coagulation in plasma. Although it remains to be established whether the CAT assay reflects the dynamics of thrombin generation *in vivo*, it is useful for predicting the thrombin generating capacity of plasma in response to a procoagulant stimulus (Al Dieri *et al.*, 2004, Hemker *et al.*, 2004). In an effort to delineate the contribution of thrombin-binding and prothrombin-binding to the inhibitory activity of HD1, we conducted CAT assays in normal and deficient plasmas. Hir^{54-}

$^{65}(SO_3^-)$ was used as a comparator in these assessments because it binds thrombin but not prothrombin (Anderson *et al.*, 2000a).

The initiation phase of thrombin generation, represented by the lag time, is described as the time required for generation of the reactants that elicit rapid thrombin generation, notably fVa and fVIIIa (Brummel *et al.*, 2002, Hemker *et al.*, 2006a, Mann *et al.*, 2006). Although HD1 and $Hir^{54-65}(SO_3^-)$ are equally effective at inhibiting thrombin-mediated activation of fV and fVIII in buffer, in the CAT assay HD1 prolongs the lag time to a greater extent than $Hir^{54-65}(SO_3^-)$ in control plasma, suggesting that the lag time is not solely respondent to thrombin feedback activation of fV and fVIII. The greater inhibitory effect of HD1 on the lag time likely reflects its capacity to also inhibit prothrombinase. Support for this concept comes from the observation that fXa inhibitors also prolong the lag time (Gerotziafas *et al.*, 2007).

In addition to determining the mechanism by which HD1 and $Hir^{54-65}(SO_3^-)$ inhibit coagulation, the use of deficient plasmas in the CAT assay provides insight into the relative importance of thrombin-mediated feedback activation of fVIII and fV to thrombin generation. Although $Hir^{54-65}(SO_3^-)$ inhibits thrombin-mediated fVIII activation in a purified system, it does not appear to affect fVIII activation during thrombin generation in plasma as evidenced by its failure to influence the lag time in defibrinated fVdp/+fVa. These results suggest that thrombin-mediated activation of fVIII is not essential for the initiation or propagation of thrombin generation in the CAT assay. This holds true even when clotting is initiated with a low concentration of TF (0.5 pM), which should maximize the reliance of thrombin generation on fVIII activation (Wakabayashi *et*

al., 2008, Wakabayashi and Fay, 2008). Further support for the concept that thrombin generation is not dependent on fVIII activation comes from the observation that the initiation phase in fVIIIdp or fIXdp is similar to that in normal plasma (Ovanesov *et al.*, 2005). These findings can be explained by the fact that only 0.1% of fX is activated during the propagation phase and ~12 nM fVa is generated 3 min after TF addition to plasma (Brummel *et al.*, 2002). Therefore, extensive fXa generation is not a prerequisite for rapid thrombin generation in plasma. The same may be true *in vivo* because other investigators have concluded that intrinsic tenase does not contribute to the initiation of thrombosis in animal models (Ovanesov *et al.*, 2005).

To determine the extent to which attenuation of thrombin-mediated fV activation contributes to the capacity of HD1 and $Hir^{54\text{-}65}(SO_3^-)$ to inhibit thrombin generation, CAT assays were performed in fVIIIdp/+fVIIIa. Both HD1 and $Hir^{54\text{-}65}(SO_3^-)$ prolong the lag time in defibrinated fVIIIdp/+fVIIIa, confirming the importance of fV activation to the lag time. Both agents also reduced the peak thrombin concentration and prolonged the lag time to a greater extent in fVIIIdp/+fVIIIa than they did in fVdp/+fVa. Furthermore, HD1 and $Hir^{54\text{-}65}(SO_3^-)$ have similar effects on the lag time and peak thrombin concentration in fVIIIdp/+fVIIIa as they do in normal plasma, highlighting the importance of thrombin-mediated fV activation in the thrombin generation assay.

At higher concentrations, $Hir^{54\text{-}65}(SO_3^-)$ does not reduce the peak thrombin concentration to the same extent as HD1 in normal or deficient plasmas. Because HD1 competes with fVa for prothrombin binding (Kretz *et al.*, 2006), the greater capacity of HD1 to reduce the peak thrombin concentration likely reflects its ability to inhibit

prothrombinase. This would explain why the inhibitory activity of HD1 is attenuated in fVdp/+Va because fVa addition accelerates prothrombinase assembly. In contrast to HD1, $Hir^{54-65}(SO_3^-)$ is a weak inhibitor of prothrombinase (Anderson *et al.*, 2000a, Anderson *et al.*, 2000b, Kretz *et al.*, 2006). Therefore, it is not surprising that $Hir^{54-65}(SO_3^-)$ is less potent than HD1 at reducing the peak thrombin concentration. Other investigators have shown that the peak thrombin concentration and ETP are sensitive to (a) mutations in fVa that impair its cofactor activity (Thorelli *et al.*, 1998), and (b) changes in plasma fV concentration (Allen *et al.*, 2004). These observations support the concept that HD1-mediated reduction in the peak thrombin concentration reflects its capacity to inhibit prothrombin activation by prothrombinase.

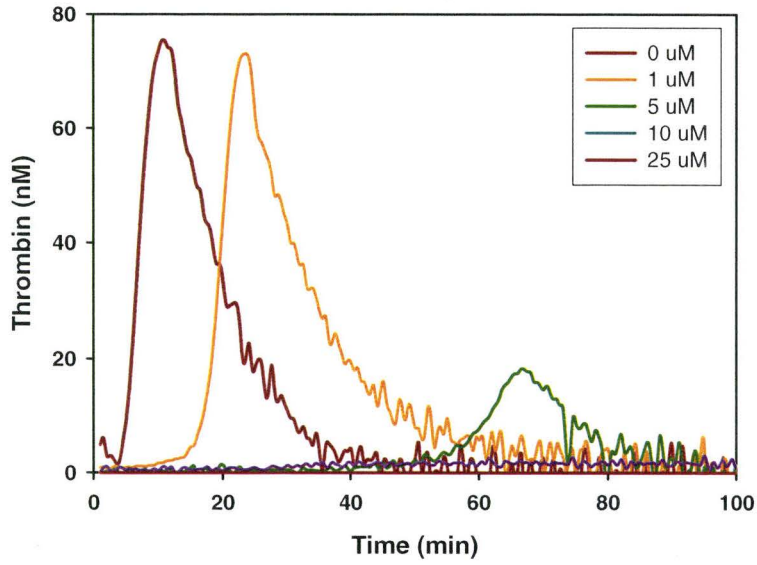
It is evident from our studies that HD1 reduces the peak thrombin concentration to a greater extent than $Hir^{54-65}(SO_3^-)$. However, the increase in peak thrombin concentration observed with $Hir^{54-65}(SO_3^-)$ requires an explanation. In a previous study, bivalirudin, another thrombin inhibitor, also prolonged the lag time and increased the peak thrombin concentration (Butenas *et al.*, 2007). To explore the mechanism responsible for this phenomenon, we examined the effect of $Hir^{54-65}(SO_3^-)$ and HD1 on the rate of thrombin inhibition by antithrombin and HCII. Both agents reduce the rate of thrombin inhibition by these serpins to a similar extent, suggesting that exosite 1-directed ligands induce an allosteric change at the active site of thrombin that impairs its interaction with the reactive center loops of the inhibitors (Croy *et al.*, 2004, Duffy *et al.*, 1997, Fredenburgh *et al.*, 1997). Our data are consistent with previous reports demonstrating that mutations in antithrombin that reduce its capacity to inhibit thrombin

or fXa result in an increase in both the peak thrombin concentration and the ETP (Hemker *et al.*, 2002, Sanchez *et al.*, 2008). The observation that low concentrations of HD1 are associated with a modest increase in peak thrombin in all plasma systems is consistent with this concept. However, higher concentrations of HD1 cause a reduction in peak thrombin because of its capacity to inhibit prothrombinase. In contrast, $Hir^{54-65}(SO_3^-)$, which is unable to inhibit prothrombinase, fails to reduce peak thrombin in the CAT assay.

Overall, our data demonstrate that the capacity of HD1 to bind prothrombin contributes to its inhibitory activity. That HD1 is effective in a dynamic system where the prothrombinase reactants are generated *in situ*, extends the observations made in a purified system (Kretz *et al.*, 2006). Based on the results of the CAT assays performed in cofactor deficient plasmas, our data indicate that (a) HD1 delays the transition from the initiation phase to the propagation phase by inhibiting fV activation by thrombin and attenuating prothrombinase assembly, and (b) thrombin-mediated fVIII activation is not an important contributor to the lag time. Building on these findings, we hypothesize that an aptamer that specifically targets prothrombin and restricts its entry into prothrombinase may be an even better anticoagulant than HD1. Such an agent would have advantages over HD1 or active site-directed thrombin or fXa inhibitors because it would prolong the clotting time and reduce the peak thrombin concentration without directly compromising either fXa or thrombin activity.

Appendix 4.1

A.



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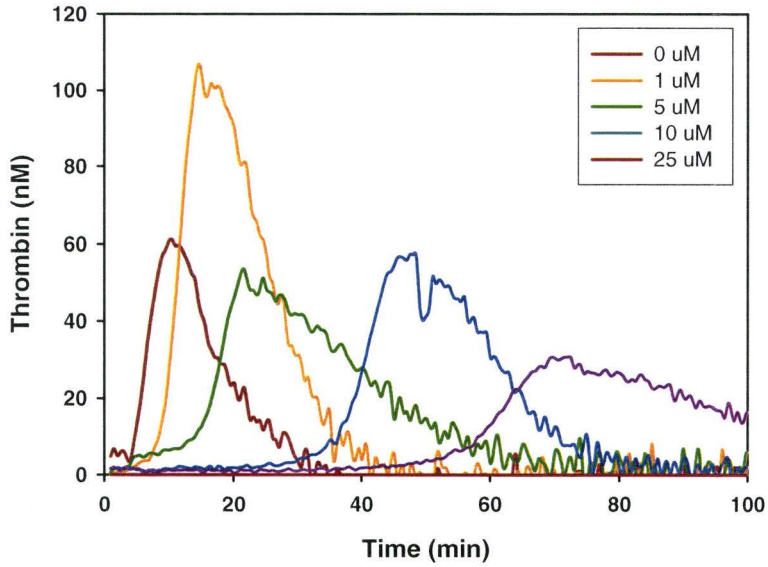


Appendix 4.1: Effect of HD1 and Hir$^{54-65}$(SO_3^-) on thrombin generation in normal PPP plasma

Thrombin generation assays were conducted using the CAT assay system. 100 µl of platelet poor plasma was added to the wells of a 96 well plate, along with 30 µM PCPS vesicles. Next, increasing concentrations (0-25 µM) of HD1 (A) or Hir$^{54-65}$(SO_3^-) (B) were added to the plasma. To the experimental wells was added 0.5 pM tissue factor, whereas the thrombin calibrator was added to the calibration wells. Thrombin generation was initiated by adding a mixture of $CaCl_2$ and fluorescent substrate. The plate was read at 30 s intervals for 2 h at 37°C.

Chapter 5: AntiHD1 neutralizes the anticoagulant activity of HD1

Foreword

This manuscript is in preparation. The authors are: Colin A. Kretz, Alan R. Stafford, James C. Fredenburgh, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz.

All of the experiments contained within this manuscript were performed by me. Furthermore, this manuscript was drafted by me. Thrombin generation experiments were conducted in collaboration with Dr. Catherine P. Hayward. Nola Fuller trained me in the use of the calibrated automated thrombography instrument.

Summary

HD1 is a DNA aptamer that binds both thrombin and prothrombin with high affinity, resulting in effective anticoagulant activity. The antidote to HD1, termed antiHD1, was developed based on complementary Watson-Crick base-pairing. In fluorescence experiments, HD1 bound antiHD1 with a K_D value of 15 nM, whereas in SPR experiments HD1 bound antiHD1 with a K_D value of 120 pM. AntiHD1 reversed the anticoagulant activity of HD1 in standard clotting assays, thrombin generation assays, and prothrombin activation experiments, at a ratio of 1:1. In contrast, a 30:1 molar excess of antiHD1 was required to neutralize HD1 in thrombin clotting assays. Investigations into the mechanism of neutralization demonstrated that HD1 was resistant to antiHD1 binding when thrombin or prothrombin was present. Thrombin and prothrombin protected HD1 from antiHD1 to a similar extent in fluorescence displacement experiments; however thrombin protected HD1 to a greater extent than prothrombin in SPR displacement experiments. Overall these data demonstrate that HD1 can be effectively neutralized by its antidote, antiHD1. Furthermore, these data suggests that aptamers are resistant to neutralization by antiaptamers in the presence of their target protein. Neutralization of bound aptamer may follow a dock-and-unzip mechanism, whereas neutralization of unbound aptamer occurs more readily.

Introduction

Anticoagulants are widely used for the prevention and treatment of venous and arterial thrombosis. Parenteral anticoagulants are often used for acute indications, whereas chronic anticoagulation usually is effected with orally active drugs. Traditionally, unfractionated heparin was the parenteral anticoagulant of choice. More recently, however, low-molecular-weight heparin (LMWH) (Weitz, 1997) and fondaparinux (Mehta *et al.*, 2005) have replaced unfractionated heparin for most indications. Although these smaller heparin fragments have pharmacological advantages over unfractionated heparin, a disadvantage of these agents is the lack of an antidote. Thus, protamine sulphate completely neutralizes the anticoagulant effects of unfractionated heparin, but only partially antagonizes those of LMWH and has no effect on the anticoagulant activity of fondaparinux (Crowther *et al.*, 2002, Gatt *et al.*, 2008). The lack of antidotes for LMWH and fondaparinux can be problematic if patients are bleeding. In addition, even though protamine sulphate neutralizes the anticoagulant effects of unfractionated heparin, patients can have allergic reactions to protamine sulphate, some of which can be fatal (Lantz *et al.*, 1991, Mousa, 2005). These considerations have prompted the development of new parenteral anticoagulants that have a rapid onset of action, produce a predictable level of anticoagulation and can be reversed with specific and safe antidotes. Anticoagulant aptamers represent a new class of drugs that fulfil these properties (White *et al.*, 2000).

Anticoagulant aptamers are single-stranded RNA or DNA oligonucleotides that have been engineered to bind to target coagulation enzymes with high affinity and

specificity (Bock *et al.*, 1992, Griffin *et al.*, 1993b, Oney *et al.*, 2007, Rusconi *et al.*, 2000, Rusconi *et al.*, 2002). Currently available anticoagulant aptamers target either factor (f) IXa (Chan *et al.*, 2008a) or thrombin (Muller *et al.*, 2008). Of these two targets, thrombin is of proven value as evidenced by the success with bivalirudin for prevention of recurrent ischemia in patients undergoing percutaneous coronary interventions (Feldman *et al.*, 2007) and with dabigatran etexilate for thromboprophylaxis in those undergoing elective hip or knee replacement surgery (Wolowacz *et al.*, 2009). HD1 is a thrombin-directed aptamer that inhibits thrombin activity by binding to exosite 1, the substrate-binding site on thrombin (Tsiang *et al.*, 1995). Recently, we demonstrated that HD1 also binds prothrombin with high affinity and attenuates its activation by prothrombinase by inhibiting factor Va binding (Kretz *et al.*, 2006). Because of this dual mechanism of action, HD1 is a more potent anticoagulant than other exosite 1-directed agents, such as $Hir^{54\text{-}65}(SO_3^-)$, which only bind thrombin (Chapter 4).

An advantage of anticoagulant aptamers is that complementary aptamers can be used as antidotes. Proof of principle comes from studies with the fIXa-directed aptamer, RB006. Administration of RB006 to animals or healthy volunteers produces a dose-dependent prolongation of the activated partial thromboplastin time (aPTT), whereas administration of the complementary aptamer, RB007, rapidly restores the aPTT to baseline levels (Dyke *et al.*, 2006). Building on the concept of aptamers/anti-aptamer pairs, we developed a complementary aptamer against HD1 based on Watson and Crick base-pairing and (a) determined its capacity to neutralize the anticoagulant activities of HD1, (b) characterized the interaction between the anti-aptamer and HD1, and (c)

compared the capacity of the anti-aptamer to interact with free HD1 and HD1 bound to thrombin or prothrombin.

Materials and Methods

Reagents:

Human prothrombin, thrombin, and fXa were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN), whereas fVa was from Haematologic Technologies, Inc (Essex Junction, VT). D-Phe-Pro-Arg chloromethyl ketone (FPRck) was obtained from Calbiochem (San Diego, CA). HD1 (5'-GGTTGGTGTGGTTGG-3') the exosite 1-directed DNA aptamer, anti-HD1 (5'-CCAACCACACCAACC-3') along with biotin-labelled HD1 and anti-HD1 (b-HD1, b-antiHD1) and fluorescein-labelled HD1 (f-HD1) were synthesized by the Molecular Biology and Biotechnology Institute at McMaster University (Hamilton, ON). Before use, HD1 was subjected to renaturation by heating to 95°C for 5 min followed by cooling on ice for 10 min (Griffin *et al.*, 1993b). Recombinant tick anticoagulant protein (TAP), a fXa-directed inhibitor, was a generous gift from Dr. G. Vlasuk, Corvas International, Inc. (San Diego, CA). The Tyr^{63}-sulfated COOH-terminal peptide of hirudin, $Hir^{54-65}(SO_3^-)$, was from Bachem (King of Prussia, PA). Tosyl-Gly-Pro-Arg p-nitroaniline (tGPR-pNA) was from Aniara (Mason, OH). Streptavidin was from Sigma-Aldrich (Oakville, Ontario, Canada). Surface plasmon resonance (SPR) experiments were conducted using a CM5 chip, containing a carboxy-dextran matrix, on a BIAcore 1000 (GE Healthcare, Piscataway, NJ). The Amine Coupling Kit, which contain solutions of 1 M ethanolamine, 11.5 mg/ml N-

hydroxysuccinimide (NHS), and 75 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was also from BIAcore. Recombiplastin and aPTT reagent were from Instrumentation Laboratory (Lexington, MA). The calibrated automated thrombogram (CAT) assay reagents, including the PPP Low tissue factor reagent, thrombin calibrator, and the fluorescent substrate reagent (FluCa), were from Thrombinoscope (Maastricht, The Netherlands). Platelet poor plasma was prepared as described previously (Chapter 4). PCPS vesicles were synthesized from bovine brain L-α-phosphatidyl-L-serine (PS) and type III-E from egg yolk L-α-phosphatidyl-choline (PC) obtained from Avanti Polar Lipids Inc. (Alabaster, AL) according to a previously described method (Kretz *et al.*, 2006). γ_A/γ_A fibrinogen was prepared as previously described (Fredenburgh *et al.*, 2008) and was used in the *in vitro* clotting assay.

Thrombin clotting experiments:

To begin to evaluate the capacity of antiHD1 to neutralize the anticoagulant activity of HD1, we tested the capacity of antiHD1 to neutralize HD1 in the thrombin clotting time in a buffer system. First, 10 nM thrombin was incubated with 200 nM HD1 (a concentration that will bind ~95% of the thrombin) for 5 min in aptamer buffer (20 mM Tris-HCl, 140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 0.1% PEG, pH 7.4) containing 0.05% Prionex in various wells of a 96-well plate. AntiHD1 was then added in concentrations ranging from 0-20 μM. After incubation for 10 min, at 23°C, 2 μM fibrinogen was added and turbidity was monitored for 30 min at 405 nm in a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA). The clotting times,

defined as the time at half maximal OD_{405}, were then determined using the instrument software.

Prothrombin activation by prothrombinase:

Previously, we demonstrated that HD1 not only inhibits thrombin activity, but also attenuates prothrombin activation by prothrombinase. To determine whether antiHD1 neutralizes this effect, a solution of 1 μM prothrombin and 6 μM PCPS was incubated with 2.5 μM HD1 (a concentration sufficient to bind ~95% of the prothrombin) for 5 min at 23°C prior to adding antiHD1 at concentrations ranging from 0 to 10 μM). After incubation for an additional 10 min at 23°C, reactions were initiated by addition of 0.25 nM fXa, 0.3 nM fVa and 6 μM PCPS (final concentrations) to each well. At intervals up to 5 min, reactions were terminated by addition of 250 nM rTAP and 2 mM EDTA (final concentrations) and the concentration of thrombin that was generated was quantified by monitoring the hydrolysis of 600 μM tGPR-pNA at 405 nm for 10 min using a plate reader. Rates of substrate cleavage (mOD/min), as determined by the instrument software, were used to calculate the extent of prothrombin activation. Relative rates of prothrombin activation were calculated by dividing rates from each condition by the rate of prothrombin activation in the absence of HD1.

Coagulation assays:

To begin to explore the capacity of antiHD1 to neutralize the anticoagulant activity of HD1, we examined the ability of increasing concentrations of antiHD1 to reverse the effect of HD1 on the prothrombin time (PT) and aPTT. Thus, 100 µl of platelet-poor citrated plasma was added to the wells of a 96-well plate in the absence or presence of 20 µM HD1. After 5 min incubation, antiHD1 was added in concentrations ranging from 0 to 40 µM and the mixture was incubated for an additional 5 min. For PT determination, 200 µl of recombiplastin was added to plasma, whereas for aPTT determination 100 µl aPTT reagent was incubated for 10 min with the plasma prior to addition of 100 µl $CaCl_2$ reagent. In both cases, plates were read at 405 nm in a plate reader set at 23°C for 20 min. Clotting times were established as the time to half-maximal OD.

Thrombin generation assay:

Because most of the thrombin is generated after clotting (Brummel *et al.*, 2002), we next examined the capacity of antiHD1 to reverse the inhibitory effects of HD1 on thrombin generation. 100 µl of plasma was added to wells of a 96-well Immulon 4 HBX microtitre plate (Thermo Scientific, Milford MA) and incubated at 37°C for 5 min in the absence or presence of 30 µM HD1. Increasing concentrations of antiHD1 (0-50 µM) were incubated with plasma containing HD1 for 10 min at 37°C. Thrombin generation was then initiated by adding PPP Low reagent (1 pM tissue factor, final concentraton) and a solution containing 16 mM $CaCl_2$ and 600 µM z-GGR-AMC (FluCa). Substrate

hydrolysis was monitored at 30 s intervals for 2 h at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a Labsystems Fluoroskan Ascent FL plate reader (MTX Lab Systems, Inc., Vienna, VA). Data were analyzed using the CAT assay software (Thrombinoscope), and the lag time, peak thrombin concentration, area under the curve or endogenous thrombin potential (ETP), and the time to peak thrombin were determined.

Fluorescence experiments:

Fluorescence was used to (a) determine the affinity of antiHD1 for fluorescein-labelled HD1 (f-HD1), and (b) evaluate the capacity of antiHD1 to displace f-HD1 that was bound to either thrombin or prothrombin. To measure affinity, 30 nM f-HD1 was diluted in aptamer buffer in a 10 x 4 mm quartz cuvette maintained at 23°C with a circulating water bath and stirred using a micro stir bar. Fluorescence was monitored at an emission wavelength of 535 nm (cut-off filter set at 520 nm) and slit width of 8 nm and an excitation wavelength of 492 nm and a slit width of 6 nm using a Perkin Elmer LS 50B luminescence spectrophotometer (Wellesley, MA). Once baseline fluorescence (I_o) stabilized, f-HD1 was titrated with 2-20 µl aliquots of a 10 µM solution of antiHD1 containing 30 nM f-HD1 to prevent fluorophore dilution, allowing the fluorescence signal to equilibrate between each injection. When the fluorescence signal reached a plateau, intensity values (I) obtained from time drive profiles were divided by the baseline intensity and the resultant I/I_o values were plotted against the antiHD1 concentration. Data were fit by nonlinear regression analysis using Table Curve (Jandel Scientific, San Rafael, CA) to the equation:

$$\frac{I}{I_o} = 1 + \frac{\alpha}{2}\left(1 + \frac{K_d + P}{A_o} - \sqrt{\left(1 + \frac{K_d + P}{A_o}\right)^2 - 4 \cdot \frac{P}{A_o}}\right)$$

where P is the concentration of antiHD1, A_o is the concentration of f-HD1, α is the maximal fluorescence change, and K_D is the apparent dissociation constant.

Because the fluorescence intensity changes when f-HD1 is titrated with antiHD1 or thrombin, competition experiments were conducted such that f-HD1 was saturated with FPR-thrombin or prothrombin prior to antiHD1 addition to simplify interpretation of fluorescence intensity changes. Thus, f-HD1 (30 nM) was titrated with FPR-thrombin or prothrombin until ~99% of the f-HD1 was bound: final concentrations of thrombin and prothrombin of 320 nM and 650 nM, respectively. Once the fluorescence signal stabilized, a 10 μM antiHD1 solution containing 30 nM f-HD1 and either 320 nM FPR-thrombin or 650 nM prothrombin was added; resulting in a final concentration of 1 μM antiHD1 in the cuvette, and fluorescence was monitored for 20 min. Then, a solution of unlabelled $Hir^{54\text{-}65}(SO_3^-)$ containing 30 nM f-HD1, 1 μM antiHD1, and either 320 nM FPR-thrombin or 650 nM prothrombin was titrated into the cuvette to a final concentration of 1 μM $Hir^{54\text{-}65}(SO_3^-)$, to determine if there was any residual f-HD1 still bound to the FPR-thrombin or prothrombin. Data are presented as (a) time drive profiles, (b) percent change in fluorescence intensity, and (c) rate of signal change.

SPR experiments:

The affinity of antiHD1 for HD1 also was determined using SPR. All SPR experiments were conducted in 10 mM Hepes-NaOH, pH 7.4, 140 mM NaCl (HBS) containing 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.005% Tween 20, pH 7.4 (SPR aptamer buffer). First, streptavidin was attached to the surface of the CM5 chip to 5000 RU according to manufacturer's instructions. Next, 500 nM of either b-HD1 or b-antiHD1 was injected into the flow cell containing the streptavidin-coated surface at a flow rate of 10 µl/min until the chip was saturated (typically 1100 RU). A solution of 10 mM NaOH was then injected into the flow cell for 30 s to remove any unbound oligonucleotides.

The affinity of antiHD1 for b-HD1 was determined in manual mode using a BIAcore 1000. Briefly, 500 nM of antiHD1 was injected into a flow cell containing a b-HD1-coated chip at a flow rate of 40 µl/min for 2 min using Kinject. Wash buffer was then passed through the flow cell for 5 min to measure dissociation. The surface was regenerated with a 5 s injection of 10 mM NaOH. To verify the binding results, the reverse experiment was conducted in an identical fashion to measure the affinity of HD1 for immobilized b-antiHD1. K_D values were calculated using Scrubber software (BioLogic Software Pty Ltd., Campbell, Australia) using the k_{on} and k_{off} rates obtained from the association and dissociation phases, respectively, of the SPR sensorgram.

The capacity of antiHD1 to displace HD1 from thrombin or prothrombin also was evaluated by SPR using the co-inject function. First, b-HD1 was immobilized onto the surface of a streptavidin-coated CM5 chip as described above. For thrombin

displacement, sample 1 of the co-injection, consisting of 100 µl of 2 µM FPR-thrombin, was injected at a flow rate of 20 µl/min. Sample 2 of the co-injection, consisting of 100 µl of 2 µM FPR-thrombin and 100 nM antiHD1, was injected at a flow rate of 20 µl/min immediately after sample 1 and displacement of FPR-thrombin was monitored for 5 min. The same methodology was used to determine the capacity of antiHD1 to displace HD1 from prothrombin except 10 µM prothrombin was substituted for FPR-thrombin. In both cases, the dissociation rate constant (k_{app}) was calculated using the Kinetic Analysis function in Scrubber. After each experiment, 10 mM NaOH was passed through the flow cell for 5 s to regenerate the b-HD1-coated surface.

The affinity of FPR-thrombin or prothrombin for immobilized b-HD1 was determined by diluting FPR-thrombin (0-500 nM) or prothrombin (0-500 nM) in SPR aptamer buffer and passing 100 µl of each sample dilution over immobilized b-HD1 at a flow rate of 20 µl/min using Kinject mode. A flow cell containing only streptavidin served as a reference to correct for non-specific binding of the proteins to the chip surface. Reference signals were subtracted from the b-HD1 binding data prior to calculating K_D values using the k_{on} and k_{off} rates.

Results

AntiHD1 neutralizes the anticoagulant properties of HD1:

We initially tested the capacity of antiHD1 to reverse HD1 inhibition of (a) thrombin-mediated conversion of fibrinogen to fibrin, and (b) prothrombin activation by prothrombinase.

When 2 μM fibrinogen is clotted with 10 nM thrombin the clotting time is 38.9 ± 0.9 s. Addition of 200 nM HD1 yields a 6-fold prolongation of the clotting time (to 294.3 ± 7.6 s; Figure 5.1A). Pre-incubation of HD1 with increasing concentrations of antiHD1 (0-20 μM) prior to fibrinogen addition resulted in a dose-dependent reduction in clotting time. With 10 μM antiHD1, a concentration 50-fold higher than that of HD1, the thrombin clotting time was restored to that measured in the absence of HD1.

We next measured the capacity of antiHD1 to neutralize HD1-induced inhibition of prothrombin activation by prothrombinase. Thus, 2.5 μM HD1 produced a 70% reduction in the rate of activation of 1 μM prothrombin by prothrombinase (Figure 5.1B), consistent with a previous report (Kretz *et al.*, 2006). With the addition of antiHD1 there was a dose-dependent increase in the rate of prothrombin activation, and at 2.5 μM antiHD1 the rate returned to that measured in the absence of HD1. These results indicate that antiHD1 neutralizes HD1 in the prothrombinase assay at a ratio of 1:1. Because HD1 binds thrombin and prothrombin with similar affinity (Kretz *et al.*, 2006), it was surprising to discover that antiHD1 neutralizes HD1 more effectively in a prothrombinase assay than in a thrombin clotting assay.

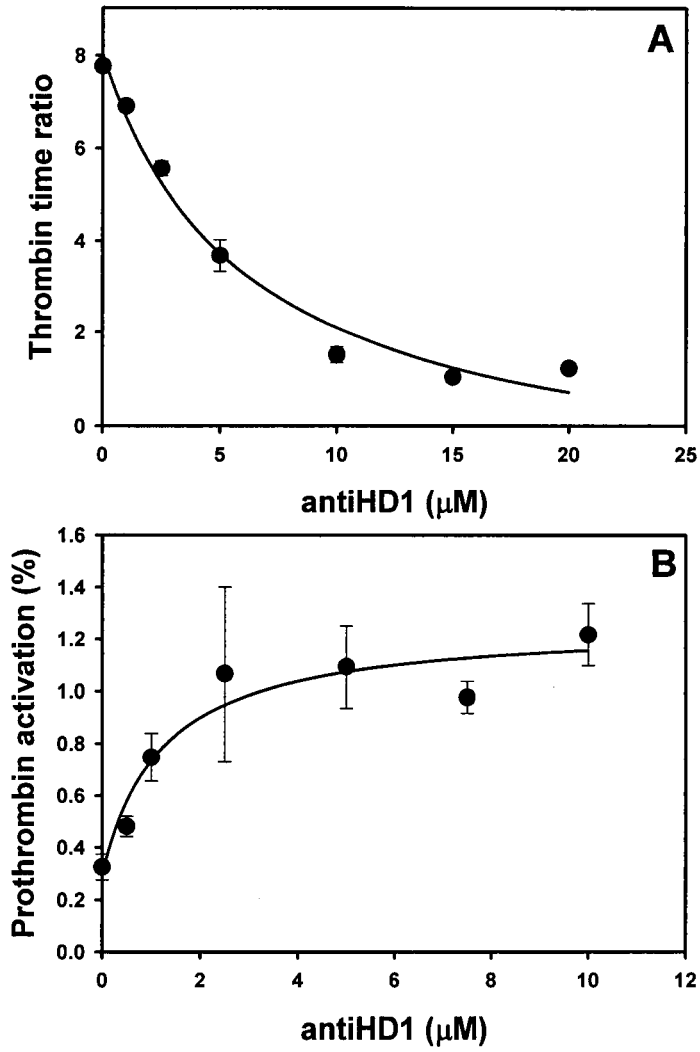

A
Thrombin time ratio
0
2
4
6
8
0
5
10
15
20
25
antiHD1 (μM)
B
Prothrombin activation (%)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
2
4
6
8
10
12
antiHD1 (μM)

Figure 5.1: Effect of antiHD1 on HD1 inhibition of thrombin clotting and prothrombin activation

A, The capacity of antiHD1 (0-20 μM) to neutralize 200 nM HD1 was measured in a thrombin clotting assay containing 10 nM thrombin and 2 μM purified fibrinogen in aptamer buffer. HD1 was incubated with thrombin for 5 min, followed by a 10 min incubation of increasing concentrations (0-20 μM) antiHD1 before the addition of fibrinogen. Plates were read at 23°C at an absorbance of 405 nm in a microplate reader. The clot time was defined as the time to half maximum OD_{405}. *B*, The initial rate of activation of 1 μM prothrombin by 0.25 nM fXa, 0.3 nM fVa, and 10 μM PCPS was measured in the absence or presence of 2.5 μM HD1 and increasing concentrations of antiHD1 (0-10 μM). Prothrombin was incubated with HD1 for 5 min, followed by a 10 min incubation with or without antiHD1 before initiating the reaction with preassembled prothrombinase. Thrombin formation was determined by measuring hydrolysis of Chz-Th. The data are presented as the rate of prothrombin activation measured in the presence of HD1 and antiHD1 relative to that determined in their absence. Each data point represents the Mean ± SEM, determined from three separate experiments. The data were fit by nonlinear regression analysis (lines).

We next tested the capacity of antiHD1 to neutralize the effects of HD1 on global tests of coagulation. The addition of 10 μM HD1 produced a 7- and 4-fold prolongation of the PT and aPTT, respectively, consistent with previous data (Chapter 4). When 20 μM antiHD1 was incubated in HD1-containing plasma for 10 min prior to initiation of clotting, the PT and the aPTT returned to baseline values. These results indicate that antiHD1 can neutralize the anticoagulant effect of HD1 when clotting is initiated through either the extrinsic or intrinsic pathway.

Prior investigation into the anticoagulant mechanism of HD1 using thrombin generation assays revealed that it prolongs the lag time and reduces the peak thrombin concentration (Chapter 4). Building on this information, we set out to determine whether antiHD1 reverses the effects of HD1 on thrombin generation in plasma. The addition of 30 μM HD1 produced a i) 6-fold prolongation of the lag time, ii) 5-fold prolongation of the time to peak thrombin, iii) 9-fold reduction in the peak thrombin concentration and iv) 5-fold reduction in the ETP. Increasing concentrations of antiHD1 produced a dose-dependent decrease in the lag time and time to peak thrombin concentration, and a dose-dependent increase in the ETP and peak thrombin concentration (Figure 5.2). At 30 μM antiHD1, equimolar to the concentration of HD1, the lag time was 6 ± 1 min, the time to peak thrombin was 8.8 ± 1.3 min, and the ETP was 1278.5 ± 93.8 nM·min, values comparable to baseline thrombin generation parameters determined in the absence of HD1. With 30 μM antiHD1, there was a 2.5-fold increase in the peak thrombin

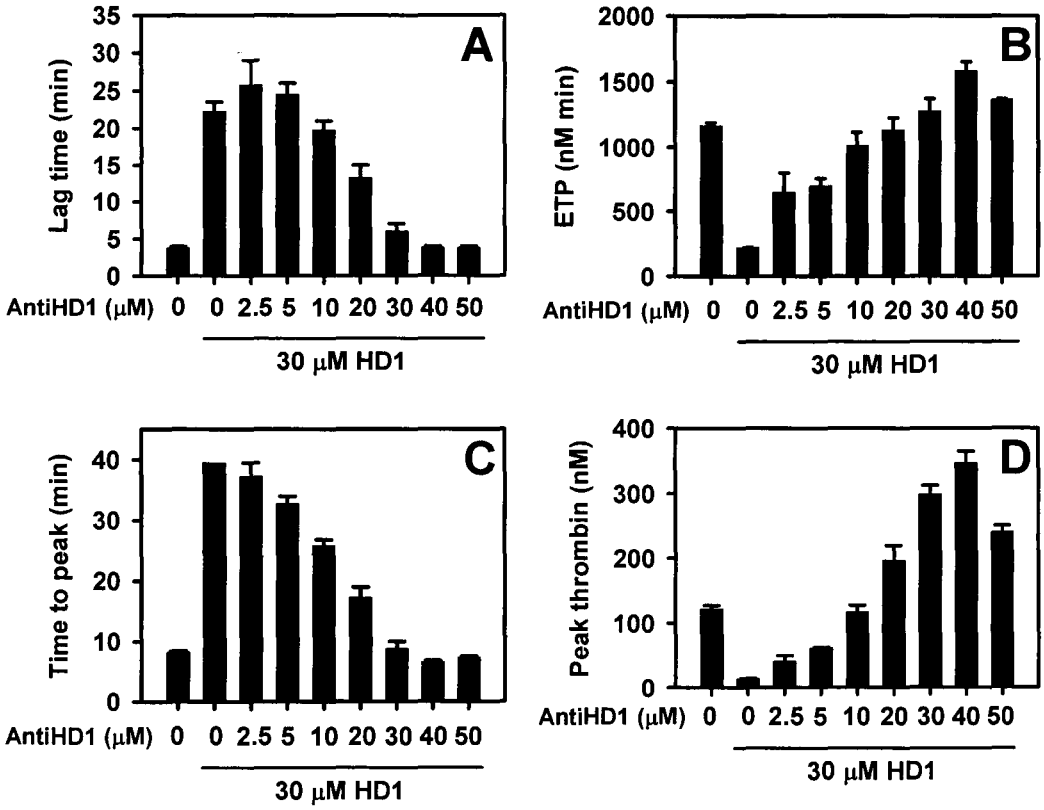

A
Lag time (min)
35
30
25
20
15
10
5
0
AntiHD1 (μM) 0 0 2.5 5 10 20 30 40 50
30 μM HD1
B
ETP (nM min)
2000
1500
1000
500
0
AntiHD1 (μM) 0 0 2.5 5 10 20 30 40 50
30 μM HD1
C
Time to peak (min)
40
30
20
10
0
AntiHD1 (μM) 0 0 2.5 5 10 20 30 40 50
30 μM HD1
D
Peak thrombin (nM)
400
300
200
100
0
AntiHD1 (μM) 0 0 2.5 5 10 20 30 40 50
30 μM HD1

Figure 5.2: Effect of antiHD1 on HD1 inhibition of thrombin generation

The effect of increasing concentrations (0-50 μM) of antiHD1 in the presence of 30 μM HD1 on thrombin generation was measured in platelet poor plasma by CAT. 1 pM tissue factor was added to the plasma before initiating with $CaCl_2$ in the form of the FluCa reagent. Cleavage of the fluorescent substrate z-GGR-AMC was monitored at 30 s intervals. The ability of antiHD1 to neutralize HD1 in thrombin generation was measured by lag time (A), ETP (B), time to peak thrombin (C), and peak thrombin concentration (D) as described in 'Methods'. The data represent the mean ± SEM from two separate experiments, each performed in duplicate.

concentration, to 298.5 ± 13.6 nM. Such an increase in peak thrombin concentration was previously observed with low concentrations of HD1 and with $Hir^{54-65}SO_3^-$ (Chapter 4). These data demonstrate that antiHD1 can neutralize the inhibitory effect of HD1 on thrombin generation and does so in a 1:1 molar ratio.

Affinity of antiHD1 for HD1:

Initially, we used fluorescence spectroscopy to study the interaction between antiHD1 and HD1. When 30 nM f-HD1 was titrated with antiHD1, there was a dose-dependent increase in the fluorescence intensity that saturated at 21% above baseline (Figure 5.3A). With each addition of antiHD1, there was a dose-dependent and saturable increase in fluorescence intensity. The fluorescence signal rapidly stabilized after each injection, suggesting that the interaction of antiHD1 with f-HD1 1 equilibrates within the 10 s intervals. AntiHD1 binds HD1 with a K_D value of 16.7 ± 4.5 nM, which is comparable to the theoretical K_D value based on the melting temperature of the double stranded aptamer/anti-aptamer complex. This predicted melting temperature is calculated assuming one of the oligonucleotides is in excess (Le Novere, 2001). Because the fluorescein probe on f-HD1 is on the 5'-guanine, the experimental K_D value determined here may reflect interaction of antiHD1 with the fluorescein moiety and may not represent total hybridization of the oligonucleotides. To account for this possibility, we used SPR to measure the affinity of antiHD1 for HD1. Approximately 1500 RU of b-HD1 were immobilized to saturate the streptavidin surface. AntiHD1, at concentrations ranging from 0-1000 nM, was injected over the b-HD1 surface at a flow rate of 40 µl/min. The

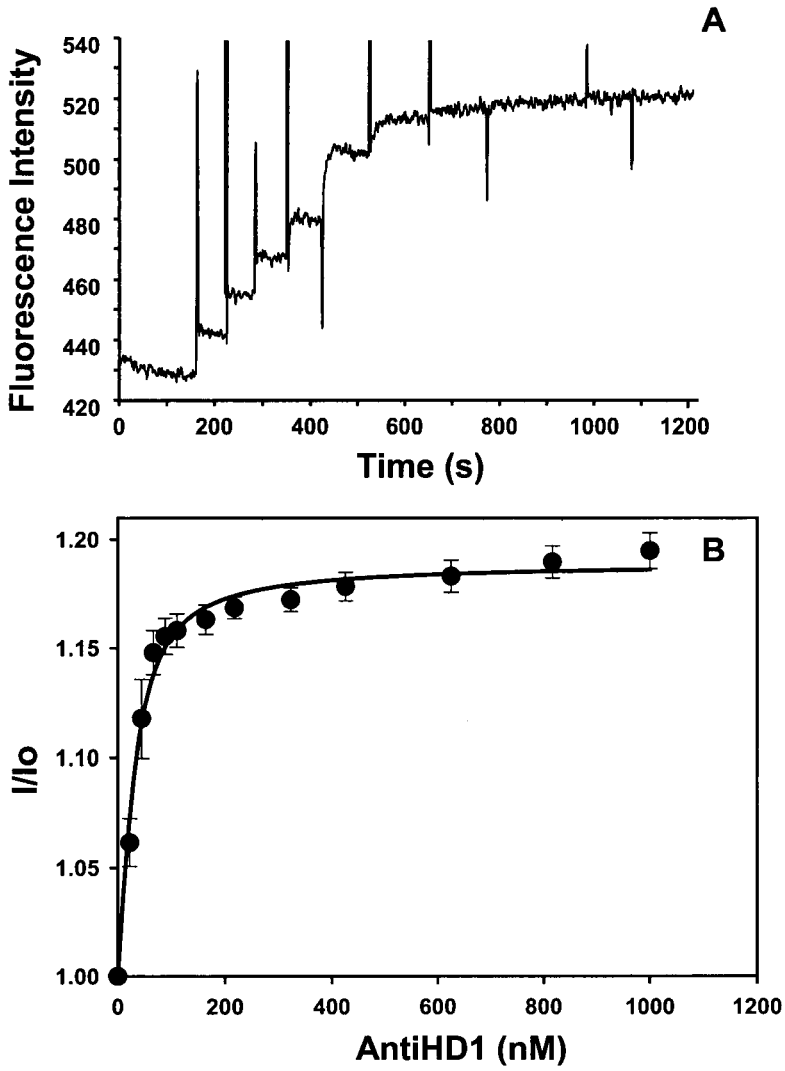

A
Fluorescence Intensity
540
520
500
480
460
440
420
0
200
400
600
800
1000
1200
Time (s)
B
I/Io
1.20
1.15
1.10
1.05
1.00
0
200
400
600
800
1000
1200
AntiHD1 (nM)

Figure 5.3: Interaction of antiHD1 and f-HD1

The fluorescence of 30 nM f-HD1 was monitored as it was titrated with aliquots of 5 μM antiHD1 (A). Fluorescence intensity values (I) determined after each addition of antiHD1 were divided by the initial fluorescence intensity (I_o). I/I_o values were then plotted as a function of prothrombin concentration (B), and the data were fit by nonlinear regression analysis (line) to obtain the K_D value.

K_D value for anti-HD1 binding to b-HD1 was 150 ± 10 pM, which was calculated based on a k_a of 4.5 ± 0.8 x 10^6 $s^{-1}$and a k_d of 7.0 ± 1.0 x 10^{-4} s^{-1}. Therefore, the high affinity interaction between antiHD1 and primarily reflects the slow rate of dissociation of the hybridized DNA oligonucleotides. In the reverse experiment, in which HD1 was passed over an immobilized b-antiHD1 surface, a similar K_D value of 120 ± 25 pM was obtained. The K_D values obtained by SPR are consistent with previous studies done in solution phase (Fang *et al.*, 2005), but are not in line with that measured by fluorescence. These data reveal that antiHD1 binds HD1 with high affinity, due in large part to a very slow off-rate.

AntiHD1 displaces HD1 from thrombin or prothrombin:

We next examined the capacity of antiHD1 to displace HD1 bound to either FPR-thrombin or prothrombin. The fluorescence of 30 nM f-HD1 was monitored as either FPR-prothrombin (Figure 5.4A) or thrombin (Figure 5.4B) was titrated into the cuvette until the fluorescence intensity was unchanged. At saturation, 320 nM FPR-thrombin or 650 nM prothrombin caused a 37 ± 2 and 31 ± 1% maximal increase in fluorescence intensity, respectively. The addition of 1 μM antiHD1 to FPR-thrombin-bound f-HD1 produced a further 6.1 ± 0.1% increase in fluorescence intensity (relative to baseline) which then decreased before reaching a plateau 33 ± 1% above baseline (Figure 5.4A). The calculated k_{off} for antiHD1-mediated displacement of f-HD1 from thrombin was 3.74 ± 0.25 x 10^{-4} s^{-1}. Likewise, addition of 1 μM antiHD1 to prothrombin-bound f-HD1 produced an immediate 5.1 ± 0.2% increase in fluorescence intensity which then decreased before reaching a plateau 27.1 ± 1.6 % above baseline (Figure 5.4B). The

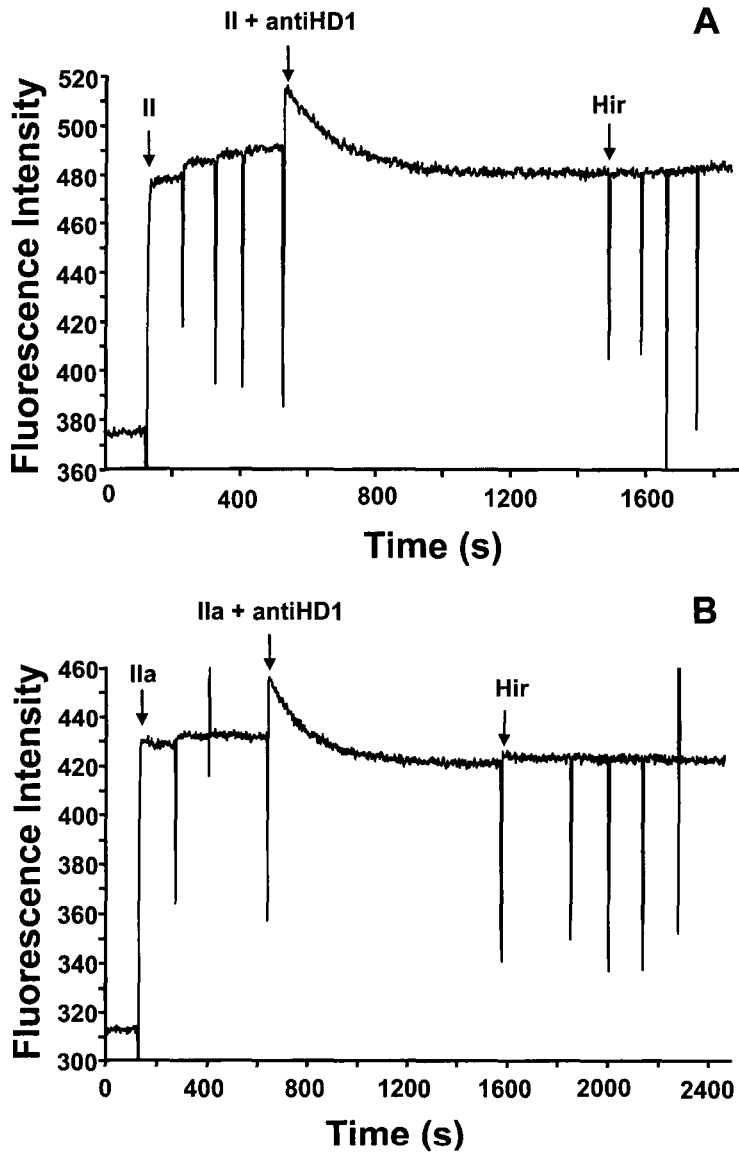

A
II + antiHD1
II
Hir
Fluorescence Intensity
520
500
480
460
440
420
400
380
360
0
400
800
1200
1600
Time (s)
B
IIa + antiHD1
IIa
Hir
Fluorescence Intensity
460
440
420
400
380
360
340
320
300
0
400
800
1200
1600
2000
2400
Time (s)

Figure 5.4: Capacity of antiHD1 to disrupt the interaction between thrombin or prothrombin with f-HD1

A cuvette containing 30 nM f-HD1 was allowed to equilibrate, and fluorescence was monitored at excitation and emission wavelengths of 492 and 535 nm, respectively. At the times indicated, a solution of 10 μM prothrombin (A) or 5 μM FPR-thrombin (B) was titrated, reaching final concentrations of 650 nM prothrombin and 320 nM FPR-thrombin, respectively. Next, 1 μM antiHD1 was added to the cuvette and the fluorescence signal was allowed to equilibrate. At the indicated time, a balanced solution of $Hir^{54-65}(SO_3^-)$ was titrated in to a final concentration of 1 μM. The presented time drive profiles illustrate typical results.

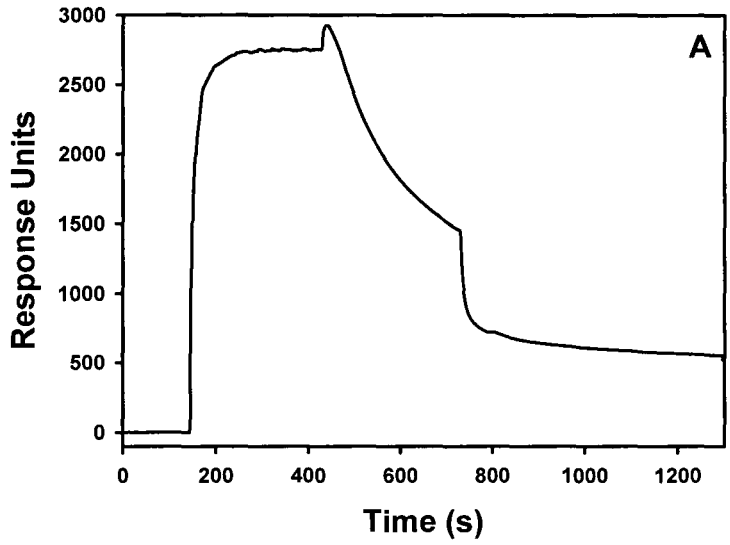
calculated k_{off} for the displacement of f-HD1 from prothrombin was $2.79 \pm 0.14 \times 10^{-4}$ s^{-1}, which is indistinguishable from the value determined with FPR-thrombin. Once the fluorescence signal stabilized after HD1 addition, $Hir^{54-65}(SO_3^-)$ was titrated into the cuvette, up to a final concentration of 1 μM. The addition of $Hir^{54-65}(SO_3^-)$ yielded no further change in fluorescence intensity (Figure 5.4 A and B), indicating that antiHD1 had completely displaced f-HD1 from both thrombin and prothrombin. Furthermore, when 20 μM antiHD1 was used in the displacement studies, there was a similar 6% increase in fluorescence intensity; however the k_{off} was 1.54×10^{-3} s^{-1}, which is 5-fold faster than with 1 μM antiHD1. This result suggests that the rate of equilibration following antiHD1 addition is dependent upon the concentration of antiHD1. Overall, these data demonstrate that antiHD1 displaces f-HD1 from thrombin and prothrombin at a similar rate. However, this is not consistent the data from functional studies which suggest that antiHD1 is better at neutralizing HD1 in prothrombinase assays than thrombin clotting assays.

Although antiHD1 displaces f-HD1 from both thrombin and prothrombin, the fluorescence intensity does not return to baseline. This can be explained by the fact that anti-HD1 causes an increase in fluorescence intensity similar to that which occurs when thrombin or prothrombin binds to f-HD1. The slow equilibration time required for antiHD1 to bind f-HD1 in these competition experiments is in contrast to the rapid equilibration between f-HD1 and antiHD1 that occurred in the absence of thrombin or prothrombin (Figure 5.3A) suggesting that HD1 bound to thrombin or prothrombin is

protected from antiHD1. We next used SPR to further explore the capacity of antiHD1 to displace HD1 from thrombin and prothrombin.

A CM5 chip was coated with streptavidin and 1100 response units of b-HD1 was immobilized. When 2 μM FPR-thrombin was passed over the b-HD1 surface, there was a saturable increase in response units, followed by a dissociation phase during which only buffer was passed through the flow cell (data not shown). The K_D value, determined from the association and dissociation rate constants, for FPR-thrombin binding to b-HD1 was 18.8 ± 4.6 nM, which is consistent with previous studies (Kretz *et al.*, 2006). When 10 μM prothrombin was injected into the b-HD1 flow cell, a saturable increase in response units was detected, indicating binding. The K_D value that was calculated from prothrombin binding to b-HD1 was 490 ± 40 nM, which is 27-fold-fold higher than the affinity of FPR-thrombin for b-HD1. This result is not consistent with the previously reported K_D value for prothrombin binding to f-HD1 (Kretz *et al.*, 2006).

To measure displacement, FPR-thrombin was first passed over immobilized b-HD1 surface until no further binding was observed. Immediately following this saturation of the b-HD1 surface, a solution containing 2 μM FPR-thrombin and 100 nM antiHD1 was injected into the flow cell and the displacement of FPR-thrombin from b-HD1 was monitored. After an initial spike in RU, there was a time-dependent and saturable decrease in response units (Figure 5.5 A and B). Kinetic analysis using Scrubber determined the rate of dissociation to be $4.9 \pm 0.3 \times 10^{-4}$ s^{-1}, which is consistent with the k_{off} determined by fluorescence. Next, the capacity of antiHD1 to displace prothrombin from b-HD1 was tested. Immediately following prothrombin saturation of


A
Response Units
3000
2500
2000
1500
1000
500
0
0
200
400
600
800
1000
1200
Time (s)

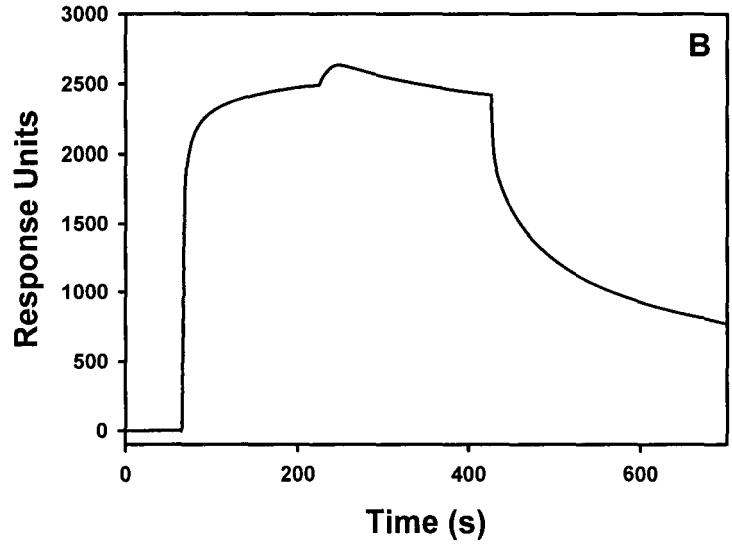

B
Response Units
3000
2500
2000
1500
1000
500
0
0
200
400
600
Time (s)

Figure 5.5: Capacity of antiHD1 to displace thrombin or prothrombin from immobilized b-HD1

A CM5 SPR biosensor chip was coated with 5000 RU of streptavidin to allow 1100 RU of biotinylated-HD1 to be immobilized to the chip surface. The co-injection function was used in manual mode at a 20 μl/min flow rate to measure the capacity of antiHD1 to displace prothrombin (A) or thrombin (B) from b-HD1. Injection 1, consisted of (A) 100 μl of 2 μM FPR-thrombin or (B) 100 μl of 10 μM prothrombin. Injection 2 of the co-injection proceeded immediately after injection 1 and consisted of 100 μl of (A) 2 μM FPR-thrombin + 100 nM antiHD1, or (B) 10 μM prothrombin + 100 nM antiHD1. Between experiments, the b-HD1 chip surface was regenerated by passing 10 mM NaOH through the flow cell for 5 s. Dissociation rate constants were calculated using Scrubber for 3 separate experiments. The presented SPR sensograms illustrate typical results.

the b-HD1 surface, a solution containing 10 μM prothrombin and 100 nM antiHD1 was injected into the flow cell. Similar to FPR-thrombin, there was an initial spike in RU, followed by a time-dependent and saturable decrease in the response units (Figure 5.5 A and B). Kinetic analysis using Scrubber calculated the dissociation constant to be 2.7 ± 0.2 x 10^{-3}, which is 5.5-fold faster than the dissociation rate constant determined using FPR-thrombin and 10-fold faster than the displacement of f-HD1 from prothrombin determined by fluorescence. These results demonstrate that antiHD1 displaces HD1 from prothrombin at a faster rate than from FPR-thrombin.

Discussion

Antidote-controlled anticoagulant aptamers offer a rapid onset and offset of action, which can be beneficial for short-term prevention and treatment of thrombosis, particularly for patients who are high risk for bleeding. Anticoagulant aptamers can be targeted against any clotting enzyme. Thrombin is an attractive target because of its established role in coagulation and the proven clinical benefit of thrombin inhibitors. HD1 is a DNA aptamer that binds both thrombin and prothrombin. As such, HD1 is a potent anticoagulant because it not only inhibits thrombin activity, but also attenuates prothrombin activation by prothrombinase. Our data demonstrate that HD1 is effectively neutralized by its complementary aptamer, termed antiHD1. Initial studies demonstrated that antiHD1 effectively neutralized HD1 in both PT and aPTT assays. In thrombin generation experiments, a 1:1 ratio of antiHD1:HD1 was required to neutralize the effects of HD1 on the lag time and time to peak thrombin. Interestingly, only 10 μM antiHD1 was required to neutralize the effects of 30 μM HD1 on the peak thrombin concentration in the CAT assay. At concentrations greater than 30 μM, antiHD1 caused an increase in the peak thrombin concentration relative to control, which has been previously demonstrated at low concentrations of HD1 (Chapter 4). As a result, antiHD1 effectively reverses the anticoagulant properties of HD1 in plasma-based thrombin generation assays.

Previous studies showed that an equimolar concentration of antidote-to-aptamer neutralized the RNA fIXa aptamer in *in vitro* and *in vivo* experiments (Dyke *et al.*, 2006, Rusconi *et al.*, 2002). Although a 1:1 ratio of antiHD1:HD1 was required to neutralize

HD1 in the prothrombinase assay, we demonstrate that a 50:1 ratio of antiHD1:HD1 was required to neutralize HD1 in the thrombin clotting assay. The ability of fVa to compete with HD1 for prothrombin binding likely contributes to the effectiveness of antiHD1 neutralizing HD1 in the prothrombinase assay (Kretz *et al.*, 2006). In contrast, fibrinogen is a much weaker competitor for HD1 binding to thrombin, due in part to its low affinity for exosite 1 (Pospisil *et al.*, 2003), which helps to explain why antiHD1 is less effective at neutralizing HD1 in the thrombin clotting assay.

Although competing ligands of HD1 contribute to the effectiveness of antiHD1, our data demonstrate that the capacity of antiHD1 to neutralize HD1 also depends upon the affinity of HD1 for thrombin or prothrombin. Thus, SPR displacement studies demonstrate that antiHD1 displaces prothrombin from immobilized HD1 better than it displaces thrombin. These differences in displacement are related to the affinity of HD1 for these proteins. Thus, antiHD1 displaced f-HD1 from thrombin and prothrombin equally, which is consistent with previous work that demonstrates thrombin and prothrombin bind f-HD1 with similar affinity (Kretz *et al.*, 2006). In contrast, our SPR data demonstrates that antiHD1 is more effective at displacing HD1 from prothrombin than thrombin, likely reflective of the finding that immobilized HD1 bound thrombin with a 27-fold lower K_D value than prothrombin; a result that is consistent with a recent report by Muller *et al* (Muller *et al.*, 2008). Thus, antiHD1 is more effective at neutralizing HD1 in the prothrombinase assay than the thrombin clotting assay because (a) HD1 binds thrombin with a higher affinity than prothrombin in solution, and (b) fVa

is a stronger competitor for HD1 binding to prothrombin than is fibrinogen for HD1 binding to thrombin.

Both fluorescence and SPR studies demonstrate that (a) HD1 binds antiHD1 rapidly in the absence of thrombin or prothrombin and (b) when HD1 is bound to thrombin or prothrombin, the addition of antiHD1 causes a rapid but transient increase in signal. Previous studies demonstrated that addition of $Hir^{54-65}(SO_3^-)$ to a solution of f-HD1 and either thrombin or prothrombin causes a dose dependent decrease in fluorescence intensity that rapidly equilibrates following each titration (Kretz *et al.*, 2006). These data suggest that antiHD1 does not compete with HD1 for binding to thrombin or prothrombin, but that it interacts with HD1 while it is still in contact with thrombin or prothrombin. Furthermore, HD1 is protected from antiHD1 bound by either thrombin or prothrombin. Previous work by Baldrich *et al* (Cho *et al.*, 2008b) and Cho *et al* (Baldrich and O'Sullivan, 2005) suggest that the secondary structure of HD1 is stabilized when it is bound to thrombin and, by extension of our studies, prothrombin. Taken together, these data suggests that the exosite 1 residues of thrombin that interact with HD1 (Tsiang *et al.*, 1995) stabilize the secondary structure of HD1, making it more resistant to antiHD1 binding. In contrast, unbound HD1 can rapidly interact with antiHD1, suggesting that the K^+ ion is much less effective at stabilizing the chair configuration than are the residues of exosite 1. This explains why there was a rapid equilibration of the fluorescence signal when antiHD1 was titrated to free f-HD1 compared with the slow equilibration when antiHD1 was added to f-HD1 bound to thrombin or prothrombin.

Previously, NMR studies demonstrated that the hydrogen bonds contained within the G-quartets are the least stable forces that support HD1's chair configuration (Mao and Gmeiner, 2005). Because of this instability, either the terminal 3'-guanine or 5'-guanine of HD1 is the most likely site for antiHD1 to initially bind in order to displace HD1 from thrombin or prothrombin. Since this guanine is not expected to make direct contacts with the surface of exosite 1 (Padmanabhan *et al.*, 1993), a transient ternary complex consisting of antiHD1-HD1-thrombin is possible. The fact that antiHD1 addition to preformed f-HD1/thrombin or f-HD1/prothrombin complexes caused an immediate increase in fluorescent signal is consistent with the 5'-guanine of f-HD1 interacting with antiHD1. The slow decrease in fluorescence signal following the spike is consistent with antiHD1 denaturing the secondary structure of HD1 as it hybridizes, starting with the 5' or 3' end, and releasing the double stranded oligonucleotide from the surface of thrombin or prothrombin.

Overall, our data demonstrate that antiHD1 is a potent neutralizing agent for HD1 in both plasma- and buffer-based assays. In addition, we demonstrate that HD1 is protected from antiHD1 when it is bound to either thrombin or prothrombin, which raises the possibility that antiHD1 will preferentially bind to unbound HD1 in plasma. Therefore the mechanism for displacement of HD1 from either thrombin or prothrombin by antiHD1 involves either (a) binding free HD1 causing a shift in the equilibrium between bound and unbound aptamer, or (b) the formation of a transient ternary complex whereby antiHD1 gradually unzips the secondary structure of HD1, resulting in gradual

release of the hybridized duplex from thrombin or prothrombin. Further work should establish the minimal sequence of antiHD1 required for efficient neutralization of HD1.

Chapter 6: General Discussion

The health of the circulatory system depends on maintaining blood in a fluid state, but primed for explosive coagulation at sites of injury. Vessel injury exposes tissue factor, which initiates a series of reactions that culminate in the formation of prothrombinase, which converts the inactive zymogen prothrombin into the active enzyme thrombin. Thrombin then converts soluble fibrinogen into fibrin, the major component of the blood clot that stems the flow of blood from the vessel. Thus, thrombin is the most important enzyme in the hemostatic system because it potentiates procoagulant, anticoagulant and anti-fibrinolytic pathways, and contributes to vascular remodelling, all of which are critical to wound healing (Hoffman and Monroe, 2007).

The studies described in this thesis were undertaken to examine the role of thrombin and prothrombin in coagulation using the DNA aptamer HD1. Prothrombin is converted to thrombin by the action of the prothrombinase complex, consisting of fXa, fVa assembled on an anionic membrane surface. Thrombin is a multifunctional serine protease that converts fibrinogen to fibrin, activates platelets and enhances its own production by activating the cofactors fV and fVIII. Critical to thrombin's physiological activity are two positively charged exosites that flank the active site and aid in the recognition of thrombin substrates and cofactors. Exosite 1, originally indentified as the fibrin-binding site, binds macromolecular substrates that interact with the active site, and also binds thrombomodulin that switches thrombin from a procoagulant enzyme to an anticoagulant enzyme. Exosite 2 binds cofactors and ligands (including glycosaminoglycans, glycoprotein 1bα, and the γ'-chain of fibrinogen) that enhance

thrombin interactions with other ligands. As a result, exosite 1 is considered to be a substrate-binding exosite, whereas exosite 2 is considered to be a cooperative-binding exosite (Lane *et al.*, 2005). Aptamers, small oligonucleotide-based molecules that are designed to bind a specific target, are useful tools for studying the role of exosites in coagulation. HD1 is a DNA aptamer that was developed to bind exosite 1 of thrombin and block its procoagulant activities (Griffin *et al.*, 1993b).

The goals of this project were to (a) elucidate the mechanism by which HD1 inhibits coagulation by examining it's capacity to bind thrombin and prothrombin, and (b) synthesize and characterize antiHD1, a complementary aptamer against HD1, to determine its utility as an antidote to HD1. We have shown that HD1 binds prothrombin with high affinity and inhibits prothrombin activation by prothrombinase by competing with fVa for prothrombin binding. Because of its capacity to block prothrombin activation as well as thrombin activity, HD1 is a more potent inhibitor of coagulation than other exosite 1-directed ligands. We also demonstrate that the anticoagulant activities of HD1 can be neutralized by antiHD1. Furthermore, we have characterized the interaction of antiHD1 with free HD1 and HD1 bound to thrombin or prothrombin. This chapter contains a detailed discussion of our studies, emphasizing the advances made in our understanding of coagulation and aptamers.

Role of proexosite 1 in prothrombin activation by prothrombinase

Exosite 1 subdomains:

Exosite 1 is composed of cationic residues surrounding a hydrophobic surface depression that leads to the active site. Examination of the crystal structure of thrombin bound to either HD1 (Padmanabhan *et al.*, 1993) or $Hir^{54-65}(SO_3^-)$ (Chen *et al.*, 1995) shows that these ligands bind to distinct subdomains of exosite 1. Whereas HD1 binds to the cationic surface residues of exosite 1, namely Arg^{409} and Arg^{413}, $Hir^{54-65}(SO_3^-)$ binds to the hydrophobic surface depression, and makes minimal contact with these charged residues. Indeed, much of the binding energy that stabilizes $Hir^{54-65}(SO_3^-)$ binding to thrombin is hydrophobic in nature (Wang *et al.*, 1995); a trait that is conserved among all exosite 1-binding peptides (Lane *et al.*, 2005). In a recent review, Huntington demonstrates that all exosite 1-binding peptides have a higher ratio of hydrophobic residues than peptides that bind to exosite 2 (Huntington, 2005). He postulates that this feature reflects the fact that exosite 1 interacts with ligands via hydrophobic forces. He goes on to propose that exosite 1 should be re-named the "apolar-binding exosite", because the role of the anion-binding residues of exosite 1 is to properly orient ligands for insertion into the hydrophobic core and not binding. Consistent with these concepts is our finding that HD1 and $Hir^{54-65}(SO_3^-)$ bind to somewhat distinct subdomains of exosite 1.

Although no known study has reported on the ratio of polar to apolar groups on HD1, we can postulate that it is primarily composed of polar groups based on the ribo-phosphate backbone of the oligonucleotide. This hypothesis is supported by the crystal

structure of HD1 bound to thrombin, which illustrates HD1 binding to arginine residues of exosite 1 (Padmanabhan *et al.*, 1993). As a result, HD1 binds exosite 1 in a fashion similar to that of ligands that bind exosite 2, which Huntington demonstrates to have at least a 2.5-fold higher proportion of charged residues relative to hydrophobic residues (Huntington, 2005). As a result, HD1 may be predisposed to bind to the charged residues that line exosite 1. The observations from our fluorescence studies demonstrating incomplete competition from the surfaces of thrombin or prothrombin is consistent with the differences in the way HD1 and $Hir^{54-65}(SO_3^-)$ are recognized by thrombin. Therefore, our studies demonstrate that there are distinct subdomains within exosite 1 of thrombin. Based on differences in their binding to exosite 1, HD1 and $Hir^{54-65}(SO_3^-)$ are useful tools for exploring differences in the binding of substrates and ligands to these subdomains.

Proexosite versus exosite:

Based upon the residues through which they bind to thrombin, we can make predictions about the binding of HD1 and $Hir^{54-65}(SO_3^-)$ to prothrombin. We show that $Hir^{54-65}(SO_3^-)$ binds prothrombin more weakly than thrombin whereas HD1 binds prothrombin and thrombin with similar affinity. Based upon experiments with $Hir^{54-65}(SO_3^-)$ and the observation that prothrombin does not bind fibrinogen, Anderson *et al* proposed that exosite 1 on prothrombin exists in an immature state, which they termed proexosite 1 (Anderson *et al.*, 2000a). Our data, as well as those of others, indicate that this is a flawed definition of exosite 1 on prothrombin. These arguments are presented in detail below.

Firstly, HD1 binds thrombin and prothrombin with equivalent K_D values, suggesting that the exosite 1 residues that bind HD1 do not undergo extensive conformational changes during the conversion of prothrombin to thrombin (Kretz *et al.*, 2006). The crystal structure of prethrombin 2 demonstrates that without cleavage at Arg^{320}, the hydrophobic pocket of exosite 1 remains hidden from solution and unavailable to bind ligands because the charged residues are highly mobile (Malkowski *et al.*, 1997). However, the cationic residues remain exposed to the solution and available to bind HD1 in the structure of prethrombin 2. In support of this concept, HD1 binds thrombin and prethrombin 2 with similar affinity, whereas $Hir^{54-65}(SO_3^-)$ binds thrombin with a 60-fold higher affinity than prethrombin 2 (Anderson and Bock, 2003). Therefore, the hydrophobic exosite 1 cleft is under-developed in prethrombin 2, whereas the charged residues surrounding the cleft are fully developed. Based on these observations, it is more accurate to state that the hydrophobic subdomain of exosite 1 is immature in prothrombin, whereas the hydrophilic subdomain is fully formed. These results may explain the differences in the capacity of HD1 or $Hir^{54-65}(SO_3^-)$ to bind prothrombin.

A second argument that the term proexosite is inappropriate is evident from examination of the properties of meizothrombin. Meizothrombin binds hirudin and $Hir^{54-65}(SO_3^-)$ with the same affinity as thrombin (Fischer *et al.*, 1998). Furthermore, meizothrombin has a fully formed active site because it cleaves chromogenic substrates as efficiently as thrombin. These properties of meizothrombin are consistent with its capacity to activate fV (Tans *et al.*, 1994) and to bind thrombomodulin and activate protein C and TAFI (Cote *et al.*, 1997). However, meizothrombin possesses only 10% of

the clotting activity of thrombin, suggesting that exosite 1 of meizothrombin is not fully capacitated for binding to fibrinogen. Therefore, the functional activity of exosite 1 cannot be fully defined by ligands such as $Hir^{54-65}(SO_3)$ or HD1, but must also be determined by the physiological substrates for which it is intended.

Thirdly, the definition of proexosite 1 is biased toward ligands and substrates of thrombin and ignores the part that exosite 1 on prothrombin plays during its activation by prothrombinase. Exosite 1 on prothrombin is fully capacitated to bind some ligands, such as fVa. Molecular modelling of prothrombin binding to fVa (Lee *et al.*, 2008), as well as functional studies that determine the points of contact between prothrombin and fVa within the prothrombinase complex, indicate that exosite 1 is a primary site for interaction. This is supported by our data, which demonstrate that HD1 inhibits prothrombin activation by prothrombinase by competing with fVa for prothrombin binding. Therefore, classifying exosite 1 on prothrombin as immature is only true in the context of ligands and substrates that interact with thrombin.

There is extensive evidence that the exosite 1 on prothrombin is different from that on thrombin. However, this does not mean that exosite 1 on prothrombin is immature or underdeveloped. Instead, exosite 1 on prothrombin may be fully capacitated to bind ligands, such as fVa, whose function is to enhance prothrombin activation by prothrombinase.

Role of exosite 1 on prothrombin during prothrombin activation:

The rate of prothrombin activation by fXa is enhanced 3000-fold in the presence of fVa and PS-membranes (Nesheim *et al.*, 1979). These cofactors augment fXa activity and bind prothrombin to co-localize the enzyme and the substrate, thereby promoting the probability of productive interactions. FVa binds prothrombin at several distinct regions, including the Gla-domain, kringle 1 of fragment 1, and exosite 1. Our data demonstrate that HD1 binds exosite 1 on prothrombin and prevents it from entering into the prothrombinase complex, thereby reducing the rate of thrombin production. Whether exosite 1 on prothrombin is the major binding site for prothrombin entry into prothrombinase is debatable because HD1 does not completely eliminate the cofactor activity of fVa. However, inhibition of this interaction reduces the catalytic efficiency of prothrombin activation by 12-fold, resulting in 90% inhibition in the rate of prothrombin conversion to thrombin. Presumably, HD1 does not interfere with fVa binding to (a) the Gla-domain of prothrombin, or (b) fXa. The fVa-fXa interaction has been shown to be the primary interaction required for efficient prothrombin activation (Guinto and Esmon, 1984). HD1 only produces a modest 12-fold reduction in the rate of prothrombin activation. In contrast, fVa contributes to a 1000-fold increase in the rate (Nesheim *et al.*, 1979). Therefore the majority of fVa cofactor activity within the prothrombinase complex remains unaffected in the presence of HD1. The ability of HD1 to attenuate prothrombin activation, without completely abrogating it, endows HD1 with potential advantages over prothrombinase inhibitors such as rivaroxaban, which target the active site of fXa, and completely inhibit prothrombinase. Agents that completely inhibit

prothrombinase function may have a narrower therapeutic window than those that target exosite 1 on prothrombin because total inhibition of thrombin generation may impair hemostasis and lead to bleeding complications.

Although our studies in buffer systems helped identify the mechanisms by which HD1 acts as an anticoagulant, they provide limited information as to how it works in more complex plasma-based systems. To address this issue, we evaluated the effects of HD1 on thrombin generation using a dynamic assay where the reactants of prothrombinase are generated *in situ* at physiologically relevant rates.

Comparing HD1 and $Hir^{54-65}(SO_3^-)$ in thrombin generation assays

Inhibiting prothrombin activation is an effective strategy for blocking thrombin generation:

Because HD1 binds both thrombin and prothrombin with high affinity, whereas $Hir^{54-65}(SO_3^-)$ only binds thrombin, we predicted that HD1 would inhibit coagulation by attenuating thrombin activity and prothrombin activation, whereas $Hir^{54-65}(SO_3^-)$ would only inhibit thrombin activity. To test this hypothesis, we compared the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on thrombin generation using the CAT (Calibrated Automated Thrombogram) assay. CAT continuously monitors thrombin activity in plasma to illustrate the dynamics of thrombin generation. This is a useful technique for studying coagulation because it yields more information than global clotting tests, such as the PT or aPTT. Our data demonstrate that HD1 is a more potent inhibitor of thrombin

generation than $Hir^{54-65}(SO_3^-)$. This is best reflected by the fact that HD1 reduces the ETP to a greater extent than $Hir^{54-65}(SO_3^-)$ in normal plasma. Surprisingly, HD1 also prolonged the lag time to greater extent than $Hir^{54-65}(SO_3^-)$, despite their similar capacities to inhibit thrombin feedback activation of fV and fVIII. These data suggest that the lag time is not only dependent on cofactor activation, but also on prothrombinase assembly.

The CAT assay has advantages over other clot-based assays because it measures the entire time course of thrombin generation, instead of a clotting end point, which occurs when only 5% of total thrombin has been generated (Brummel *et al.*, 2002). As a result, the CAT assay provides more detailed information of the dynamics of thrombin generation. The enhanced sensitivity of the TGA is reflected by the fact that the lag time does not necessarily coincide with the clotting time. Indeed, prolongation of the clotting time (as measured by the PT or aPTT) is not always indicative of a prolongation of the lag time (Beilfuss *et al.*, 2008). This is reflected in our data. For example, 20 μM HD1 caused a 3-fold prolongation of the aPTT and a 7-fold prolongation of the PT. In contrast, the same concentration of HD1 caused a ~10-fold prolongation of the lag time in the TGA. Although direct comparisons between the lag time and clotting time cannot be made, an agent that prolongs the lag time would also be expected to delay the clotting time. The CAT assay provides additional information including peak thrombin, which indicates prothrombinase function relative to serpin function, and ETP, which is a measure of the thrombin load. Therefore the CAT assay is a useful method for comparing the effects of HD1 and $Hir^{54-65}(SO_3^-)$ on the dynamics of thrombin generation.

Role of cofactor activation in thrombin generation:

In addition to studying the capacity of HD1 and $Hir^{54-65}(SO_3^-)$ to inhibit thrombin generation, we were interested in understanding the contribution of thrombin feedback activation of fV and fVIII to thrombin generation. To this end, we used plasma deficient in either fV, fVIII, or both, that was supplemented with the activated form of the missing cofactor(s), to evaluate their contribution to the lag time, peak thrombin concentration, and ETP.

In the absence of inhibitors, the addition of fVa to fV-deficient plasma shortened the lag time by 1 min and increased the peak thrombin concentration by 100 nM compared with normal plasma. The moderate reduction in the lag time in fVdp/+fVa relative to normal plasma suggests that the generation of fXa is primarily responsible for the transition from initiation phase to propagation phase, which is consistent with previous studies (Butenas *et al.*, 1997, Cawthern *et al.*, 1998). In fVdp/+fVa, neither HD1 nor $Hir^{54-65}(SO_3^-)$ prolonged the lag time. However, HD1 and $Hir^{54-65}(SO_3^-)$ inhibited fVIII activation to a similar extent, suggesting that fVIII activation by thrombin does not contribute to the lag time in the CAT assay. Interestingly, only HD1 reduced the peak thrombin concentration in fVdp/+fVa, evidence of its capacity to attenuate prothrombin activation by prothrombinase.

In fVIIIdp/+fVIIIa, both HD1 and $Hir^{54-65}(SO_3^-)$ prolonged the lag time and reduced the peak thrombin concentration. HD1 and $Hir^{54-65}(SO_3^-)$ inhibited thrombin generation in fVIIIdp/+fVIIIa to a similar extent as they did in normal plasma, consistent with results in fVdp/+fVa that suggest thrombin feedback activation of fVIII does not

contribute to the lag time in the CAT assay. During the initiation phase, fXa formation occurs primarily through the extrinsic pathway. As reactants are generated and TFPI inhibits the extrinsic pathway, the intrinsic tenase complex becomes the predominant activator of fX (van't Veer and Mann, 1997). This switch occurs as thrombin generation transitions into the propagation phase; therefore, deficiencies in fVIIIa typically manifest as a reduced rate of thrombin generation during propagation (Cawthern *et al.*, 1998). However, the absence of fVIIIa in fVIIIdp did not reduce the rate of thrombin generation in the CAT assay. This can be explained by the fact that we used fVIII-deficient plasma, which may contain small amounts of residual fVIII. Any fVIII remaining in the plasma could influence fX activation by intrinsic tenase and contribute to prothrombinase assembly. However, the observation that HD1 and $Hir^{54-65}(SO_3^-)$ prolonged the lag time in fVIIIdp/+fVIIIa to a similar extent as they did in normal plasma confirms the observation that fXa-mediated activation of fV does not contribute to the initiation phase (Butenas *et al.*, 1997). Furthermore, the fact that HD1 prolongs the lag time to a greater extent than $Hir^{54-65}(SO_3^-)$ suggests that simultaneous inhibition of thrombin feedback activation of fV and prothrombin entry into prothrombinase produces a synergistic prolongation of the lag time.

In fVfVIIIdp/+fVa+fVIIIa, HD1 inhibited the peak thrombin concentration and prolonged the lag time, whereas $Hir^{54-65}(SO_3^-)$ only moderately reduced the peak thrombin concentration. These data provide insights into the mechanism of HD1 inhibition of thrombin generation. The prolongation of the lag in the absence of cofactor activation indicates that HD1 binding to prothrombin restricts its entry into

prothrombinase, thereby extending the initiation phase. Interestingly, HD1 was unable to prolong the lag time in fVdp/+fVa, but was able to prolong it in fVfVIIIdp/+fVa+fVIIIa. This is likely because in the presence of fVIIIa, more fXa is formed and is available to interact with the supplemented fVa. As a result of higher concentrations of fXa, there is less competition between HD1 and fVa for prothrombin within the prothrombinase complex. The inability of $Hir^{54-65}(SO_3^-)$ to bind prothrombin, especially in the presence of fVa, is also confirmed in this experiment where it has no significant effect on either the lag time or the peak thrombin concentration.

The effect of current anticoagulants on thrombin generation:

The goal of this study was to use HD1 and $Hir^{54-65}(SO_3^-)$ to evaluate the role of exosite 1 on thrombin and prothrombin in a dynamic thrombin generation assay. However, a commentary about the effectiveness of these agents to block thrombin generation relative to clinically approved anticoagulants is warranted.

Thrombin inhibitors that are approved for clinical applications and that have been studied using the CAT assay, include: (a) argatroban- a small molecule active site inhibitor, (b) lepirudin- a recombinant form of the leech protein hirudin, and (c) bivalirudin- a cleavable hirudin derivative. Although it is not licensed for clinical use, the effect of PPAck, a tripeptide that binds the active site of thrombin in a covalent fashion, also has been examined using the CAT assay. FXa inhibitors that have been studied using the CAT assay include: (a) fondaparinux, the synthetic pentasaccharide, (b) ZK-807834, a reversible small molecule active site inhibitor of fXa, and (c) rivaroxaban, a reversible small molecule active site inhibitor of fXa. In addition, enoxaparin- a low

molecular weight heparin that targets both fXa and thrombin has also been studied using the CAT assay. RB006/RB007, the fIXa aptamer/antidote pair, has also been studied using CAT, but these results will be discussed in a subsequent section dealing with antiaptamers. I will compare the results from studies that used these anticoagulants in the CAT assay below.

Interesting similarities and differences exist in the manner by which these various anticoagulants inhibit thrombin generation. For example, argatroban is similar to enoxaparin and fondaparinux because they all inhibit thrombin generation by reducing the peak thrombin, but not by prolonging the lag time (Robert *et al.*, 2009). In contrast, lepirudin, PPAck, and bivalirudin produce dose-dependent prolongations of the lag time and reductions in the peak thrombin concentration (Beilfuss *et al.*, 2008, Robert *et al.*, 2009), similar to HD1. Interestingly, lepirudin was able to reduce the ETP, whereas PPAck and bivalirudin did not, even at high concentrations. These findings are indicative of the potent anticoagulant properties of lepirudin. The observations that argatroban only reduced the peak thrombin, whereas PPAck reduced peak thrombin and prolonged the lag time, suggest that covalent inhibition of thrombin's active site results in a prolongation of the lag time, whereas reversible binding to the active site does not. This is interesting given our findings with $Hir^{54\text{-}65}(SO_3^-)$, which has minimal effects on peak thrombin, but prolongs the lag time. These results may suggest that the capacity of $Hir^{54\text{-}65}(SO_3^-)$ to bind exosite 1 and induce allosteric changes at the active site contributes in a major way to its capacity to inhibit thrombin generation.

Of the fXa inhibitors, ZK-807834 and rivaroxaban reduce the peak thrombin concentration and moderately prolong the lag time, whereas fondaparinux reduces the peak thrombin without prolonging the lag time (Gerotziafas *et al.*, 2007, Robert *et al.*, 2009). The concentrations of ZK-807834, rivaroxaban, and fondaparinux required to completely inhibit thrombin generation were 5 μM, 100 nM, and 15 μM, respectively. Thus, of these agents, rivaroxaban is the most potent inhibitor of coagulation. If we compare our findings with previously published work, HD1 inhibits thrombin generation with a similar potency as fondaparinux.

The results of these CAT assays provide insights into the mechanism of inhibition by anticoagulants. Thus, lepirudin, PPAck, and bivalirudin would be expected to prolong clotting time because they inhibit the lag time more than the peak thrombin, thereby delaying the onset of thrombin generation and prolonging the clotting time. In contrast, enoxaparin, fondaparinux, rivaroxaban, and ZK807834 would be expected to prolong clotting times because they reduce the peak thrombin concentration below the threshold required for clot formation. The major difference between these mechanisms of inhibition is that thrombin generation eventually occurs in the presence of thrombin inhibitors, but not with fXa inhibitors. Compared with these results, HD1 is better at prolonging the lag time and does so before it reduces peak thrombin concentration below the threshold required for clot formation. These findings suggest that the clotting time would be prolonged before thrombin generation is completely abolished. The capacity of anticoagulants to prolong the lag time without reducing peak thrombin concentration, may provide a window of safety because although thrombin generation would be

delayed, it would eventually occur, thereby maintaining hemostasis. Interestingly, activated protein C causes a reduction in the peak thrombin concentration without prolonging the lag time (Nicolaes *et al.*, 1997). This result may provide an explanation for the bleeding complications associated with Xigris (recombinant APC) administration in patients with sepsis (Bernard *et al.*, 2001), because high doses of this agent may completely abolish thrombin generation. Therefore, careful dosing of Xigris may be necessary when the utility of this agent is explored in patients with ischemic stroke (Wang *et al.*, 2009b).

Neutralization of the anticoagulant activity of HD1 with antiHD1

DNA aptamers have advantages over traditional anticoagulants. First, aptamers bind their targets with high specificity, resulting in a predictable level of anticoagulation (Nimjee *et al.*, 2005a). Second, the half-life of aptamers is short, but can be extended depending on the application (Dougan *et al.*, 2000). Third, there are no reports of allergic reactions to aptamers, unlike protein- or carbohydrate-based drugs, which can elicit immune reactions (Dyke *et al.*, 2006). Fourth, antidotes can be rationally designed that specifically and safely neutralize aptamers (Nimjee *et al.*, 2006). Because of these features, several aptamer-based drugs have been developed or are under development (Nimjee *et al.*, 2005b). However, a detailed biochemical characterization of paired aptamer/anti-aptamer interactions is lacking. Our studies were undertaken to evaluate the kinetics of HD1 neutralization by anti-HD1.

The mechanism of anti-aptamer neutralization of aptamer activity:

Fluorescence spectroscopy experiments confirmed that fluorescein-HD1 bound prothrombin and thrombin with similar affinity. However, studies using SPR suggested that HD1 bound prothrombin with 27-fold lower affinity than that for thrombin. This disparity raises the possibility that fluorescein may influence the binding of HD1 to prothrombin but not to thrombin. Fluorescein is a hydrophobic group, which may insert into the immature hydrophobic pocket of exosite 1 on prothrombin, thereby enhancing the affinity of HD1 for prothrombin. In contrast, the biotin that was used to immobilize HD1 onto the SPR streptavidin biosensor chip binds to streptavidin and is not available

for interaction with prothrombin. Under these circumstances, HD1 may bind to prothrombin with reduced affinity. Another potential explanation for the reduced affinity of HD1 for prothrombin observed in the SPR experiments is that biotin-HD1 is conjugated on a 2-dimensional surface, which may induce steric constraints on its interaction with prothrombin. However, this is less likely because biotin-HD1 and fluorescein-HD1 bind thrombin with similar affinities. Despite the disparity in the affinity of HD1 for prothrombin measured using these techniques, our functional data demonstrates that HD1 is a potent inhibitor of prothrombin activation, consistent with the high affinity measured using the fluorescence technique.

Because of the differences in affinity of HD1 for prothrombin between these two assays, the capacity of antiHD1 to displace HD1 from prothrombin was also different. Thus, antiHD1 displaced prothrombin from biotin-HD1 at a rate that was 10-fold faster than the rate at which it displaced prothrombin from fluorescein-HD1. From these data we can conclude that the capacity of antiHD1 to displace HD1 from thrombin or prothrombin is dependent upon the affinity of HD1 for its target. This is somewhat surprising given that HD1 binds antiHD1 with a ~1000-fold higher affinity than it binds thrombin or prothrombin. Based on the high affinity of antiHD1 for HD1 (K_D in the pM range, as measured by SPR), antiHD1 should readily outcompete thrombin or prothrombin (K_D in the nM range). Consequently, additional insights into the mechanism of displacement are needed.

Spectroscopy experiments demonstrated that antiHD1 binds HD1 very rapidly because the fluorescence intensity equilibrated in less than 10 s after each injection. In

contrast, when the interaction of antiHD1 with HD1 was studied in the presence of thrombin or prothrombin, there was a time-dependent displacement following an initial spike in fluorescence intensity. These findings suggest that HD1 is less accessible to antiHD1 when it is bound to thrombin or prothrombin. In contrast, $Hir^{54-65}(SO_3^-)$ can readily displace f-HD1 from thrombin or prothrombin, as described in Chapter 3. Thus, increasing concentrations of $Hir^{54-65}(SO_3^-)$ yielded a dose-dependent reduction in the fluorescence intensity that equilibrated very quickly. Based on these differences, we propose a dock-and-unzip mechanism, whereby antiHD1 initially docks on HD1 that is bound to prothrombin or thrombin, forming a transient ternary complex. Once antiHD1 hybridizes with HD1, the HD1-antiHD1 complex dissociates from the protein surface. Because of this mechanism, antiHD1 binds more readily to free HD1. Neutralization of bound HD1 occurs more slowly through this dock-and-unzip mechanism.

This would explain why antiHD1 is more effective at neutralizing HD1 in the prothrombinase assay than it is in the thrombin clotting assay. Based on the SPR data, HD1 binds to thrombin with higher affinity than it does to prothrombin. As a result, antiHD1 must neutralize a greater proportion of bound HD1 when thrombin is present and a greater proportion of unbound HD1 when prothrombin is present. Because unbound HD1 is more rapidly neutralized, antiHD1 is more effective at inhibiting the activity of HD1 in the prothrombinase assay than it is in the thrombin clotting assay.

Examples of aptamer/anti-aptamer pairs:

That antiHD1 is an effective antidote for HD1 is not surprising given the success of the Regadol system consisting of the fIXa-directed aptamer, RB006, and its antidote,

RB007. RB006 was developed to inhibit coagulation upstream to prothrombinase. Recently, phase 1b clinical studies were reported on the effectiveness of Regadol in patients with stable coronary artery disease (Chan *et al.*, 2008a). Thus, RB006 produced dose-dependent anticoagulation that was rapidly reversed with RB007. A recent report demonstrated the effects of RB006 on thrombin generation using the CAT assay. Thus, increasing concentrations of RB006 caused a dose-dependent decrease in peak thrombin and prolongation of the lag time (Tanaka *et al.*, 2009). A 2:1 ratio of RB007 to RB006 completely reversed the anticoagulant activity of RB006. However, it must be noted that no information was provided as to the incubation time or the order of addition of RB006 or RB007. This information is important when evaluating the effectiveness of the antidote in reversing anticoagulation. Therefore, in-depth analysis of the kinetics of neutralization of RB006 by RB007 is still required.

The antidote, RB007, was developed to hybridize only a portion of the fIXa-directed aptamer, RB006 (Rusconi *et al.*, 2002). In contrast, antiHD1 hybridizes with the entire HD1 aptamer. Based on its mechanism of neutralization, it is possible that an anti-aptamer sequence can be optimized to neutralize its target aptamer by identifying the minimal sequence that hybridizes with the aptamer on a protein surface. To neutralize HD1, it may be possible to develop an anti-aptamer that specifically targets the T-T loops that are exposed to solution when HD1 binds to thrombin (Padmanabhan *et al.*, 1993). The solvent exposure may limit steric hindrance by thrombin or prothrombin and yield more rapid hybridization.

Our data also provide insights into the mechanism of aptamer neutralization by an anti-aptamer. If our findings findings with antiHD1 can be extrapolated to RB007, the antiaptamer may neutralize RB006 in 2 ways (a) by binding free aptamers, and/or (b) by displacing bound aptamer. Furthermore, RB006 has been shown to bind fIX and to block its activation by extrinsic tenase (Gopinath *et al.*, 2006). Based on our studies, RB007 may be more effective at neutralizing RB006 bound to fIX than RB006 bound to fIXa. As a result, a lower concentration of RB007 could be used to adjust the dose of RB006 before coagulation is initiated, because the anti-aptamer will readily hybridize with unbound aptamer.

HD1-22 is a recently described bivalent aptamer that was developed by linking HD1 to HD22 with a poly-adenine spacer (Muller *et al.*, 2008). This exosite 1 and 2-binding aptamer binds thrombin with a 10-fold higher affinity than HD1 and prolongs the thrombin clotting time. Interestingly, HD1-22 binds prothrombin with the same affinity as HD1, confirming our data that HD1 binds prothrombin. An antisense oligonucleotide directed against HD1 completely reversed the anticoagulant properties of HD1-22. This anti-aptamer is identical to anti-HD1, and shows a similar capacity to reverse the anticoagulant properties of HD1. However, in contrast to our studies, the investigators did not fully explore the mechanism of neutralization.

Future directions

In the course of this research, numerous preliminary observations were made. As a result, there are several avenues of investigation that extend beyond the boundaries of this thesis. This section contains a brief explanation of work conducted to-date and outline directions for future experiments.

Prothrombin as a target for anticoagulant development:

Rationale: Throughout this body of work, we have repeatedly mentioned that prothrombin is a novel target for new anticoagulants. Prothrombin may be a safer target than thrombin, because coagulation can be attenuated without directly inhibiting enzymatic activity. Continuing our work on the validation of prothrombin as an effective and safe target for new anticoagulants, we have collaborated with Dr. Feng Ni at the Biotechnology Research Institute of the National Research Council Canada (BRI-NRCC). Dr. Ni's group has developed a prothrombin-binding peptide based on the variable heavy chain of a prothrombin-directed antibody, termed V_H-Pro. The following statement was provided by the BRI-NRCC as a rationale for the development of this peptide:

> "Docking interactions between prothrombin and the prothrombinase can be targeted to reduce the production of thrombin at vascular sites decorated with elevated prothrombinase activity (Bock *et al.*, 2007, Kalafatis *et al.*, 2003, Kretz *et al.*, 2006). Indeed, a DNA aptamer exhibits a potent inhibitory activity toward the prothrombinase-catalyzed generation of thrombin from prothrombin as a

result of high-affinity binding to both thrombin and prothrombin (Kretz *et al.*, 2006). However, the current generation of such DNA-based ligands of prothrombin may suffer from significantly reduced in-vivo efficacy as a result of high-affinity binding to high levels of circulating prothrombin. In particular, an anticoagulant agent targeting prothrombin exhibits a moderate in-vivo activity (Griffin *et al.*, 1993a) despite an intrinsically high binding affinity toward prothrombin (Kretz *et al.*, 2006). In fact, this is an intrinsic limitation for all prothrombin-specific ligands unless they can be made to act only on prothrombin-prothrombinase interactions."

Furthermore the BRI-NRCC has provided 2 figures for inclusion into this thesis to establish a rationale for their decision to collaborate with Dr. Weitz and myself. Our contribution to this collaboration was to establish a mechanism for the inhibitory activities of the V_H-Pro protein (Appendix 6.1) using purified proteins in buffer-based systems.

Completed work: V_H-Pro inhibited prothrombin activation by prothrombinase with an IC_{50} value of 22 μM (Figure 6.1). Although this protein is significantly less potent than HD1, it is more potent than $Hir^{54\text{-}65}(SO_3^-)$ at inhibiting prothrombin activation by prothrombinase. To begin to characterize the mechanism of inhibition, I tested the capacity of V_H-Pro to inhibit prothrombin activation by fXa and components of prothrombinase. I demonstrated that V_H-Pro inhibited prothrombin activation by fXa/fVa and fXa/PCPS, but not by fXa alone (Figure 6.2). These data suggest that V_H-

Pro binds to the Gla-domain of prothrombin because this is the only site on prothrombin that binds both fVa and PCPS vesicles, but not fXa.

Future studies: Although V_H-Pro is a poor inhibitor of prothrombin activation relative to HD1, affinity optimization studies are currently being performed by Dr. Ni's group to develop a higher affinity derivative. Once obtained, the high-affinity V_H-Pro peptide will be tested in prothrombinase assays as well as in thrombin generation assays, using the CAT system to determine its effectiveness as an inhibitor of coagulation and to identify its site of action.

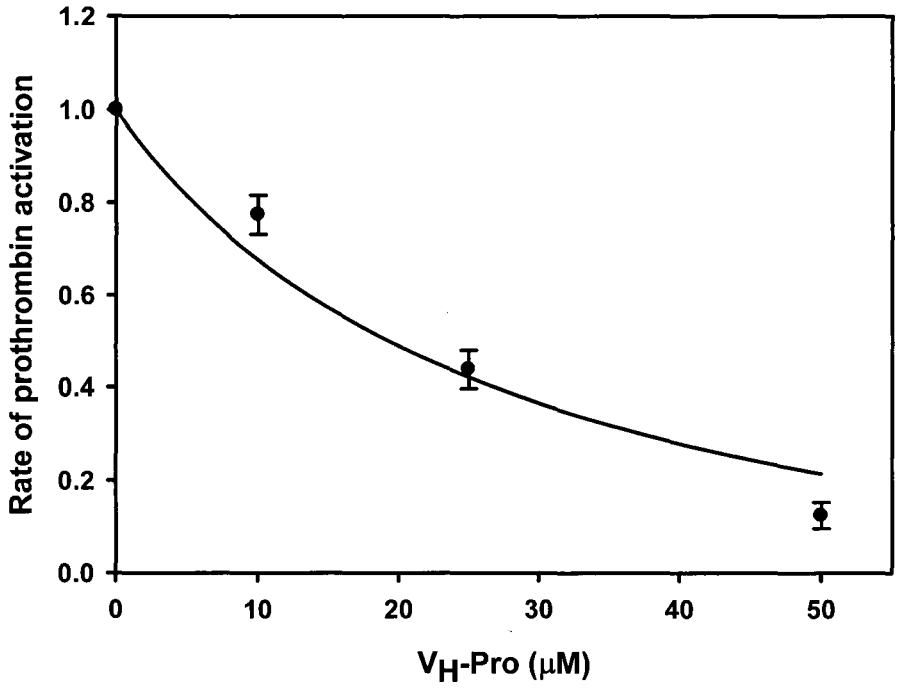

Rate of prothrombin activation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
V_H-Pro (μM)

Figure 6.1: Effect of V_H-Pro on the rate of prothrombin activation by prothrombinase

The capacity of V_H-Pro to inhibit prothrombin activation was tested in a discontinuous prothrombinase assay. Thus, 1 μM prothrombin and 6 μM PCPS vesicles in aptamer buffer with 0.1% PEG were incubated for 10 min at room temperature with 0-50 μM V_H-Pro. Pre-assembled prothrombinase consisting of 0.25 nM fXa, 0.3 nM fVa, and 6 μM PCPS (final concentrations) was then added to initiate the reaction. At intervals up to 2 min, aliquots were removed and the reaction was stopped by addition of 250 nM TAP and 4 mM EDTA, as previously reported (Kretz *et al.*, 2006). Generated thrombin was quantified by measuring the hydrolysis of 600 μM S2366 at 405 nm for 10 min using a plate reader. Rates of substrate cleavage, as determined by instrument software, were used to calculate thrombin concentration. The relative rates of prothrombin activation were calculated by dividing the rate of prothrombin activation determined at each concentration of V_H-Pro by the control.

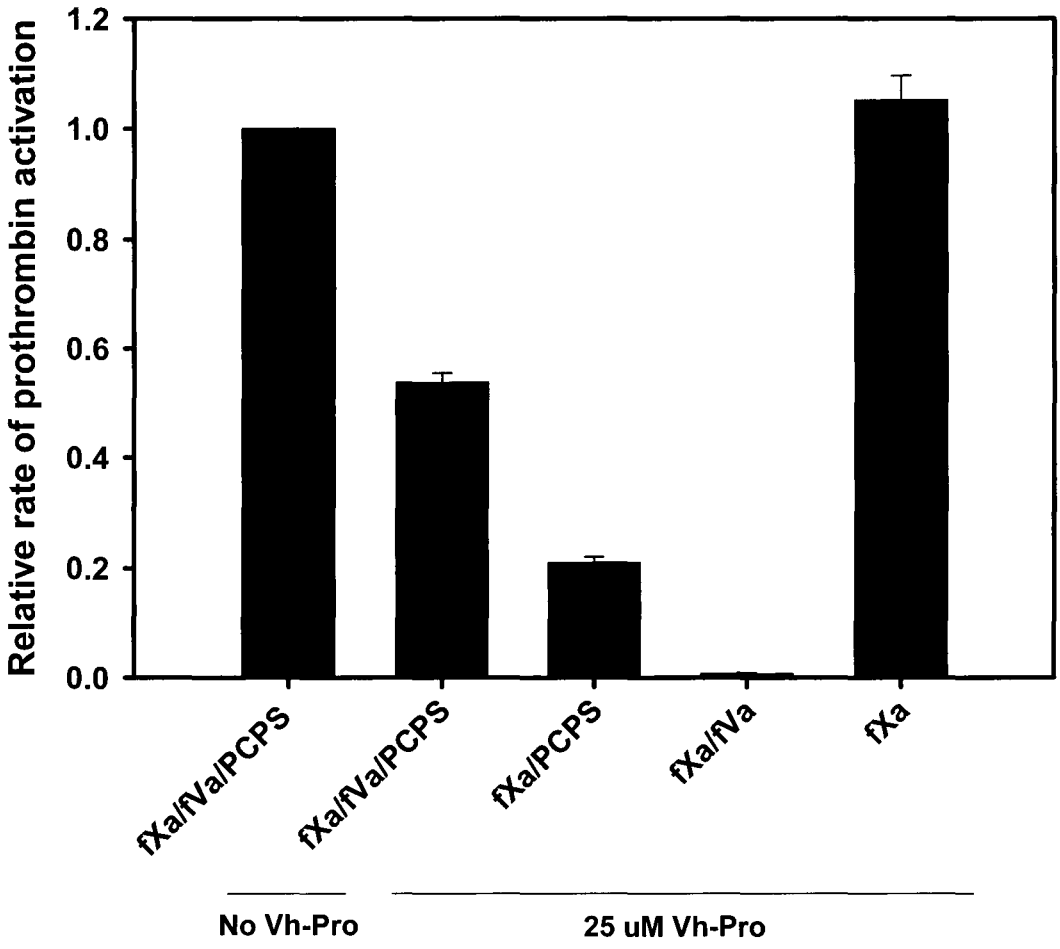

Relative rate of prothrombin activation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
fXa/fVa/PCPS
fXa/fVa/PCPS
fXa/PCPS
fXa/fVa
fXa
No Vh-Pro
25 uM Vh-Pro

Figure 6.2: Effect of V_H-Pro on prothrombin activation by components of prothrombinase

To begin to elucidate the mechanism by which V_H-Pro inhibits prothrombin activation, prothrombinase experiments were performed with various components of the prothrombinase complex omitted. Thus, we tested the capacity of 25 μM V_H-Pro to inhibit the activation of 1 μM prothrombin by (a) 0.25 nM fXa, 0.3 nM fVa, and 6 μM PCPS, (b) 150 nM fXa and 6 μM PCPS, (c) 2 nM fXa and 5 nM fVa, or (d) 1 μM fXa. In each case, thrombin generation was measured in a discontinuous manner, as described in Figure 6.1.

Defining the contribution of exosites 1 and 2 to fV and fVIII activation:

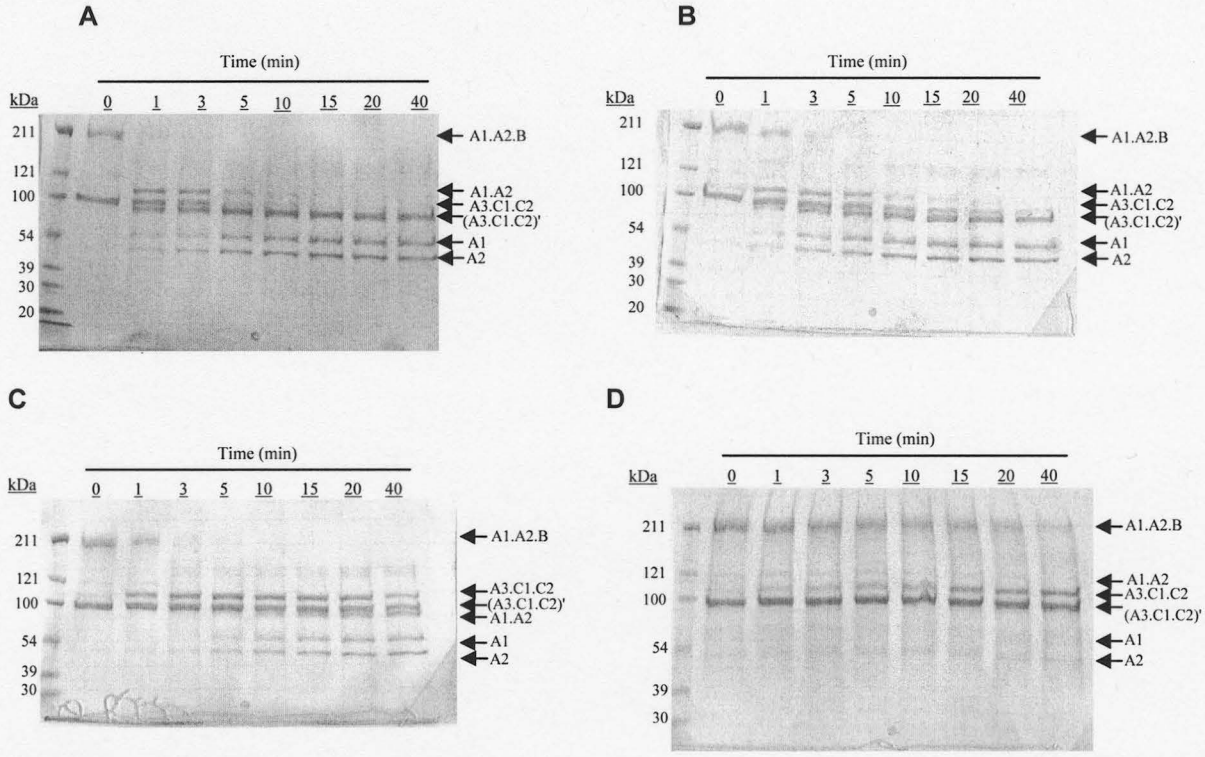
Rationale: It is clear from our work, as well as that of others, that exosite 1 is critical to thrombin activation of fV and fVIII. However, several studies have also identified a role for exosite 2 in cofactor activation by thrombin (Esmon and Lollar, 1996, Segers *et al.*, 2007). Therefore, HD1 and HD22 can be used to better define the contribution of exosites 1 and 2 to thrombin-mediated activation of fV and fVIII. Furthermore, the role of exosite 2 in amplifying thrombin generation can be assessed using the CAT assay in normal and cofactor-deficient plasma.

Work completed: SDS-PAGE analysis demonstrates that HD22 alone cannot inhibit thrombin activation of fV or fVIII. In the presence of HD22, however, fVIII activation by thrombin (Figure 6.3) proceeds in a slightly altered activation pattern compared with thrombin alone. Although cleavage at Arg^{740} or Arg^{372} is unaffected by HD22, there is a 20 min delay in cleavage at Arg^{1689} on the A3 subunit of the light chain. These data are in contrast to the dramatic effect of HD1 on thrombin activation of fVIII, as previously discussed (Figure 4.2). When both HD1 and HD22 are included in the reaction (Figure 6.3), there is a synergistic delay in the cleavage at Arg^{372} that releases the B domain from the heavy chain. This synergistic effect appears specific to Arg^{372} because cleavage at Arg^{1689} is not delayed any more than it is with HD1 alone. In the presence of either HD1 or HD22, thrombin readily cleaves this bond, suggesting that either exosite is sufficient for cleavage at Arg^{372}. These results demonstrate the dual role of exosite 1 and 2 in fVIII activation by thrombin. These qualitative SDS-PAGE data were confirmed in functional experiments by measuring the effect of HD1, HD22, or

both on the generation of fVIIIa cofactor activity during fVIII activation by thrombin (Figure 6.4)

Like its effect on fVIII activation, HD22 also affects fV activation by thrombin. Thus, HD22 alone delays the formation of the light chain by attenuating cleavage at Arg^{1545}, but has little effect on the formation of the heavy chain (Figure 6.5). This is in contrast to HD1, which delayed the formation of both the heavy chain and the light chain by inhibiting cleavages at Arg^{709}, Arg^{1018}, and Arg^{1545} (Figure 4.1). In combination, HD1 and HD22 caused a synergistic inhibition of fV activation, illustrated by the marked delay in the formation of the heavy and light chains of fVa (Figure 6.5). These data are consistent with previous findings (Segers *et al.*, 2007). However, the combination of HD1 and HD22 did not cause a synergistic inhibition of cofactor activity during fV activation by thrombin (Figure 6.6). Therefore, more work is needed to better define the effects of HD1 and HD22 on fV activation by thrombin. Any synergistic effect of HD1 and HD22 on fV activation by thrombin would suggest that either exosite is sufficient for proteolysis.

Although the above experiments suggest that exosite 2 of thrombin plays a supporting role in cofactor activation by thrombin, both HD1 and HD22 also modulate the active site of thrombin. Thus, HD1 and HD22 attenuate thrombin inhibition by α_1-antitrypsin Pittsburgh (Figure 6.7). As a result, the effects of HD1 and HD22 on cofactor activation may reflect (a) blocking fV and fVIII interaction with exosites, and/or (b) allosteric changes at the active site. As a result, the effects of HD1 and HD22 on fV and fVIII activation by thrombin cannot be solely attributed to blocking exosite interactions.


A
Time (min)
kDa
0 1 3 5 10 15 20 40
211
121
100
54
39
30
20
A1.A2.B
A1.A2
A3.C1.C2
(A3.C1.C2)'
A1
A2
B
Time (min)
kDa
0 1 3 5 10 15 20 40
211
121
100
54
39
30
20
A1.A2.B
A1.A2
A3.C1.C2
(A3.C1.C2)'
A1
A2
C
Time (min)
kDa
0 1 3 5 10 15 20 40
211
121
100
54
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30
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A3.C1.C2
(A3.C1.C2)'
A1.A2
A1
A2
D
Time (min)
kDa
0 1 3 5 10 15 20 40
211
121
100
54
39
30
A1.A2.B
A1.A2
A3.C1.C2
(A3.C1.C2)'
A1
A2

Figure 6.3: Effect of HD1 and HD22 on factor VIII activation by thrombin

We used SDS-PAGE to evaluate the capacity of 5 nM thrombin to activate 1 mg/ml fVIII in the (*A*) absence or (*B*) presence of 5 μM HD1, (*C*) 5 μM HD22, or (*D*) 5 μM of both HD1 and HD22 (>99% thrombin bound by either aptamer alone). 5 μl aliquots were removed into 10 μl sample buffer at 0, 1, 3, 5, 10, 15, 20, 40 min time points and were subjected to SDS-PAGE analysis on a 4-15% acrylamide Ready Gel (Bio-Rad, Hercules, CA) under reducing conditions. The gels were then fixed and stained with Fast Stain.

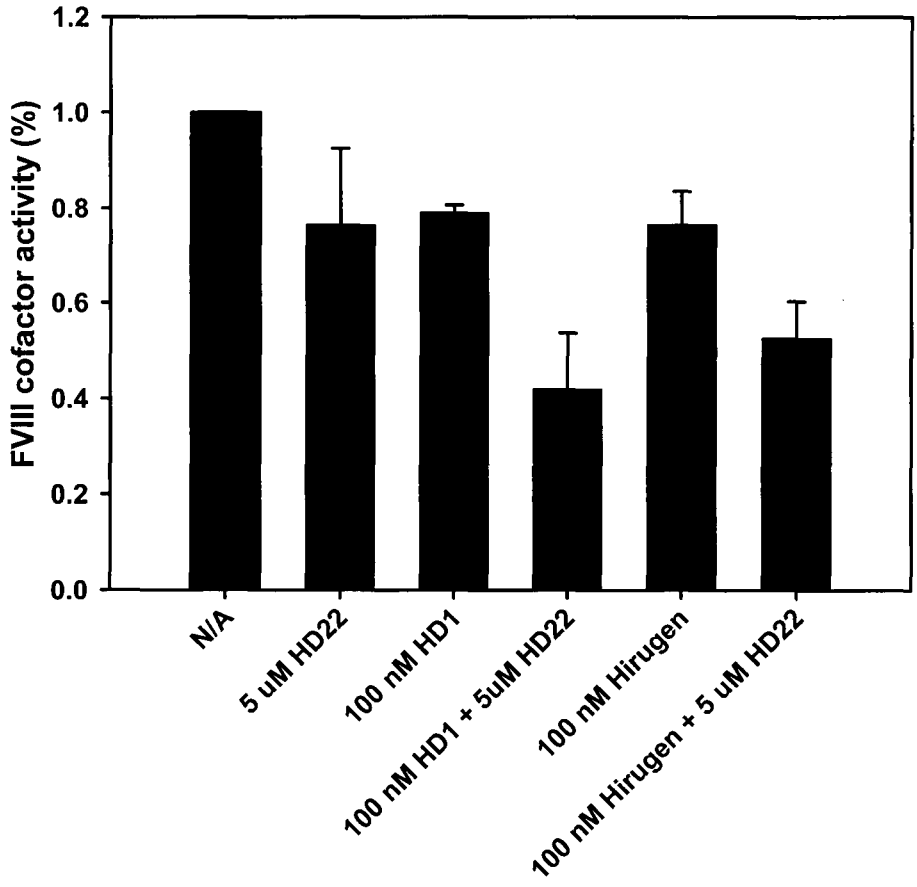

FVIII cofactor activity (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N/A
5 uM HD22
100 nM HD1
100 nM HD1 + 5uM HD22
100 nM Hirugen
100 nM Hirugen + 5 uM HD22

Figure 6.4: Effect of HD1 and HD22 on the kinetics fVIII activation by thrombin

To test the capacity of HD1 and HD22 to inhibit fVIII activation by thrombin, we assayed the formation of fVIIIa cofactor activity in a continuous tenase assay. Thus, 30 nM thrombin was incubated with 5 μM HD22, 100 nM HD1, 100 nM hirugen ($Hir^{54-65}(SO_3^-)$), 5 μM HD22 + 100 nM HD1, or 5 μM HD22 plus 100 nM $Hir^{54-65}(SO_3^-)$, before initiating the reaction by adding 40 nM fVIII. After 5 min, the fVIII activation reaction was terminated by addition of 120 nM hirudin. To determine the extent of cofactor formation, the fVIII reaction mixture was diluted to 32 nM by addition of 40 nM fIXa and 20 μM PCPS. Following 5 min of incubation to allow tenase assembly, a mixture of fX and S2765 was added to final concentrations of 600 nM and 900 μM, respectively. The rate of fXa generation was measured continuously for 10 min at 23°C in a plate reader read a 405 nm. The initial rate of fX activation was determined by taking point-to-point slopes of the chromogenic substrate hydrolysis plotted as a function of time. The rate of fX activation was an indication of fVIII activation. The results are presented as extent of cofactor activity relative to control (no aptamer). Each condition reflects the average of 4 separate experiments ± SEM.

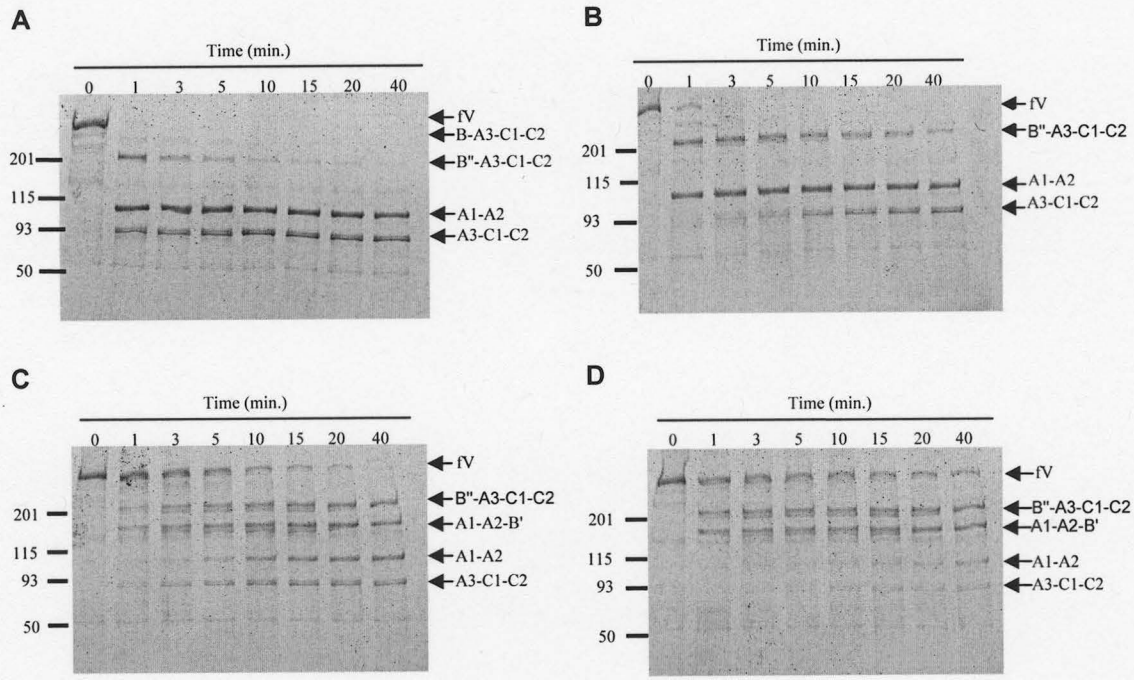

A
Time (min.)
0 1 3 5 10 15 20 40
201
115
93
50
fV
B-A3-C1-C2
B"-A3-C1-C2
A1-A2
A3-C1-C2
B
Time (min.)
0 1 3 5 10 15 20 40
201
115
93
50
fV
B"-A3-C1-C2
A1-A2
A3-C1-C2
C
Time (min.)
0 1 3 5 10 15 20 40
201
115
93
50
fV
B"-A3-C1-C2
A1-A2-B'
A1-A2
A3-C1-C2
D
Time (min.)
0 1 3 5 10 15 20 40
201
115
93
50
fV
B"-A3-C1-C2
A1-A2-B'
A1-A2
A3-C1-C2

Figure 6.5: Effect of HD1 and HD22 on factor V activation by thrombin

We used SDS-PAGE to evaluate the capacity of 5 nM thrombin to activate 0.2 mg/ml fV in the (*A*) absence or presence of (*B*) 5 μM HD1, (*C*) 5 μM HD22, or (*D*) 5 μM of HD1 plus HD22 (>99% thrombin bound by either aptamer alone). 5 μl aliquots were removed into 10 μl sample buffer at 0, 1, 3, 5, 10, 15, 20, 40 min time points and were run on a 4-15% acrylamide Ready Gel (Bio-Rad, Hercules, CA) under reducing conditions. The gels were then fixed, stained with Sypro Ruby and imaged using a Typhoon 9410 (Amersham Biosciences, Piscataway, NJ).

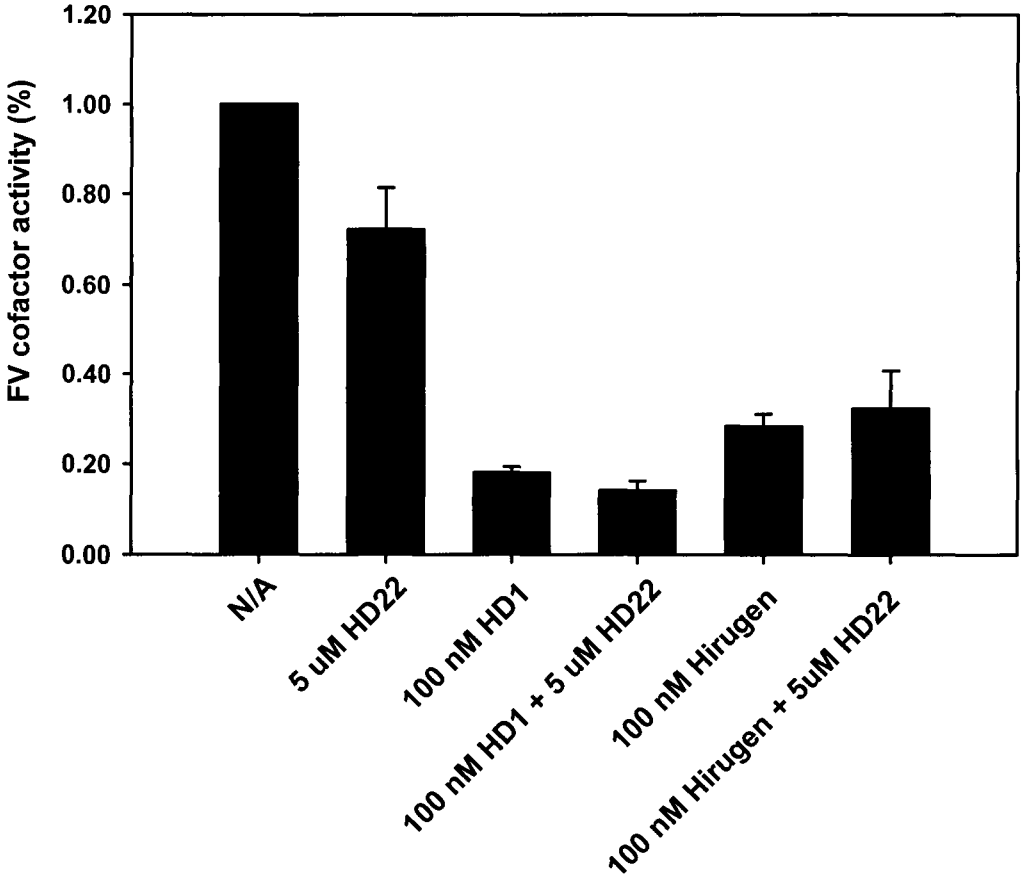

FV cofactor activity (%)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
N/A
5 uM HD22
100 nM HD1
100 nM HD1 + 5 uM HD22
100 nM Hirugen
100 nM Hirugen + 5uM HD22

Figure 6.6: Effect of HD1 and HD22 on kinetics of factor V activation by thrombin

To test the capacity of HD1 and HD22 to inhibit the rate of fV activation by thrombin, we assayed the formation of fVa cofactor activity in a continuous prothrombinase assay. Thus, 10 nM thrombin was incubated with 5 μM HD22, 100 nM HD1, 100 nM $Hir^{54-65}(SO_3^-)$ ($Hir^{54-65}(SO_3^-)$), 5 μM HD22 + 100 nM HD1, or 5 μM HD22 + 100 nM $Hir^{54-65}(SO_3^-)$, before initiating the reaction by adding 600 nM fV. At various times (0-10 min) aliquots were removed into a solution containing hirudin and Prionex to yield 50 nM fV. To determine the extent of cofactor activation, the reaction mixture was diluted to 1 nM fV by addition of 0.1 nM fXa and 20 μM PCPS. Following 5 min of incubation, a mixture of prothrombin and S2238 was added to final concentrations of 1 μM and 800 μM, respectively. The rate of thrombin formation was measured continuously for 10 min at 23°C in a plate reader. The initial rate of thrombin formation was determined by taking point-to-point slopes of chromogenic substrate hydrolysis plotted as a function of time. The rate of prothrombin activation at each time is an indication of fVa cofactor activity. For each condition, fVa activity was plotted as a function of time to yield a rate of fV activation. The results are presented as rates of fV activation relative to control (no aptamer). Each condition is an average of 4 separate experiments ± SEM.

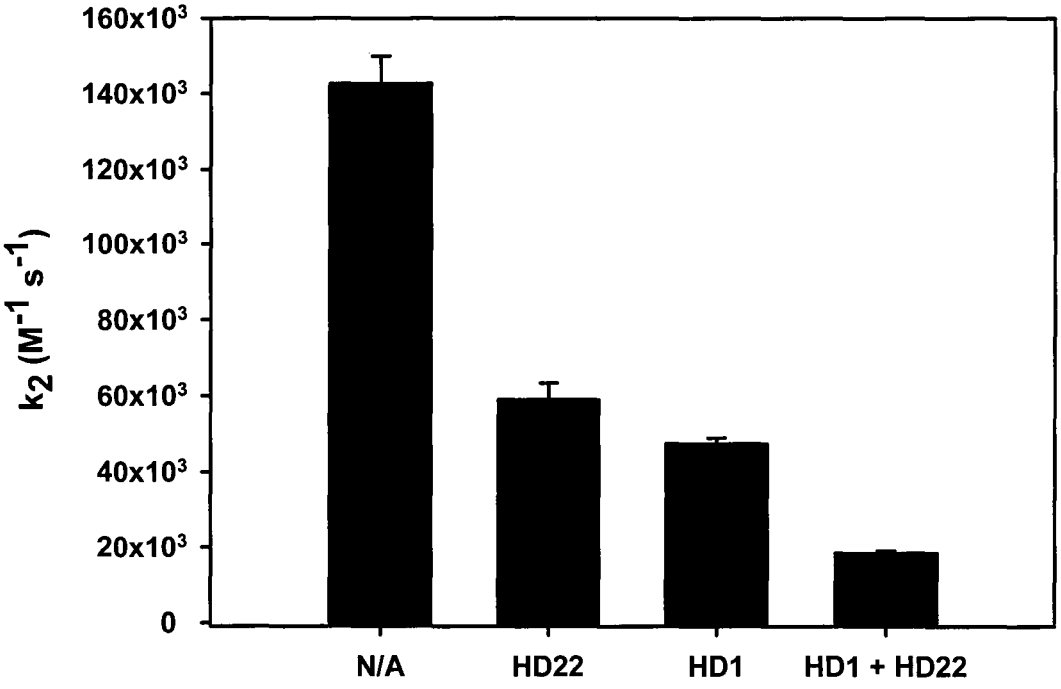

160x10³
140x10³
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
k2 (M-1 s-1)
N/A
HD22
HD1
HD1 + HD22

Figure 6.7: Effect of HD1 and HD22 on rate of thrombin inhibition by α_1-antitrypsin Pittsburgh

We tested the capacity of HD1 and HD22 to attenuate the rate of thrombin inhibition by α_1-antitrypsin Pittsburgh. Thus, 20 nM thrombin was incubated with 200 nM α_1-antitrypsin Pittsburgh at room temperature in the absence or presence of 5 μM HD1, HD22 or both in aptamer buffer. At various times (0-90 s), 200 μM Chz-Th was added to the reaction well to measure residual thrombin activity. Thrombin activity was monitored at room temperature for 10 min in a plate reader. The initial rate of substrate cleavage was divided by the specific activity of thrombin for Chz-Th, as determined in a separate experiment, to obtain the concentration of thrombin present at each time point, as mentioned above.

The natural log of the residual thrombin concentration was then plotted as a function of time to obtain the pseudo-first order rate constant for inhibition (k_1) as per the equation:

$$V_t/V_o = e^{-k_1 t}$$

where V_o represents thrombin activity at 0 s, V_t represents thrombin activity at time (t), and k_1 represents the apparent first-order rate constant. The second order rate (k_2) constant was then obtained by dividing the k_1 by the molar concentration of α_1-antitrypsin Pittsburgh (2×10^{-7} M).

Effects of HD22 on thrombin generation in plasma:

Rationale: Our preliminary data (above) as well as the results of others suggest that exosite 2 of thrombin may play a role during cofactor activation. As a result, we hypothesized that HD22 is an inhibitor of thrombin generation.

Work conducted: We tested the effectiveness of HD22 as an inhibitor of thrombin generation using the CAT assay. Increasing concentrations of HD22 caused an increase in the peak thrombin concentration, but did not affect the lag time (Figure 6.8). When the concentration of HD22 was increased in the presence of 5 μM HD1, there was a dose dependent increase in the peak thrombin concentration, similar to that produced by HD22 alone (Figure 6.9). These results suggest that the combination of HD1 and HD22 does not have a synergistic effect on thrombin generation compared with either aptamer alone.

Future studies: Modifications to the CAT assay conditions may be required to fully explore the role of exosite 2 in plasma thrombin generation. A recent report by Spronk *et al* demonstrated that Corn Trypsin Inhibitor (CTI), a fXIIa inhibitor that blocks contact activation, may be needed at the low tissue factor concentrations required to study thrombin feedback reactions (Spronk *et al.*, 2009). As a result, the above thrombin generation experiments (Figures 6.8 and 6.9) should be repeated in the presence of CTI. Furthermore, thrombin interactions with platelets are suspected to involve exosite 2 (De Cristofaro *et al.*, 2000). Therefore the effect of HD22 on thrombin generation in platelet-rich plasma should also be investigated. These experiments may be important in identifying exosite 2 of thrombin as a useful target for anticoagulant development.

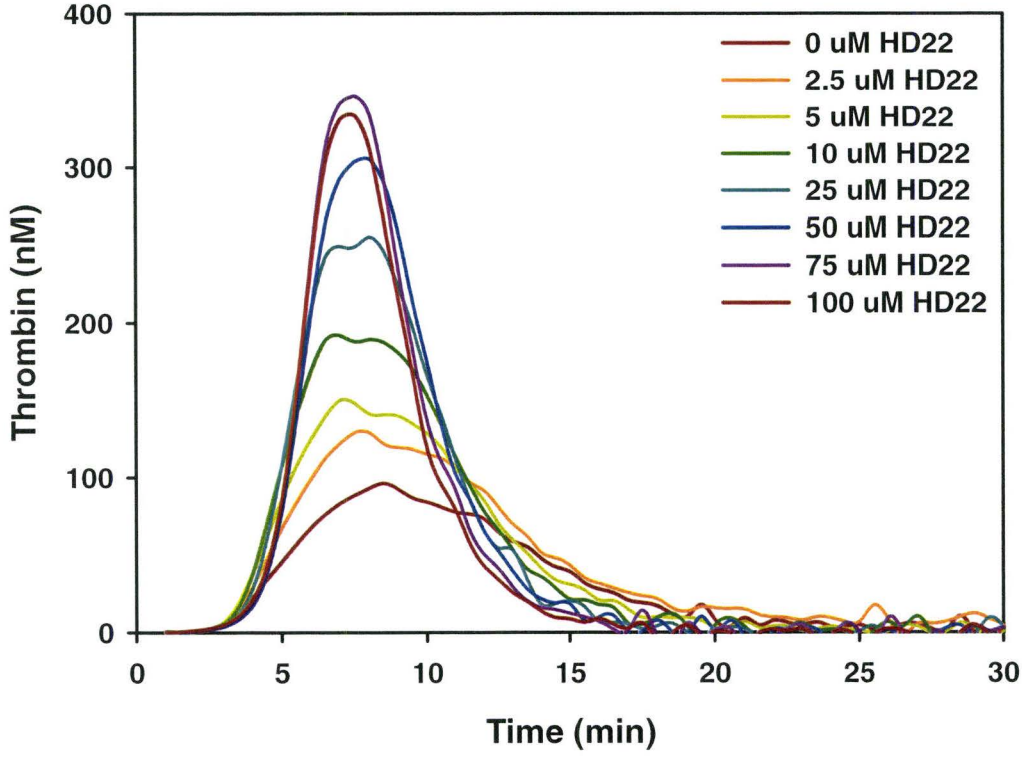

400
300
200
100
0
Thrombin (nM)
0
5
10
15
20
25
30
Time (min)
0 uM HD22
2.5 uM HD22
5 uM HD22
10 uM HD22
25 uM HD22
50 uM HD22
75 uM HD22
100 uM HD22

Figure 6.8: Effect of HD22 on thrombin generation in plasma

The effect of increasing concentrations (0-100 μM) of HD22 on thrombin generation in defibrinated normal plasma was determined using the CAT assay. Briefly, 100 μl of defibrinated plasma was added to round-bottom wells of a 96-well plate together with 30 μM PCPS and 0.5 pM TF from 1:2 diluted PPP Low reagent (Thrombinoscope, Maastricht, The Netherlands). The thrombin generation reactions were initiated by adding FluCa, a mixture of $CaCl_2$ and fluorescent substrate (Thrombinoscope). Appropriate reference wells containing thrombin calibrator were prepared for each condition and HD22 was added where appropriate. Plates were read on a Labsystems Fluoroskan Ascent FL plate reader (MTX Lab Systems, Inc, Vienna, VA) for 2 h at 30 s intervals at excitation and emission wavelengths of 360 nm and 460 nm, respectively. The thrombin concentrations were calculated and plotted versus time by instrument software. The data represent the average result of 2 separate experiments, each performed in duplicate.

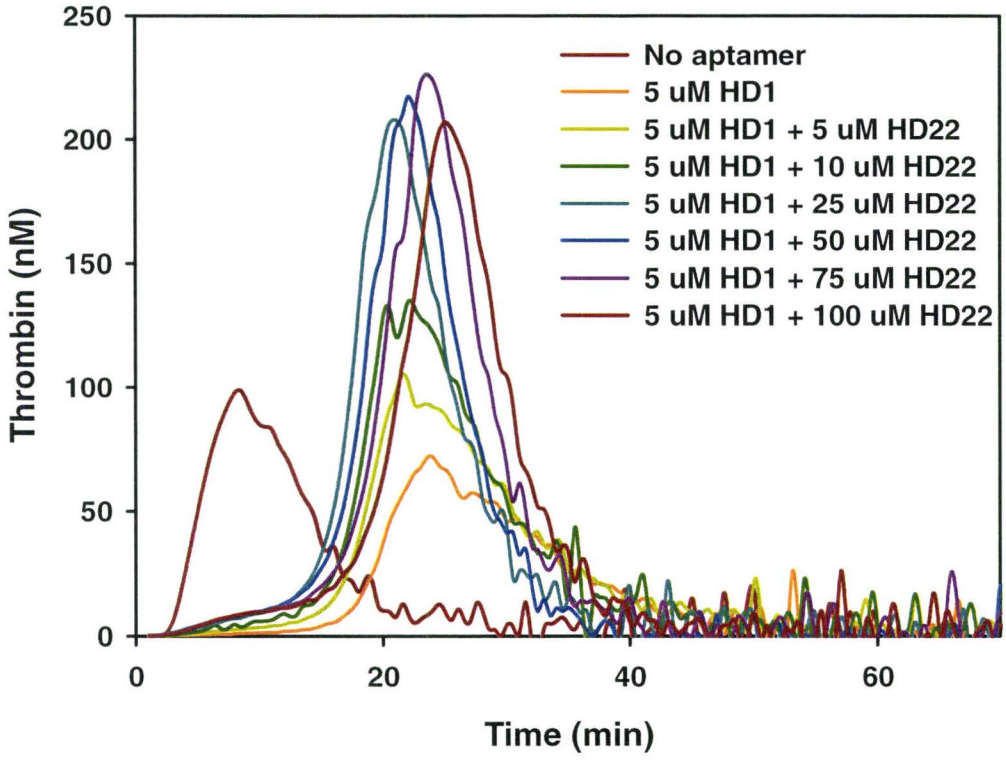

250
200
150
100
50
0
Thrombin (nM)
0
20
40
60
Time (min)
No aptamer
5 uM HD1
5 uM HD1 + 5 uM HD22
5 uM HD1 + 10 uM HD22
5 uM HD1 + 25 uM HD22
5 uM HD1 + 50 uM HD22
5 uM HD1 + 75 uM HD22
5 uM HD1 + 100 uM HD22

Figure 6.9: Effect of HD1 and HD22 on thrombin generation in plasma

The effect of increasing concentrations (0-100 μM) of HD22 in the presence of 5 μM HD1 on thrombin generation in defibrinated normal plasma was determined using the CAT assay. Briefly, 100 μl of defibrinated plasma was added to round-bottom wells of a 96-well plate together with 30 μM PCPS and 0.5 pM TF from 1:2 diluted PPP Low reagent (Thrombinoscope, Maastricht, The Netherlands). The thrombin generation reactions were initiated by adding FluCa, a mixture of $CaCl_2$ and fluorescent substrate (Thrombinoscope). Appropriate reference wells containing thrombin calibrator were prepared for each condition and HD22 and/or HD1 were added where appropriate. Plates were read on a Labsystems Fluoroskan Ascent FL plate reader (MTX Lab Systems, Inc, Vienna, VA) for 2 h at 30 s intervals at excitation and emission wavelengths of 360 nm and 460 nm, respectively. The thrombin concentrations were calculated and plotted versus time by instrument software. The data represent the average result of 2 separate experiments, each performed in duplicate.

Functional maturation of exosite 1 as recognized by fV and fVa:

Rationale: The development of exosites 1 and 2 during prothrombin activation has been described in terms of small molecule binding, namely $Hir^{54-65}(SO^{-}_{3})$ (Anderson and Bock, 2003) and HD1 (Kretz *et al.*, 2006) for exosite 1, and heparin (Husi and Walkinshaw, 1999) and HD22 for exosite 2 (Kretz *et al.*, 2006). However, these probes do little to describe the functional maturation of these exosites because, aside from heparin, they are not natural thrombin ligands. Studies have shown that thrombin binds fV and fVa equally (Dharmawardana *et al.*, 1999), similar to what was observed for HD1, whereas prothrombin only binds fVa (Dharmawardana *et al.*, 1999). Consequently, we expect that the development of the fVa recognition sites parallels maturation of the HD1 binding site, because fVa does not need to bind the hydrophobic pocket of exosite 1 to gain access to the active site. In contrast, we hypothesize that the development of the fV recognition site will parallel maturation of the $Hir^{54-65}(SO^{-}_{3})$ binding site because fV requires access to the active site of thrombin to become activated. Therefore fV is expected to bind the hydrophobic surface depression of exosite 1, like $Hir^{54-65}(SO^{-}_{3})$.

Future studies: Each of the prothrombin activation intermediates (Figure 1.2) could be biotinylated and immobilized on the surface of a streptavidin-coated biosensor chip for SPR studies. Next, fV or fVa could be passed over the immobilized proteins to establish binding affinities for each of the prothrombin-derivatives.

Define the residues on prothrombin that contribute to fVa binding:

Rationale: Our studies have demonstrated that HD1 and $Hir^{54-65}(SO_3^-)$ bind somewhat distinct subdomains of thrombin, which may explain their different capacities to bind prothrombin. Since fVa binds prothrombin, we hypothesize that fVa and HD1 share more similar binding sites on prothrombin than fVa and $Hir^{54-65}(SO_3^-)$.

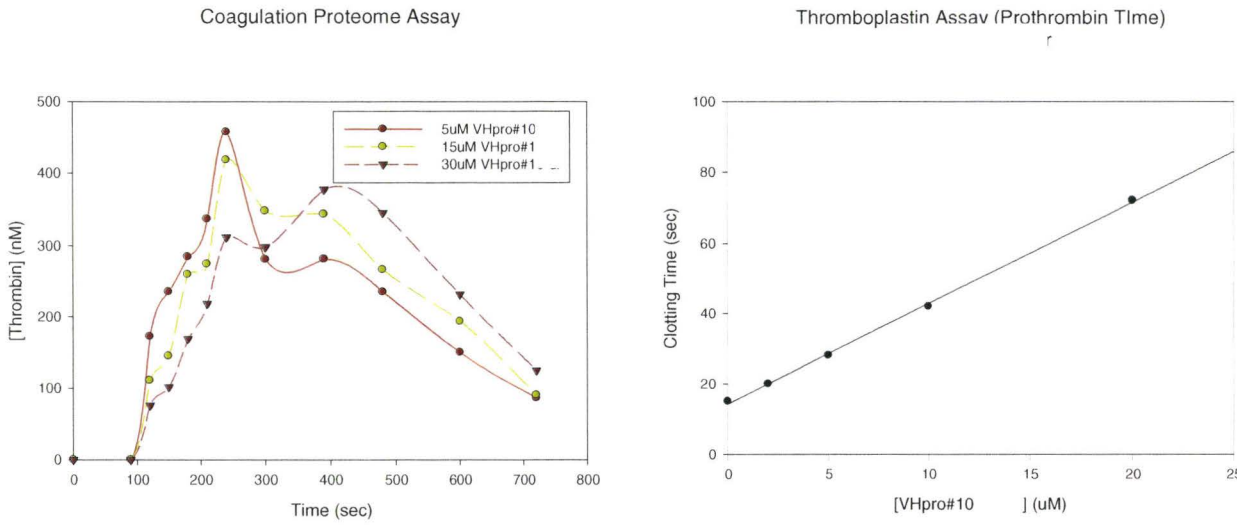
Mutational analysis identified the exosite 1 residues that mediate the thrombin-HD1 interaction (Tsiang *et al.*, 1995). We anticipate that the same residues that mediate thrombin's interaction with HD1 also are responsible for its interaction with prothrombin. As a result we have acquired a series of thrombin mutants from Dr. Timothy Myles, which demonstrate reduced binding to HD1. These mutants were supplied in the form of prothrombin-expressing cells lines, and are therefore well-suited for our studies.

Future studies: Surface plasmon resonance experiments can be done to measure the affinity of each prothrombin mutant to immobilized HD1 or $Hir^{54-65}(SO^-_3)$. These experiments will be useful in determining if the same residues that are important of HD1 binding to thrombin, are also important for its binding to prothrombin.

Those prothrombin mutants that exhibit reduced affinity for HD1 will then be tested for their capacity to bind fVa. Thus, prothrombin mutants will be immobilized to the SPR chip surface, and tested for their capacity to bind to fVa injected into the flow cell. Also, these prothrombin mutants could be tested in prothrombinase assays to determine the rate of activation in the absence and presence of fVa compared to WT control.

Appendix 6.1: Inhibition Activities of the VHpro Protein Using Human Coagulation Proteome and Prothrombin Time Assays

Note: This figure and the corresponding figure legend were supplied by Dr. Feng Ni of the BRI-NRCC and they appear in this thesis in the exact form they were provided to me.



Legend:

The sample is exactly the same for both the coagulation proteome and prothrombin time (PT) assays. There is not a clear dose-dependency for the delay of thrombin burst, but clearly a delay in thrombin peak level. This lack of burst delay for thrombin generation is consistent with prior experiments where the protein sample is extensively dialyzed to remove phosphate ions (>1000x). If the dialysis is only 50-100x, as in earlier experiments, there is a large delay effect, which may be explained by Ca++-chelating effect of residual phosphate. Note that peak levels of thrombin generation are consistently lower with prothrombin-specific ligands than with hirudin-based inhibitors of thrombin.

Conclusions

This thesis discusses the use of aptamers as anticoagulants, specifically the thrombin-binding aptamer HD1. In addition, we have used HD1 as a tool to study previously unexplored mechanisms of prothrombin activation and thrombin generation. Thus, HD1 binds to a mature site on exosite 1 of prothrombin, whereas the $Hir^{54-65}(SO_3^-)$ binding site is an under-developed region of exosite 1 on prothrombin. Because of its greater capacity to bind prothrombin, HD1 is a more potent inhibitor of prothrombin activation by prothrombinase than $Hir^{54-65}(SO_3^-)$. To take advantage of this difference, we compared HD1 and $Hir^{54-65}(SO_3^-)$ in thrombin generation assays demonstrate that thrombin feedback activation of fV contributes to the lag phase, whereas thrombin feedback activation of fVIII does not. In addition, we show that the rate of prothrombin activation by prothrombinase dictates the magnitude of the peak thrombin concentration, and prothrombin entry into prothrombinase contributes to the lag time. In order to fully explore the utility of HD1 as an anticoagulant, antiHD1 was developed and observed to fully reverse the anticoagulant activity of HD1. AntiHD1 preferentially binds to free HD1, but will dock to bound HD1 and slowly hybridize with it to release it from thrombin or prothrombin. The preceding studies fulfill the goals of this thesis by (a) determining the specificity of HD1 to thrombin, (b) fully characterizing the anticoagulant properties of HD1 in *in vitro* clotting and thrombin generation assays, (c) identifying prothrombin as a potentially useful target for anticoagulant development, and (d) development and characterization of the antidote to HD1, antiHD1.

Regardless of the initiator of the coagulation system, the prothrombinase complex must be assembled to rapidly generate the thrombin required to produce a hemostatic plug. As a result, anticoagulants that either target thrombin alone or prothrombinase alone have been developed. HD1 acts at the nexus of the coagulation system, inhibiting both prothrombinase and thrombin activities. As a result, HD1 is an effective anticoagulant that could be useful in the clinical setting.

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