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Factor Va Directs Catalysis by Factor Xa During Prothrombin Activation

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Factor Va Directs Catalysis by Factor Xa During Prothrombin Activation

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Factor Va Directs Catalysis by Factor Xa During Prothrombin Activation

MICHAEL A. BUKYS

Abstract

Hemostasis occurs through the controlled activation and inactivation of clotting factors resulting in the arrest of bleeding without blockage to the vasculature, while thrombosis is the undesired formation of vascular blood clots, which adversely affects millions of people annually. Clotting factors circulate in blood as inactive zymogens, which are proteolytically activated in response to vascular injury, assembled into enzymatic complexes and in turn activate additional coagulation factors culminating in the production of thrombin from the enzymatic complex prothrombinase. The prothrombinase complex is composed of the activated enzymatic component factor Xa (fXa) complexed to the activated cofactor portion factor Va (fVa) assembled on a membrane surface in the presence of calcium. Prothrombinase enzymatic activity results in the conversion of prothrombin, the inactive precursor, to thrombin, the active enzyme following two sequential proteolytic cleavages. Thrombin in turn converts fibrinogen, a soluble precursor, to fibrin, the insoluble product which composes the meshwork of a fibrin clot. Therefore, the regulation of prothrombinase will result in the inhibition of clot formation.

Incorporation of fVa into the prothrombinase complex increases the catalytic rate of fXa by five orders of magnitude and increasing kinetic studies have suggested that prothrombinase has two equilibrating forms which function in conjunction in the production of thrombin. The prothrombinase complex is unique in its function of
activating thrombin making it the ideal enzyme to target for regulating thrombin production, however the molecular events governing prothrombinase assembly and function are not well understood, and a better understanding of these events will provide novel targets for therapeutic compounds capable of regulating thrombin production. The long-term goal of this study is to determine the molecular interactions governing prothrombinase assembly/function, because the specific inhibition of thrombin formation will be realized through the disruption of prothrombinase assembly. Our central hypothesis is that discrete amino acid binding motifs present on fVa for the components of prothrombinase represent a potential source of anticoagulant molecules. Thus, defining binding regions present on fVa for fXa and or prothrombin is necessary in the development of specific inhibitors of prothrombinase that could be used in treatment of individuals with thrombotic tendencies. This proposal plans to define regions present in fVa to target disruption of prothrombinase assembly by addressing the following specific aims.

**Specific Aim 1:** Determine the relative contribution of the binding of fVa’s heavy chain to fXa in the overall function of prothrombinase.

**Specific Aim 2:** Determine the role of the carboxyl-terminal portion of the heavy chain of fVa in prothrombinase catalyzed activation of prothrombin.

**Specific Aim 3:** Determine the discrete amino acid residue(s)/region(s) present in the light chain of fVa which are involved in the recognition of fXa.
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CHAPTER I

INTRODUCTION

One of the most important systems within the human body is the vasculature, because all organs and tissues are dependent on it for survival. Blood transports all of the nutrients and removes all of the metabolic wastes from the tissues of the body. Therefore maintenance of the vascular system, termed hemostasis, is vital for survival. Hemostasis is the controlled arrest of bleeding and maintains vessel integrity with minimal blood loss. Hemostasis ensures the balance between the opposing undesired conditions of thrombosis and hemophilia. Thrombosis is the unneeded formation of a clot within the vasculature and can result in deep vein thrombosis (DVT), pulmonary embolism, stroke or heart attack (1). Hemophilia is uncontrolled bleeding and results in severe blood loss. Both thrombosis and hemophilia have contributing genetic components as well as acquired causes and better treatment methods are needed for both of these conditions.

The entire vasculature is lined with a single layer of endothelial cells which form the walls of the vessels and veins which normally function as an anticoagulant surface
ensuring no blockage to the vasculature occurs (2). An injury occurs when the endothelial lining of the vasculature is disrupted allowing the circulating blood to leak outside of the vasculature resulting in the blood being exposed to the sub-endothelial environment. The sub-endothelial environment has many procoagulant components within it which function to promote clot formation (3). Of these procoagulant factors collagen and the protein tissue factor are essential. Exposure of the blood to collagen results in the activation of platelets and exposure of the blood to tissue factor initiates the coagulation response (4,5). An injury to the vasculature triggers many other responses all of which result in blockage of the injured site as a means to minimize blood loss. The combination of all of these responses is termed hemostasis and is divided into two interconnecting processes termed primary and secondary hemostasis.

1.1 PRIMARY HEMOSTASIS

The initial response to vascular injury is termed primary hemostasis. Primary hemostasis involves two interrelated processes; first a narrowing of the injured vessel minimizes blood flow to the damaged area while the formation of an initial platelet plug seals the injured site (6). Vasoconstriction is the first response to an injury that occurs in a damaged blood vessel and is a narrowing of the vessel to restrict blood flow to the injured area resulting in a minimization of blood loss. Exposure of blood to the sub-endothelial environment causes platelets to adhere at the injured site resulting in the formation of a primary platelet plug. Exposure of platelets to the collagen present in the sub-endothelial environment stimulates the activation of platelets (7).

Activated platelets release the contents from their interior alpha and dense granules resulting in the secretion of the vasoconstrictors serotonin, prostaglandin and
thromboxane (8). An enzymatic flipping of inner leaflet phosphatidylserine phospholipids to the outer leaflet upon activation of platelets creates an enzymatic surface capable of sustaining the formation of enzymatic complexes composed of coagulation factors which are attracted to the negatively charged surface (9,10). The activation of platelets also results in the release of many molecules from within the platelet needed for clot formation, including fibrinogen, factor V and von Willebrand factor (11). All sub-endothelial tissues express the cellular membrane protein tissue factor which is exposed to the circulation when the endothelial layer is disrupted. The exposure of tissue factor to the circulation is the initiating event in the coagulation portion of hemostasis, termed secondary hemostasis (9,10). The initial platelet plug is a temporary blockage to the site of injury and is strengthened by the deposition of fibrin, the end product of the coagulation cascade. The polymerization of fibrin leads to the stabilization of the platelet plug needed to ensure hemostasis.

1.2 COAGULATION RESPONSE

Secondary hemostasis is also referred to as the coagulation cascade and culminates in the formation of fibrin deposition upon the initial platelet plug resulting in a stable blockage to the injured site (12, 13). Platelets are actively involved in the coagulation response known as secondary hemostasis because they generate the negatively charged catalytic surface needed for formation of all enzymatic complexes (3). The coagulation cascade is composed of the sequential activation of clotting factors culminating in the production of fibrin from the zymogen fibrinogen. Coagulation enzymes are serine proteases with highly specific substrate recognition determined by exosites distant from their active sites (14). Coagulation factors circulate in inactive forms that are
proteolytically activated in response to vascular injuries. Activated clotting factors assemble into sequential enzymatic complexes on the membrane surfaces provided by activated platelets (9,10,15). The association between activated membrane surfaces and the components of the enzymatic complexes are dependent on Gla domains. Gla domains are regions present within the proteins that have several gamma carboxylated glutamic acid residues (16). The gamma carboxylation of glutamic acid residues are vitamin K dependent post-translational modifications necessary for protease recognition of the membrane surface.

The initiating event in the coagulation cascade is the exposure of tissue factor to the circulation resulting in the formation of the extrinsic Xase complex (17). A small fraction of fVII circulates in an active form and will complex with tissue factor when it is exposed to the blood (9). The extrinsic Xase complex is formed when circulating factor VIIa comes in contact with tissue factor. The extrinsic Xase complex recognizes the substrates factor IX and factor X proteolytically converting them to their active forms factor IXa (fIXa) and factor Xa (fXa), respectively (18). However the extrinsic Xase complex recognizes the substrate fIX with a much higher affinity than it recognizes fX and consequently the production of fIXa is necessary, needed for formation of the intrinsic Xase complex, is necessary for efficient production of fXa.

The intrinsic Xase complex is formed when the enzymatic component fIXa, generated from the extrinsic Xase complex, comes in contact with its activated cofactor portion, factor VIIIa, on a catalytic surface provided by activated platelets (19). The importance of the intrinsic Xase complex in proper fibrin formation is demonstrated by the genetic disorder hemophilia which occurs in individuals missing the gene for fVIII. The intrinsic
Xase complex recognizes the substrate fX, with a far greater specificity than the extrinsic Xase complex and is thus obligatory for sufficient generation of the active form of fX and consequently normal fibrin formation. Formation of the intrinsic Xase complex results in the rapid generation of fXa, the enzymatic component of the prothrombinase complex which is responsible for the activation of prothrombin to its active form thrombin.

The prothrombinase complex is composed of the activated enzymatic component, fXa, associated with the activated cofactor portion, factor Va (fVa), on a catalytic surface supplied by activated platelets in the presence of calcium ions (20,21). Prothrombinase proteolytically activates prothrombin through two sequential activating cleavages at Arg\textsuperscript{320} and Arg\textsuperscript{271} to produce the enzyme product, thrombin. Thrombin is responsible for the conversion of fibrinogen, the soluble precursor, to fibrin the insoluble monomer which composes the meshwork that stabilizes the fibrin clot.

1.3 THROMBIN REGULATES ITS OWN PRODUCTION

Thrombin recognizes its substrates through exosites, these are regions exposed to plasma in the activate form of thrombin which are composed of extended basic amino acid residues that facilitate substrate recognition (22,23). Thrombin has several substrates which are recognized by its two anion binding exosites referred to as ABE I and ABE II. Thrombin interacts with the substrates thrombomodulin, fibrinogen, heparin cofactor II, PAR1, and the hirudin peptides through ABE I, while ABE II is involved in the recognition by thrombin with the substrates heparin cofactor II, protease nexin and antithrombin III. Both ABE I and ABE II have been implicated in interacting with the cofactors for the intrinsic Xase complex and the prothrombinase complex, fV and fVIII respectively (24).
The initial production of thrombin up-regulates thrombin production by activating the cofactors needed for the intrinsic Xase and the prothrombinase complexes, fVIII and fV respectively (25-27). The proteolytic activation of these zymogen cofactors by thrombin results in greatly increasing thrombin production, because the active cofactors greatly up-regulates the activity of the intrinsic Xase and the prothrombinase complexes. The increased fVIII generated for the intrinsic Xase complex results in a greatly increased fXa production coupled with a greatly increased fVa production produces a surge in the concentration of the prothrombinase complex and consequently greatly accelerates thrombin generation. Thrombin is the enzyme responsible for the conversion of fibrinogen, the inactive soluble zymogen, to fibrin, the insoluble meshwork of the fibrin clot. Once formed fibrin is attracted to the platelet plug mediated through platelet receptors and polymerizes resulting in the strengthen platelet plug. An additional way that thrombin is responsible for greatly accelerating a fibrin clot formation is by the activation of factor XIII. The active form of fVIII, fXIIIa, functions in cross linking the fibrin monomers that are deposited on the platelet plug as an additional way of stabilizing the fibrin clot (9).

The fact that thrombin also down-regulates the coagulation response by the proteolytic activation of protein C (PC) shows the tight regulation of the coagulation cascade. The active form of protein C (APC) is involved in down regulating thrombin production by the enzymatic degradation of the cofactors involved in the intrinsic Xase and prothrombinase complexes, fVIIIa and fVa respectively (28). As thrombin is generated it will complex with thrombomodulin, a protein present on the surface of the endothelial lining of the vasculature, resulting in the formation of the APCase enzymatic
complex (29,30). The APCase complex is responsible for the activation of PC. Once generated APC down regulates thrombin production through the proteolytic degradation of fVIIIa through three sequential cleavages at Arg$^{336}$, Arg$^{562}$ and Arg$^{740}$ resulting in the loss of the intrinsic Xase complex ability to generate fXa. The enzymatic degradation of fVa is also mediated by APC through three sequential proteolytic cleavages of fVa at Arg$^{506}$, Arg$^{306}$, and Arg$^{679}$ destroying the affinity of fVa for the prothrombinase complex.

1.4 PROTHROMBINASE REGULATES THROMBIN PRODUCTION

As previously described coagulation occurs by a series of limited enzymatic cleavages of sequential clotting factors culminating in the formation of the prothrombinase complex (20,21). Both the intrinsic and extrinsic pathways lead to the formation of the prothrombinase complex, also known as the common pathway because of its central role in thrombin production. Prothrombinase is unique in its function of activating prothrombin to generate thrombin. The prothrombinase complex consists of an assembly of activated clotting factor X (fXa) and activated clotting factor V (fVa) on a platelet surface in the presence of calcium ions (20,21). Factor Xa is the enzymatic portion of prothrombinase and is capable of catalyzing prothrombin activation by initially cleaving Arg$^{271}$ producing the two inactive intermediates, prethrombin 2 (residues 272-579), and fragment 1-2 (residues 1-271) (31). Prethrombin 2 is sequentially cleaved by membrane-bound fXa at Arg$^{320}$ to produce thrombin. This process proceeds through inactive intermediates and has an overall reaction rate insufficient for timely thrombin production.

The association of fVa with fXa in the prothrombinase complex causes a reversal of the observed cleavage pattern of prothrombin in addition to a 300,000 fold increase in the
Figure 1.1 – The activation of prothrombin. Pathway I - Membrane-bound fXa catalyzes prothrombin activation by initially cleaving at Arg<sub>271</sub> producing the inactive intermediate prethrombin 2 and fragment 1-2. Prethrombin 2 is then sequentially cleaved at Arg<sub>320</sub> to produce thrombin and fragment 1-2. Pathway II – In the presence of a fully assembled prothrombinase complex the rate of thrombin generation is greatly enhanced and the order of the prothrombin activating cleavages are reversed. Prothrombinase initially cleaves prothrombin at Arg<sub>320</sub> generating the active enzymatic intermediate, meizothrombin. Meizothrombin is then cleaved at Arg<sub>271</sub> to produce thrombin and fragment 1-2.
rate of thrombin production. Prothrombinase catalyzed activation of prothrombin proceeds through initially cleaving prothrombin after Arg\textsuperscript{320}, producing meizothrombin (residues 1-320 covalently attached through disulfide linkage to residues 321-579), an enzymatic active intermediate (32). Prothrombinase rapidly consumes the meizothrombin intermediate by catalyzing the additional activating cleavage at Arg\textsuperscript{271} producing thrombin and fragment 1-2 (33,34). In addition to the greatly accelerated rate of thrombin generation realized through prothrombinase as compared to membrane-bound fXa, the enzymatic intermediate meizothrombin is capable of activating additional clotting factors.

1.5 FACTOR Va INTERACTS WITH ALL COMPONENTS OF PROTHROMBINASE

The physiological importance of fVa is demonstrated by the increased cases of DVT attributed to a single point mutation in its gene and the fact that its loss results in severe hemophilia. The zymogen factor V (fV) has a MW of 330,000kDa and circulates through plasma at a concentration of 20nM. FV is composed of three homologous A domains; the A1 domain composed of residues 1-303, the A2 domain composed of residues 317-656 and the A3 domain composed of residues 1546-1877, an interconnecting B region composed of residues 710-1545 and two C domains; the C1 domain composed of residues 1878-2036 and the C2 domain composed of residues 2037-2196 (35). The zymogen fV has the domain composition of A1-A2-B-C1-C2-A3 and zymogen activation is realized through the removal of the interconnecting B-domain from the rest of the molecule. Both thrombin and meizothrombin activate fV through three sequential cleavages occurring after Arg\textsuperscript{709}, Arg\textsuperscript{1018} and Arg\textsuperscript{1545} resulting in an active cofactor, fVa,
Figure 1.2

The activation of the zymogen factor V occurs through three sequential thrombin mediated cleavages occurring at $\text{Arg}^{709}$, $\text{Arg}^{1018}$ and $\text{Arg}^{1545}$ resulting in the active form of the cofactor composed of a heavy chain derived from the carboxyl-terminal portion and a light chain derived from the amino-terminal portion. The heavy chain is composed of residues 1-709 while the light chain is composed of residues 1546-2196 and the two chains are associated with each other through calcium ions.
composed of an amino-terminal derived heavy chain (residues 1-709) of Mr 105,000kDa
and a carboxyl-terminal derived light chain (1546-2196) of a Mr of 74,000kDa. The
association of the heavy and light chains of fVa is non-covalent and dependent on the
presence of Ca\textsuperscript{2+} ions (36).

Both the heavy and light chains of fVa are needed in binding the components of the
prothrombinase complex. The heavy chain of fVa interacts with prothrombin through an
acidic region present at the carboxyl-terminal portion (37,38). The heavy chain of fVa is
also involved in recognition of fXa through residues present within the A2 domain at
positions 323-331 (39,40). It has been demonstrated that the light chain of the cofactor
bonds both the membrane surface and fXa. The recognition of the membrane surface by
the light chain of fVa occurs through residues present within the C2 domain and has been
thoroughly characterized (41-43). Previous studies have implicated the A3 domain of the
light chain of fVa in binding fXa, however little is known on which amino acid residues
are involved in the association (44-45).

1.6 THE INACTIVATION OF FVa

Due to the central role fVa plays in thrombin generation both the generation and
degradation of fVa is highly regulated and the existence of the active form of fV is very
transient because it is degraded shortly after it is generated. Thrombin not only up-
regulates fVa production, but starts the down regulation of fVa by activating protein C
(PC) the serine protease responsible for the enzymatic degradation of fVa (46). PC
circulates in plasma at a concentration of 60nM and is activated by an enzymatic complex
composed of thrombin and thrombomodulin (47). The activation of PC occurs through a
single proteolytic cleavage occurring at Arg\textsuperscript{169} to produce the active form, APC. APC
down regulates thrombin production by inactivating the cofactor fVa through three sequential cleavages occurring at Arg$^{506}$, Arg$^{306}$ and Arg$^{679}$ (28).

The importance of APC for the down regulation of thrombin production is illustrated through the commonly occurring fV$^{\text{LEIDEN}}$ mutation. The fV$^{\text{LEIDEN}}$ mutation has been correlated to an increased occurrence of DVT and results from a single point mutation in the gene encoding fV which causes a R506Q substitution in the protein (48). This substitution removes the APC mediated cleavage site within the A2 domain of fVa and consequently decreases the rate of APC mediated inactivation of fVa 10-fold (49). Increased occurrence of DVT is associated with the decreased ability of APC to degrade the active form of the fV$^{\text{LEIDEN}}$ mutation.
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CHAPTER II
INCORPORATION OF FACTOR Va INTO PROTHROMBINASE IS REQUIRED
FOR COORDINATED CLEAVAGE OF PROTHROMBIN BY FACTOR Xa

2.1 ABSTRACT

The interaction of fXa with fVa promotes prothrombinase assembly and is governed by two distinct regions within fVa. It has been demonstrated on several occasions that one important region governing the fXa/fVa interaction and proper prothrombinase assembly is present within the A2 domain of the heavy chain of fVa. We have used overlapping synthetic peptides representative of residues 307-348 of the A2 domain to screen their ability to inhibit prothrombinase activity in a fXa dependent manner. Peptide studies indicated amino acid residues 323, 324, 330 and 331 of the A2 domain are crucial for fVa recognition of fXa. The present study employs recombinant mutations within these residues consisting of $^{323}\text{DY}^{324} \rightarrow ^{323}\text{FF}^{324}$ (fVa$^{\text{FF}}$), $^{330}\text{DV}^{331} \rightarrow ^{330}\text{MI}^{331}$ (fVa$^{\text{MI}}$) and the combined mutation of $^{323}\text{DY}^{324}$ and $^{330}\text{DV}^{331} \rightarrow ^{323}\text{FF}^{324}$ and $^{330}\text{MI}^{331}$ (fVa$^{\text{FF/MI}}$). All recombinant molecules were assayed for their ability to accelerate fibrin clot formation in
clotting based assays. Kinetic studies determined $K_d$ values for the fVa/fXa interaction showing an approximate 4-5 fold decrease for the ability of fXa to interact with rfVa$^{FF}$ and rfVa$^{MI}$ and an approximate 50-fold decrease in the ability of fXa to interact with the rfVa$^{FF/MI}$ mutant. Determined $K_{cat}$ values generated for prothrombinase catalyzed reactions performed with fXa fully saturated with the rfVa$^{MI}$ and rfVa$^{FF}$ mutants demonstrated values comparable to rfVa$^{WT}$. While prothrombinase assembled with the combined rfVa$^{FF/MI}$ mutant demonstrated a severely decreased Kcat value when compared to rfVa$^{WT}$. The present study demonstrated drastic changes in the kinetic parameters governing prothrombinase assembled with the rfVa$^{FF/MI}$ molecule demonstrating the effects the loss of the fXa/fVa interaction has on the normal function of prothrombinase.

2.2 INTRODUCTION

The arrest of bleeding is dependent on the generation of thrombin from the enzymatic complex prothrombinase (1-3). Though the enzymatic component of prothrombinase, activated factor X (fXa) is capable of activating the zymogen prothrombin, the rate of this reaction is insufficient for timely thrombin generation in response to vascular injury. The incorporation of activated factor V (fVa) into the prothrombinase complex accelerates the rate of thrombin generation by five orders of magnitude, as well as reverses the order of the two activating cleavages that occur within prothrombin (3,4). It has been well-established that membrane-bound fXa initially cleaves prothrombin at Arg$^{271}$ resulting in a non-enzymatic intermediate, prethrombin 2 (residues 271-579 of prothrombin) which is then subsequently cleaved at Arg$^{320}$ to generate thrombin (4). The association of fXa with fVa, results in a reversal of these two
proteolytic events with initial prothrombin cleavage occurring at Arg$^{320}$ followed by cleavage at Arg$^{271}$ (3,5) This latter pathway to thrombin generation has an enzymatic intermediate, meizothrombin, and occurs at a greatly accelerated rate (6).

The zymogen factor V (fV) circulates in plasma at a concentration 20nM and has no cofactor effect before proteolytic processing resulting in the formation of the active form of fV. Both thrombin and fXa are capable of activating fV through three sequential cleavages occurring at Arg$^{709}$, Arg$^{1018}$ and Arg$^{1545}$ generating an amino-terminally derived heavy chain composed of two homologous A domains (A1-A2), a carboxyl-terminally derived light chain composed of an A domain followed by two C domains (A3-C1-C2) and the release of the interconnecting B domain (7,8). The association of the heavy and light chains of fVa is calcium dependent and necessary for the generation of a functional cofactor (9). The association of fVa with fXa is highly specific with a Kd of approximately 0.2nM and has been shown to be mediated through two distinct regions present within the A2 and A3 domains of fVa (9).

Experimental evidence for the A2 domain’s involvement in recognition of fXa has been demonstrated by many research groups. Experiments employing activated protein C (APC), an anticoagulant molecule which degrades the heavy chain of fVa, correlates the loss of the cofactor effect to the removal of the A2 domain from fVa (10,11). APC degrades fVa through three consecutive cleavages occurring within the heavy chain of fVa at Arg$^{506}$, Arg$^{306}$ and Arg$^{679}$ and completely inactivates the cofactor. Peptide studies of the A2 domain have shown that N42R (a synthetic peptide designed with the corresponding sequence from amino acid residues 307-348 of the heavy chain of fVa) is capable of mimicking the loss of function associated with APC degradation of
fVα by strongly inhibiting prothrombinase activity in a fXα dependent manner (11). Further peptide studies have indicated that residues 323-324 and 330-331 from fVα are involved in binding fXα and are required for optimal cofactor effect for the prothrombinase catalyzed activation of prothrombin (12,13). The present recombinant study was undertaken to identify the contribution of these residues to the overall function of the prothrombinase molecule.

2.3 EXPERIMENTAL PROCEDURES

Materials, Reagents and Proteins. L-α-phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL) and prepared as previously described (14) and quantified by assaying for phosphorus. The chromogenic substrate Spectrozyme-TH was purchased from America Diagnostica, Inc (Greenwich, CT). Human α-thrombin, prothrombin, human fXα and the fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (Dapa) were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The monoclonal antibody αhFV1 coupled to Sepharose, and the two monoclonal antibodies against factor V (against the heavy and the light chains respectively, αHFVHC#17 and αHFVLC#9) were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT). Diisopropyl fluorophosphates (DFP), O-phenylenediamine dihydrochloride (OPD), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Trizma, Coomassie Blue R-250 and factor V-deficient plasma were purchased from Sigma. Polyethylene glycol (Mr 8000) was purchased from J. T. Baker (Danvers MA). The secondary anti-mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). The chemiluminescent
reagent ECL+ was purchased from Amersham Biosciences. The thromboplastin reagent (recombinoplastin) used in clotting assays was purchased from Beckman (Fullerton, CA). The cDNA encoding fV was purchased from American Type Tissue Collection (ATCC 40515 pMT2-V, Manassas, VA). All restriction Enzymes were purchased from New England Biolabs (Beverly, MA). Human fV and fVa were purified and concentrated using methodologies previously described employing the monoclonal antibody αhFV#1 coupled to sepharose and heparin-sepharose respectively (11,15,16).

**Generation of Recombinant Mutations.** The recombinant fV mutations used throughout this series of experiments were generated using PCR based mutagenesis. The heavy chain mutations were generated using the primers 5'-GAAGAGGTGGTTCTTCTTCATTGCTGC-3' (sense) and 5'-GCAGCAATGAAGAAGGAAACCACCTTTGCTGC-3' (antisense) for the rfVaFF mutation, the primers 5'-CATTGCTGCAGAGATGAATGTTTGGGACTATGC-3' (sense) and 5'-GCATAGTCCAAATGATCATCTCTCAGCAATG-3' (antisense) were used to generate the rfVaMI mutation (the underlined portion of the primers are the mismatched bases). PCR reactions were performed in the presence of outer primers encompassing the upstream XcmI restriction site (5'-ACATCCACTACCGCAATATGAC-3') and the down stream Bsu361 site (5'-CCTCAGGCAGGAACACACCATGA-3'). PCR products were purified from agarose gels and subjected to a third PCR reaction performed in the presence of the purified products. The product from the third PCR reaction and pMT2-fV were both subjected to XcmI and Bsu361 digestion followed by a ligation reaction to insert the desired mutations. Ligation products were transformed into chemically
competent bacteria cells and colonies were screened for the presence of the desired insert through DNA sequencing.

*Generation of Recombinant Proteins.* All of the respective mutagenic plasmids were transiently transformed into Cos 7 cells using the transformation reagent fugene 6. Cos 7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and incubated at 37°C in the presence of 5% CO₂ atmosphere. Transfection was performed on plates approximately 70% confluent by the addition of 2 µg of fugene 6 treated DNA per plate. Plates of transfected Cos 7 cells were incubated for 48 hours before being washed twice with a phosphate buffered saline solution followed by incubation in the presence of VP-SFM medium supplemented with 5mM L-glutamine. Harvesting of the cultured VP-SFM medium was performed for 4 consecutive 24 hour intervals and either concentrated or frozen for future use.

*Quantification of Recombinant fV Molecules within the Harvested Medium.* Harvested medium from the transfected Cos 7 cells were concentrated by centrifugation on YM-30 centrics to a final volume of approximately 1 ml followed by three consecutive washes with trizma buffered saline solution containing 5mM CaCl₂. ELISA specific for fV was performed on the washed concentrated medium containing the fV heavy chain mutants. Briefly, 96-well plates were coated with a polyclonal anti-fV antibody diluted in a carbonate buffer and incubated overnight at 4°C, ELISA plates were then washed three times with a trizma buffered saline (TBS) solution containing 5% tween 80 followed by an hour incubation at 37°C in the presence of 5% milk diluted in TBS + 5% tween 80. Serial dilution of the concentrated harvested medium containing the respective rfV heavy chain mutants were incubated along side a plasma derived fV serial
dilutions performed in triplicate with concentrations ranging from 0.01\(\mu\)g to 10\(\mu\)g as a standard. The monoclonal anti-human fV antibody, \(\alpha\)-hfV\#17, was incubated on the washed ELISA plate at a concentration of 5\(\mu\)g/ml for an hour at 37\(^{\circ}\)C followed by an additional hour incubation of the ELISA plate in a 1:2000 dilution of a secondary anti-mouse coupled with HRP antibody. ELISA plates were developed using OPD (5mg) tablets dissolved in 12ml 0.1M citric acid and 0.1M Na\(_2\)HPO\(_4\) for 15 minutes before the addition of 150\(\mu\)l of 2.5M H\(_2\)SO\(_4\) to stop the reaction. The standard was graphed as log of the concentration of the plasma derived fV vs. the absorbance at 490nm. The absorbance of the serial dilutions of the concentrated medium was compared to that of the standard for the determination of the concentration of the respective rfV molecules.

*Activation of Recombinant Molecules.* Throughout the research, activation of the respective recombinant molecules was accomplished by incubating the molecules with thrombin at a 1/100 (enzyme to substrate) ratio for ten minutes at 37\(^{\circ}\)C. Addition of 2mM DFP, a specific serine protease inhibitor, was performed to stop the activation reaction followed by 10 minute incubation on ice before use. Experiments showed that these conditions assured complete activation of the cofactor with minimal thrombin degradation of the activated molecule. However, for all protein preparations western blots using monospecific antibodies recognizing the heavy and light chains of fVa respectively was performed to confirm the generation of the active cofactor.

*Assaying the Activity of the Recombinant Molecules.* Equivalent concentrations of activated recombinant proteins were used in a discontinuous assay measuring thrombin production monitored through chromogenic substrate. Reaction mixtures were composed of 1.4\(\mu\)M prothrombin being activated by 5nM fXa in the presence of 0.5nM fVa species,
5μM Dapa, 10μM PCPS in a HEPES buffered saline solution containing 5mM CaCl$_2$ at a pH of 7.40. Reactions were initiated by the addition of prothrombin and aliquots of the reaction mixture were removed at various time points during the first 5 minutes of the reaction and quenched in a two-fold volume of a HEPES buffer containing EDTA within a 96-well plate. Plates were then incubated with a final concentration of 0.4mM chromogenic substrate, Spectrozyme TH, for 15 minutes before optical density readings were taken at 405nm. Control reactions performed in the absence of fVa and with plasma derived fVa were performed to establish minimal and optimal parameters for this series of experiments. The reactions employing the recombinant proteins fVa$^{WT}$, fVa$^{FF}$, fVa$^{MI}$ and fVa$^{FF/MI}$ were performed simultaneously with the control reactions to demonstrate that any decrease in prothrombinase activity was due to the mutations present in the fVa$^{FF}$, fVa$^{MI}$ and fVa$^{FF/MI}$ molecules. Reactions with increased concentrations of fVa$^{FF}$, fVa$^{MI}$ and fVa$^{FF/MI}$ molecules were performed to determine if normal prothrombinase function could be restored using higher concentrations of the respective recombinant mutant molecules. All reactions had aliquots removed from the mixture prior to initiation of the reaction to determine if any increase in absorption was due to the reaction mixture. All experiments were graphed mOD verses time (seconds) using Prism graphing software.

**Determination of Kinetic Parameters Governing Mutant Molecules** – Assays measuring the kinetic parameters consisted of prothrombinase assembled in the presence of prothrombin and measured the appearance of thrombin by the secondary event of cleavage of a chromogenic substrate. This discontinuous assay was monitored at several time points within the first minute or two of the reaction. A limiting amount of fXa was titrated with increasing concentrations of fVa to determine the apparent Kd value for the
interaction. Titrations were performed in 96 well plates and measured the initial velocities of product formation from the different concentrations of the titrant (fVa). Time points of the different reactions were stopped by addition of EDTA. Prism graphing software was used to graph the data and generate apparent $k_d$ values for the titrations. In all cases mutants were compared to wild type and plasma derived fVa and measurements were done in triplicate with different protein purifications. The obtained $k_d$ values were used to determine proper ratios of the respective fVa mutants to fXa used in subsequent prothrombin titration reactions ensuring similar concentrations of the assembled prothrombinase complex were used. The degree of fXa saturation was calculated using equation 1 (17),

$$[E_b] = (i\cdot[T_t] + [E_t] + K_d) - \sqrt{((i\cdot[T_t] + [E_t] + K_d)^2 - 4i\cdot[T_t]\cdot[E_t])}/2$$  

(Eq. 1)

where $E_b$ is the percentage of bound enzyme, $i$ is the stoichiometry between fXa and fVa (i.e., 1), $T_t$ is the total concentration of the titrant (i.e., fVa), $E_t$ is the total concentration of the enzymatic component (i.e., fXa), and $K_d$ is the observed disassociation constant determined from the respective fVa titration.

Prothrombin titrations were performed to determine the apparent $K_m$ and $K_{cat}$ values for the fully assembled enzymes. Prothrombin titrations were performed in 96 well plates and reactions consisted of limiting concentrations of fXa in the presence of saturating concentrations of fVa as determined by $k_d$ values obtained from the fVa titrations. Prism graphing software was used to determine measurements of the initial velocity of the varied prothrombin concentrations and used to generate a secondary graph measuring maximum velocity and km.
Effects of the Mutant fVa Molecules in the Activation of Prothrombin. – Monitoring prothrombin consumption using SDS PAGE allowed for the determination of prothrombin cleavage patterns. Prothrombinase assembled with the various fVa mutants was used in prothrombin activating reactions monitored through SDS-PAGE followed by coomassie blue staining. Aliquots of reaction mixtures at various time points were placed in 0.2M acetic acid to stop the reaction followed by preparation for SDS-PAGE as previously described (13).

Measurement of Clotting Times – One stage and two stage clotting times were performed with all of the recombinant molecules at identical concentrations. One-stage clotting assay consisted of addition of the procofactor (fV) into fV deficient plasma, while two stage clotting assay consisted of the activated cofactor (fVa) being added to fV-deficient plasma. In both cases coagulation was initiated by the addition of the thromboplastin reagent and time to clot formation measured and transformed into specific activity. Deficiency in mutant molecules to accelerate fibrin clot formation in fV-deficient plasma implies a functional defect. All purified protein preparations were assayed in triplicate by this manner.

Measurement of the Interaction of the Two Mutation Sites – Since the point mutations generated in the recombinant fVa molecules decrease the stability of the enzymatic complex, prothrombinase, the change in the free energy of the system can be evaluated to determine whether the two sets of mutations (i.e., rfVa\textsuperscript{MI} and rfVa\textsuperscript{FF}) energetically interact (18-20). The following general equation is used to define the change in prothrombinase function due to the mutations (state B) as compared to the wild type fVa molecule (state A),
\[ \Delta \Delta G_B = \Delta \Delta G_B - \Delta \Delta G_A \]  
(Eq. 2)

Evaluation of each of the three recombinant mutants presented in this study was accomplished by comparison to the rfVa^{WT} using the following equation,

\[ \Delta \Delta G_{A \rightarrow B} = \frac{R T \ln [(k_{cat}/K_m)_B/(k_{cat}/K_m)_A]} \]  
(Eq. 3)

where \( R \) is the universal gas constant (2 cal·K^{-1}·mol^{-1}), \( T \) is the temperature in Kelvin (298K), \( k_{cat} \) is the turnover number, and \( K_m \) is the Michaelis-Menten constant for prothrombinase assembled with the various recombinant fVa molecules. The free energy of the interaction between two mutations can be calculated with the following general equation,

\[ \Delta \Delta G_{\text{int}} = \Delta \Delta G_{A \rightarrow B/C} - \left( \Delta \Delta G_{A \rightarrow B} + \Delta \Delta G_{A \rightarrow C} \right) \]  
(Eq. 4)

where the term \( A \rightarrow B \) and \( A \rightarrow C \) represent either of the two mutations (i.e., rfVa^{MI} and rfVa^{FF}), the term \( A \rightarrow B/C \) represents the double mutant (i.e., rfVa^{MI/FF}) and the term \( \Delta \Delta G_{\text{int}} \) reflects energy exchange between the individual amino acids being studied. A \( \Delta \Delta G_{\text{int}} > 0 \) implies that the two mutation sites interact with each other reducing the catalytic efficiency of the enzyme and a \( \Delta \Delta G_{\text{int}} < 0 \) implies that the two mutation sites interact with each other in a fashion that enhances the catalytic efficiency of the enzyme.

2.4 RESULTS

Previous studies have indicated that binding of the heavy chain of fV to fXa involves residues 323, 324, 330 and 331. In order to determine the contribution of these residues to the activity of prothrombinase a recombinant study mutating these residues was undertaken. PCR based mutagenesis was performed, as described in the method section to generate the following mutants; rfVa^{FF} (\(^{323}DY^{324} \rightarrow ^{323}FF^{324}\)), rfVa^{MI} (\(^{330}DV^{331} \rightarrow ^{330}MI^{331}\)) and the combined mutation of rfVa^{FF/MI} (\(^{323}DY^{324}, ^{330}DV^{331} \rightarrow ^{323}FF^{324}\).
Figure 2.1

- No mutation
- $E^{323} \rightarrow F$ and $Y^{324} \rightarrow F$
- $E^{330} \rightarrow M$ and $V^{331} \rightarrow I$
- $E^{323} \rightarrow F/Y^{324} \rightarrow F$ and $E^{330} \rightarrow M/V^{331} \rightarrow I$

factor $Va^{w^t}$

factor $Va^{FF}$

factor $Va^{MI}$

factor $Va^{FF/MI}$

---

Figure 2.1 – Mutations created for determination of heavy chain binding.

Previous studies using peptides representative of the heavy chain sequences of fVa were used to determine binding regions. Recombinant proteins mutated at amino acid residues indicated in binding of fXa were generated to further test the results generated using synthetic peptides. The four recombinant proteins used throughout this study were: rfVa$^{WT}$ (no mutations), rfVa$^{FF}$ (residues $^{323}EY^{324} \rightarrow FF$), rfVa$^{MI}$ (residues $^{330}EV^{331} \rightarrow MI$) and the combined mutant rfVa$^{FF/MI}$ ($^{323}EY^{324} \rightarrow FF$ and $^{330}EV^{331} \rightarrow MI$ both within the same construct).
Figure 1 shows a schematic outlining the mutations described above demonstrating the sites where the mutations were introduced.

To investigate the possibility that the introduced mutations had a detrimental effect on zymogen activation, SDS PAGE followed by Western blot was performed on all of the recombinant mutations. Figure 2 shows Western blotting of the activated recombinant molecules. Lane 1 is plasma derived fV that has been activated through incubation with thrombin and serves as a control sample demonstrating the relative positions of both the heavy and light chains of the cofactor. Lanes 3 – 6 are samples of the recombinant molecules used throughout the study after incubation with thrombin to generate their active forms, they are arranged rfVa\textsuperscript{WT}, rfVa\textsuperscript{FF}, rfVa\textsuperscript{MI} and rfVa\textsuperscript{FF/MI} respectively. All of the recombinant molecules generated both heavy and light chains following thrombin mediated activation, though there was a light chain degradation product detected within all of them.

To assess the ability of the respective recombinant mutants to function as a cofactor, identical concentrations of the recombinant mutants (500pM) were assembled into a prothrombinase complex and assayed for their ability to generate thrombin. Figure 3 demonstrates that identical concentrations of plasma derived fVa (open circles) and rfVa\textsuperscript{WT} (closed circles) both accelerate the ability of fXa to generate thrombin to the same degree showing that there is no functional defect in the wild type cofactor molecule. Both the rfVa\textsuperscript{FF} (inverse filled triangles) and the rfVa\textsuperscript{MI} (filled triangles) displayed diminished thrombin formation, though the prothrombinase assembled with rfVa\textsuperscript{MI} was more deficient in producing thrombin. Combining both mutations sites in the rfVa\textsuperscript{FF/MI}, produced a cofactor that was greatly defective in promoting thrombin production when
**Figure 2.2** – Western Blot of Recombinant Heavy Chain Mutations. Western blotting with two monoclonal antibodies was performed on all of the recombinant molecules used throughout this series of experiments. Lane 1 shows activated plasma derived fV, lane 3 shows rfVa^{WT}, lane 4 shows rfVa^{FF}, Lane 5 shows rfVa^{MI} and lane 6 shows rfVa^{FF/MI}. Arrows indicate position of the heavy and light chain of the active cofactor as well as a low molecular weight degradation product which occurred upon thrombin incubation of the various recombinant molecules.
Figure 2.3

**Figure 2.3 – Activity assays comparing recombinant fVα molecules.** The cofactor activity of the various recombinant fVα molecules were assayed in the presence of 5nM fXa as described in the experimental procedures. The various reactions shown above are: filled circles, 500pM rfVα\textsubscript{WT}; open circles, 500pM plasma derived fVα; filled inversed triangles, 500pM rfVα\textsubscript{FF}; filled triangles, 500pM rfVα\textsubscript{MI}; open squares, 1nM rfVα\textsubscript{FF/MI}; open triangles, 2nM rfVα\textsubscript{FF/MI}; open inverse triangles, 5nM rfVα\textsubscript{FF/MI}; open diamonds, 5nM fXa in the absence of cofactor; stars, 5nM fXa in the presence of concentrated medium from plates of Cos 7 cells that were not transfected. All reactions were performed in triplicate and error bars are representative of standard deviation. Graphs were generated using Prisma 2.01 graphing software.
assembled into prothrombinase, however thrombin production was still accelerated over lipid-bound fXa in the absence of a cofactor molecule (open diamonds). Increasing the concentration of the rfVa^{FF/MI} to 5nM increased the ability of prothrombinase assembled with rfVa^{FF/MI} (open triangles) to generate thrombin demonstrating a rate similar to that of prothrombinase assembled in the presence of 1nM rfVa^{MI} (filled diamonds). Since all the recombinant molecules were assayed in concentrated medium the possibility that the observed increased catalyst by fXa was a result of an unknown factor present in the cultured medium had to be assayed. This possibility was assayed using an equal volume of concentrated cultured medium from plates of Cos 7 cells that were not transfected. The addition of the conditioned medium (stars) to a reaction mixture containing lipid-bound fXa displayed similar capabilities for thrombin generation as that of the reaction performed by lipid-bound fXa in the absence of fVa (open diamonds).

Since the rfVa^{FF}, rfVa^{MI} and rfVa^{FF/MI} were generated to diminish fVa association with fXa, activity assays in the presence of an increased concentration of the recombinant cofactors were performed to determine if the impaired binding could be overcome by an increased cofactor concentration. Figure 4 shows the results of activity assays performed with 1nM rfVa^{WT}, rfVa^{FF} and rfVa^{MI} in the presence of 5nM fXa. What we observe was that both the rfVa^{FF} (circles) and the rfVa^{MI} ability to accelerate thrombin generation by fXa increased in a fashion similar to that of 500pM rfVa^{WT} cofactor. This demonstrates that the weaken interaction between the recombinant mutants and fXa can be partially compensated for by increasing the concentration of the cofactor. Since the rfVa^{FF/MI} combined mutations within the interactive site for fXa displays the greatest diminished activity in assays measuring thrombin generation, the concentration of this mutant was
Figure 2.4 – Activity assays with increased concentrations of the recombinant fVa molecules. Initial rates of thrombin generation were determined at ambient temperature employing 5nM fXa in activity assays as described in the experimental procedure section. The various reactions are: filled squares, 1nM rfVa\textsuperscript{WT}; filled circles, 1nM rfVa\textsuperscript{FF}; filled diamonds, 1nM rfVa\textsuperscript{MI}; filled triangles, 10nM rfVa\textsuperscript{FF/MI}. All reactions were performed in triplicate and error bars are representative of standard deviation. Graphs were generated using Prisma 2.01 graphing software.
increased to 10nM (Diamonds) to determine if the fXa interaction could be restored. What is observed when 10nM rfVa\textsuperscript{FF/MI} is assembled into prothrombinase the ability to accelerate the generation of thrombin is restored to a rate similar of the prothrombinase assembled with 1nM rfVa\textsuperscript{MI}. Since the ability of the rfVa\textsuperscript{FF/MI} to properly interact with fXa is compromised figure 4 also demonstrates the contribution of the light chain to the fVa/fXa interaction and the importance of the heavy chain of fVa for the proper interaction with fXa.

To visualize the activation pattern of prothrombin by prothrombinase assembled with the various recombinant mutants SDS PAGE was employed. Figure 5 compares the ability of prothrombinase assembled with the various recombinant molecules to activate prothrombin as compared to the control reactions catalyzed with prothrombinase assembled with plasma-derived fVa (panel B) and lipid-bound fXa in the absence of cofactor (panel A). The reaction catalyzed by lipid-bound fXa demonstrate the inability of timely prothrombin activation in the absence of cofactor, showing little consumption of prothrombin after 1 hour of incubation and initial cleavage occurring at Arg\textsuperscript{271} as evident by the formation of the prethrombin 2 intermediate. When 10nM plasma derived fVa is added into the reaction mixture all of the prothrombin is consumed within the first 10 minutes of the reaction demonstrating the optimal rate of thrombin generation and a reversal of the cleavage pattern with an initial activating cleavage occurring at Arg\textsuperscript{320} as evident by the generation of fragment 1·2A. Panel C shows the reaction catalyzed by prothrombinase assembled with 10nM rfVa\textsuperscript{WT} and demonstrates a rate of thrombin production comparable to that of the prothrombinase assembled with plasma-derived fVa. Reactions catalyzed with prothrombinase assembled with 10nM rfVa\textsuperscript{FF}, rfVa\textsuperscript{MI} or
Figure 2.5 – SDS PAGE analysis of prothrombinase assembled with recombinant fVa mutants. To investigate the effects of the various rfVa mutants in the activation of prothrombin, prothrombinase was assembled under the following conditions: 0.5nM fXa, 10nM fVa species (when present), 10μM DAPA, 20μM PCPS and 1.4μM prothrombin. Panel A shows the activation of prothrombin by lipid-bound fXa in the absence of fVa. Panel B shows the activation of prothrombin by prothrombinase assembled with plasma derived fVa. Panel C-F shows the activation of prothrombin assembled with the recombinant fVa molecules, rfVa\textsuperscript{WT}, rfVa\textsuperscript{FF}, rfVa\textsuperscript{MI} and rfVa\textsuperscript{FF/MI} respectively. Reaction mixtures were assembled in trizma buffered saline solutions in the presence of 5mM CaCl\textsubscript{2} and aliquots were removed and prepared for SDS PAGE before and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 300, 360, 600, 1200, 1800 and 3600 seconds after the addition of fXa. The prothrombin derived fragments are labeled: II, prothrombin (residues 1-579); F1·2A, fragment 1·2 A chain (residues 1-320); F1·2, fragment 1·2 (residues 1-271); P2, prethrombin 2 (residues 271-579); B, B chain of thrombin (residues 321-579) and F1, fragment 1 (residues 1-155).
Table 2.1

<table>
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<tr>
<th>Enzyme</th>
<th>Prothrombinase Consumption</th>
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<tr>
<td></td>
<td>moles consumed/moles fXa</td>
<td></td>
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<td>Lipid-Bound fXa</td>
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<td>6.7 ± 0.03</td>
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<tr>
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<td>0.92 ± 0.08</td>
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Table 2.1 – Densitometry scanning of SDS PAGE in figure 5 of the rate of prothrombin consumption. Densitometry scanning of the acrylamide gels presented in figure 5 was performed to determine the rates of prothrombin consumption by prothrombinase assembled in the presence of the respective fVα mutants. Rates are presented as moles of prothrombin consumed per mole of fXa and the fold decrease in the observed rates as compared to the reaction performed with prothrombinase assembled with fVα<sup>WT</sup> is presented.
rfVa$^{FF/MI}$ are shown in panels D – F respectively. All of the recombinant mutants when assembled into a prothrombinase complex generate initial prothrombin activating cleavage at Arg$^{320}$ as evident by generation of fragment 1·2A, though production of the meizothrombin intermediate is delayed for all of them. The prothrombinase catalyzed reactions assembled with rfVa$^{FF}$ and rfVa$^{MI}$ took over 30 minutes to consume all of the prothrombin present within the reaction, while prothrombinase assembled with rfVa$^{FF/MI}$ was incapable of consuming all of the prothrombin present within the reaction mixture even after an hour incubation.

Densitometry scanning of SDS PAGE presented in figure 5 was performed to generate rates of prothrombin consumption presented in table 1. The optimal rate of prothrombin consumption under the condition employed throughout this series of experiments was 15.4 moles of prothrombin consumed per second per mole of fXa. The fold decrease of prothrombin consumption for the prothrombinase assembled with rfVa$^{FF}$ and rfVa$^{MI}$ mutants were approximately the same being 3.2 and 2.3 respectively. While the fold decrease for the prothrombinase assembled with rfVa$^{FF/MI}$ was much greater being 16.7 times slower in generating thrombin as compared to the prothrombinase assembled with the rfVa$^{WT}$.

To assay the defective association between the recombinant fVa mutants with fXa in a more quantitative fashion the recombinant proteins were titrated against a limiting concentration of fXa to determine the apparent Kd values for the association of the fVa mutants with fXa. Figure 6 shows the results of this kinetic study using an activity based assay monitored through the cleavage of the chromogenic substrate S-2338, specific for thrombin. Prizma graphing software was used to generate the graph presented in figure 6.
Figure 2.6

**Figure 2.6 – Titration of rFVa molecules.** To determine the apparent $k_d$ values for the recombinant rFVa molecules for fXa, fVa titrations were performed. Increasing concentrations of the respective rFVa molecules were assembled into prothrombinase in the presence of 15pM fXa, 10μM DAPA, 20μM PCPS and 1.4μM prothrombin. Initial velocity of thrombin generation was assayed through the cleavage of the chromogenic substrate S-2238 and quantified by comparison to a thrombin standard. The empirically derived $k_d$ values are presented in table 2.
Table 2.2 – Apparent $k_d$ values for the rfVa molecules for fXa. The empirically derived $k_d$ values for the association of the various rfVa molecules with fXa. Values were determined by using Prisma 2.01 Graphing software to analyze the data presented in figure 6.

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<th>fVa Species</th>
<th>$K_d^{\text{App}}$</th>
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<tr>
<td>fVa$^{\text{WT}}$</td>
<td>$0.18 \pm 0.02$</td>
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<tr>
<td>fVa$^{\text{FF}}$</td>
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</tbody>
</table>
as well as the apparent Kd values that are presented in table 2. Comparison of the apparent Kd values for the rfVaWT and the plasma-derived fVa demonstrate similar values for the interaction between these two molecules with fXa and is consistent with Kd values presented throughout the literature. The rfVaFF and rfVaMI displayed impaired association with fXa as demonstrated by the 4-fold and 10-fold increase in the apparent Kd values respectively. When the two sets of mutations within the A2 domain of fVa are combined within the rfVaFF/MI mutant the increase in the apparent Kd value becomes 60-fold showing a greatly hampered interaction between the rfVaFF/MI mutant and fXa.

The determination of the apparent Kd values for the recombinant mutants enabled us to study the effects of the induced mutations to the overall function of the prothrombinase complex. The degree of saturation of fXa in the presence of a known concentration of the respective recombinant fV molecule was determined using the apparent Kd value. Equation 1 allows us to predict the percentage of fXa associated with the respective recombinant fVa molecules depending on the concentration of fVa used in the reaction thus allowing us to match the concentration of the prothrombinase complex (17). Matching the concentration of the prothrombinase complex, fXa associated with the respective recombinant fVa mutants, allowed us to measure the catalytic efficiencies of the different prothrombinase molecules. Figure 7 shows the results of matching concentrations of prothrombinase assembled with the various recombinant fVa molecules titrated with prothrombin as a means of measuring the apparent Kcat and Km values for the different prothrombinase molecules. The prothrombinase assembled with rfVaWT displayed Km and Kcat values comparable to that of the prothrombinase assembled with plasma derived fVa demonstrating that there was no functional defect with the wild type
Figure 2.7 – Determination of apparent $K_m$ and $K_{cat}$ values. Prothrombinase assembled with saturating concentrations of the respective rfVa molecules as determined by the fVa titrations performed in figure 6 were performed to determine the respective $K_m$ and $K_{cat}$ values for each of the recombinant mutations. Reactions mixtures composed of 0.5nM fXa, 20µM PCPS, 10µM DAPA and 5nM rfVa$^{WT}$ (filled squares), 15nM rfVa$^{WT}$ (filled circles), 5nM plasma derived fVa (filled triangles), 5nM rfVa$^{FF}$ (open diamonds), 5nM rfVa$^{MI}$ (opened circles), and 30nM rfVa$^{FF/MI}$ (filled inverse triangles) were initiated with increasing concentrations of prothrombin. Initial velocities were measured and quantified against a thrombin standard to determine the thrombin (nM) produced per minute for the respective prothrombinase molecules.
Table 2.3

<table>
<thead>
<tr>
<th>Species</th>
<th>$V_{\text{Max}}^{\text{App}}$</th>
<th>Km</th>
<th>Kcat</th>
<th>Kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nMIIa/Min</td>
<td>μM</td>
<td>Min$^{-1}$</td>
<td>S$^{-1} \cdot$ M$^{-1}$</td>
</tr>
<tr>
<td>fV$a_{\text{Plasma}}$</td>
<td>9.72 ± 0.6</td>
<td>0.12 ± 0.03</td>
<td>1944 ± 120</td>
<td>2.7 X 10$^8$</td>
</tr>
<tr>
<td>fV$a_{\text{WT}}$</td>
<td>9.85 ± 0.4</td>
<td>0.16 ± 0.02</td>
<td>1970 ± 80</td>
<td>2.1 X 10$^8$</td>
</tr>
<tr>
<td>fV$a_{\text{FF}}$</td>
<td>9.03 ± 0.2</td>
<td>0.1 ± 0.01</td>
<td>1806 ± 40</td>
<td>3.0 X 10$^8$</td>
</tr>
<tr>
<td>fV$a_{\text{MI}}$</td>
<td>10.2 ± 0.24</td>
<td>0.14 ± 0.01</td>
<td>2040 ± 48</td>
<td>2.4 X 10$^8$</td>
</tr>
<tr>
<td>fV$a_{\text{FF/MI}}$</td>
<td>2.03 ± 0.07</td>
<td>0.15 ± 0.02</td>
<td>406 ± 14</td>
<td>4.5 X 10$^7$</td>
</tr>
</tbody>
</table>

Table 2.3 – Empirically derived kinetic values from prothrombin titrations presented in figure 7. The apparent $V_{\text{max}}$, $K_m$ and $K_{\text{cat}}$ values as determined using Prisma 2.01 Graphing software to analyze the prothrombin titrations presented in figure 7 are shown for all of the prothrombin titrations performed.
protein. These values were also consistent with the kinetic values well established throughout the literature. Both the rfVa\textsuperscript{FF} and the rfVa\textsuperscript{MI} demonstrated similar K\textsubscript{cat} and K\textsubscript{m} values when the concentration of the cofactor molecules were increased to produce similar concentration of fXa associated with the respective mutant cofactor. However prothrombinase assembled with saturating concentrations of the rfVa\textsuperscript{FF/MI} molecule failed to recover the catalytic efficiency displayed by the other molecules used throughout this study. This deficiency is the result of the decreased K\textsubscript{cat} displayed by prothrombinase assembled with the rfVa\textsuperscript{FF/MI} and can be attributed to the weakened interaction of the rfVa\textsuperscript{FF/MI} molecule with fXa. Table 3 shows the kinetic values obtained from figure 7 and demonstrate that none of the recombinant fVa molecules, when assembled into prothrombinase, affect the Km of the enzyme as evident by the small variance in the apparent Km values obtained. However the effect of the K\textsubscript{cat} of the enzyme due to the weakened fVa/fXa interaction caused by the mutations within the proposed binding site varied greatly, suggesting that the proper interaction of the A2 domain of fVa with fXa is a prerequisite for optimal catalytic efficiency of prothrombinase.

The kinetic study provided within figures 6-7 and tables 1-2 enable the calculation of the thermodynamic cycle presented in figure 8. As figure 8 demonstrates, the interaction between the two mutated regions demonstrated an additive effect suggesting that the two mutated regions (i.e., rfVa\textsuperscript{FF} and rfVa\textsuperscript{MI}) have a cooperative effect between each other when the heavy chain of fVa associates with fXa. The overall exchange in free energy between the two altered sites as determined by equation 4 is 1.21 kcal/mol demonstrating that the two sites interact with each other when the associating with fXa.
Figure 2.8 – Thermodynamic cycle for prothrombinase assembled with the various rfVa molecules. The $\Delta \Delta G_{int}$ is the free energy of the interaction between the $^{323}\text{EY}^{324}$ and $^{330}\text{EV}^{331}$ residues and was calculated according to equation 4 as presented in the materials and methods section.
To assay the effects of the decreased fVa/fXa interaction on the individual prothrombin activating cleavages independently of each other, SDS PAGE was employed on prothrombinase catalyzed activation of the recombinant prothrombin mutants rP2-II (R^{155} \rightarrow A, R^{284} \rightarrow A and R^{320} \rightarrow A) and rMZ-II (R^{155} \rightarrow A, R^{271} \rightarrow A and R^{284} \rightarrow A) which are only capable of being cleaved at Arg^{271} and Arg^{320} respectively. Figure 9 shows the results from reactions using the different prothrombin mutants as substrate for the enzymes; lipid-bound fXa (panel A), prothrombinase assembled with plasma-derived fVa (panel B), prothrombinase assembled with rfVa^{WT} (panel C), prothrombinase assembled with rfVa^{FF} (panel D), prothrombinase assembled with rfVa^{MI} (panel E) and prothrombinase assembled with rfVa^{FF/MI} (panel F). Prothrombinase assembled with rfVa^{WT} (panel C) displayed comparable catalysis of both of the prothrombin mutants when compared to prothrombinase assembled with plasma-derived fVa. Prothrombinase assembled with the rfVa^{FF} (panel D) and rfVa^{MI} (panel E) displayed slower consumption of both rP2-II and rMZ-II approximately 8 and 5 fold respectively as shown in table 4. While the prothrombinase assembled with rfVa^{FF/MI} showed the greatest deficiency when using rP2-II or rMZ-II as substrates. Densitometry scanning of SDS PAGE presented in figure 9 was performed and the results are presented in table 4 establishing that the fold decrease in the ability of prothrombinase assembled with rfVa^{FF} or rfVa^{MI} respectively to cleave both substrates rP2-II and rMZ-II are similar. Interestingly the reactions performed in the presence of prothrombinase assembled with rfVa^{FF/MI} showed a dissimilar fold decrease in its ability to cleave rP2-II (an 8.8-fold decrease) or rMZ-II (a 17.6-fold decrease) suggesting that the interaction of the A2 domain with fXa is more important for the catalyzed cleavage at Arg^{320}.
Figure 2.9 – Prothrombinase assembled with the various rfVa mutants were assayed for their ability to cleave the recombinant prothrombin mutants rP2-II and rMZ-II which are only capable of being cleaved at one of the two activating sites as described in the experimental procedures section. Reactions mixtures were composed of by 0.5nM fXa, 10nM fVa species (when present), 10μM DAPA, 20μM PCPS and 1.4μM of the respective recombinant prothrombin molecules in a HEPES buffered saline solution. In all cases lanes 1-9 shows reactions using the rMZ-II mutant as the substrate, while lanes 10-18 are reactions which employed the rP2-II mutant as the substrate. Panel A shows the results from a fXa catalyzed reactions, panel B shows the results from a reactions catalyzed by prothrombinase assembled with plasma derived fVa, panel C shows the reactions catalyzed by prothrombinase assembled with rfVaWT, Panel D shows the reactions catalyzed by prothrombinase assembled with rfVaFF, panel E shows the reactions catalyzed by prothrombinase assembled with rfVaMI and panel E shows the reactions catalyzed by prothrombinase assembled with rfVaFF/MI. For all of the reactions lanes 1-9 and 10-18 are aliquots removed from reactions mixtures and quenched at the following time points after the addition of fXa: 0 min (before the addition of fXa), 0.5 min, 1 min, 2.5 min, 4 min, 6 min, 10 min, 20 min and 30 min. The prothrombin derived fragments are the same as explained in the figure legend to figure 5.
Table 2.4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>rMZ-II (Cleavage at Arg320) (moles consumed • sec-1 • mole factor Xa-1)</th>
<th>Fold decrease</th>
<th>rP2-II (Cleavage at Arg271) (moles consumed • sec-1 • mole factor Xa-1)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xa alone</td>
<td>0.07 ± 0.11</td>
<td>--</td>
<td>0.05 ± 0.08</td>
<td>--</td>
</tr>
<tr>
<td>Prothrombinase with wild type</td>
<td>24.6 ± 2.8</td>
<td>--</td>
<td>2.72 ± 0.23</td>
<td>--</td>
</tr>
<tr>
<td>Prothrombinase with factor Va^{FF}</td>
<td>2.8 ± 0.2</td>
<td>8.8</td>
<td>0.33 ± 0.04</td>
<td>8.2</td>
</tr>
<tr>
<td>Prothrombinase with factor Va^{Mi}</td>
<td>4.6 ± 0.5</td>
<td>5.3</td>
<td>0.52 ± 0.04</td>
<td>5.2</td>
</tr>
<tr>
<td>Prothrombinase with factor Va^{FF/Mi}</td>
<td>1.4 ± 0.1</td>
<td>17.6</td>
<td>0.31 ± 0.06</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Table 2.4 – Densitometry scanning of the consumption of recombinant prothrombin molecules. The SDS PAGE presented within figure 9 were subjected to densitometry scanning to determine the rate of prothrombin consumption by prothrombinase assembled in the presence of the various fVa molecules. The fold decrease of the rate of consumption of the recombinant prothrombin molecules were measured by comparison to the rate of prothrombin consumption by prothrombinase assembled with rfVa^{WT}. 
2.5 DISCUSSION

Previous studies demonstrated that activated protein C (APC) degrades fVa in a fashion resulting in the removal of the A2 domain from fVa and a loss of the fVa/fXa interaction (21,22). Synthetic peptides designed to study this region have shown that N42R (a synthetic peptide designed with the corresponding sequence from residues 307-348 of the heavy chain of fVa) is capable of mimicking the APC degradation of fVa loss of function by strongly inhibiting prothrombinase activity (23). Further peptide studies have shown that residues 323-324 and 330-331 in fVa are involved in binding fXa and are required for the coordinated cleavage of prothrombin by fXa (11-13). All of the above studies imply the involvement of the A2 domain of fVa in the recognition fXa. The present study explores these residues involvement in both the assembly and function of prothrombinase through the generation of recombinant molecules with mutations in these residues.

The elevated $K_d$ values for prothrombinase assembled with the rfVa$^{FF}$, rfVa$^{MI}$ and rfVa$^{FF/MI}$ molecules illustrate a decreased interaction between the respective molecules with fXa and demonstrate the necessity of these residues for the proper interaction of fVa with fXa. All of the recombinant molecules when assembled into prothrombinase displayed similar $K_m$ values indicating that the induced mutations had no detrimental effect on the ability of the different prothrombinase molecules in recognizing the substrate prothrombin and showing the selective nature of the damaging effects of the mutated sites. Both the rfVa$^{FF}$ and rfVa$^{MI}$ mutants were able to fully recover their catalytic efficiency when their respective concentrations were increased to compensate for their weakened interaction with fXa. This was not the case when the two mutated
regions were joined together into a single construct, the catalytic efficiency of prothrombinase assembled with rfVa$^{FF/MI}$ was not restored when fXa was fully saturated with the rfVa$^{FF/MI}$. The loss of multiple binding sites for fXa could have caused a damaging in fVα’s ability to function as a cofactor molecule for fXa, one that could not be compensated for by simply increasing the concentration of rfVa$^{FF/MI}$, though the possibility that the induced mutations altered the conformation of fV in a manner that was detrimental to function of prothrombinase cannot be excluded as an opposing cause of the result presented within this study.

Presently this recombinant study demonstrates that decreasing the affinity of fVα for fXa causes a decrease in the ability of prothrombinase to efficiently generate thrombin, suggesting that the interaction of fVα with fXa increases the catalytic capability of fXa. This study shows that residues 323, 324, 330 and 331 of the A2 domain of fVα are important for the association between fVα and fXa and that the loss of any of these sites will result in a weakened interaction between fVα and fXa that can be compensated for by increasing the concentration of the cofactor molecule, but a loss of all of these residues result in a cofactor molecule that is incapable of interacting properly with fXa and consequently incapable of properly functioning as a cofactor.
2.6 REFERENCES


CHAPTER III

A CONTROL SWITCH FOR PROTHROMBINASE

3.1 ABSTRACT

Previous studies have demonstrated that residues 695-698 of the A2 domain of the heavy chain of fVα are involved in recognition of prothrombin by prothrombinase and that a synthetic peptide representative of this region strongly inhibits prothrombinase by substrate depletion. We have shown that a pentapeptide representative of this region (DYDYQ (D5Q)) strongly inhibits thrombin generation by prothrombinase by specifically inhibiting the initial prothrombinase catalyzed cleavage of prothrombin at R\textsuperscript{320}. In contrast, the interaction of D5Q with prothrombin accelerates the rate of initial cleavage at R\textsuperscript{271} of prothrombin in reactions catalyzed by lipid-bound fXα, however thrombin production is inhibited due to a slower rate of the subsequent cleavage at R\textsuperscript{320} of the prethrombin2/fragment 1-2 intermediate. Recombinant prothrombin molecules only capable of being cleaved at one of the activation sites were used to confirm results obtained with plasma-derived prothrombin; rMZ-II (R\textsuperscript{155} → A, R\textsuperscript{271} → A and R\textsuperscript{284} → A)
and rP2-II (R^{155} \rightarrow A, R^{284} \rightarrow A and R^{320} \rightarrow A). Comparison of prothrombinase inhibition by D5Q with the hirudin derived peptides demonstrated dissimilar inhibitory patterns. While the hirudin peptides strongly inhibited the prothrombinase catalyzed activation of prethrombin 1 in the absence and the presence of fragment 1, D5Q demonstrated little inhibition in these reactions. In contrast the prothrombinase catalyzed activation of the prethrombin 2/fragment 1-2 intermediate was strongly inhibited in the presence of D5Q, while the hirudin peptide showed little inhibition in this reaction. In conclusion, D5Q strongly inhibits thrombin generation by both prothrombinase and lipid-bound fXa in a manner independent from the hirudin peptides.

3.2 INTRODUCTION

The maintenance of blood vessel integrity is dependent on the timely generation of thrombin in response to vascular injury. Thrombin is generated through two sequential activating cleavages occurring at Arg^{271} and Arg^{320} catalyzed by membrane-bound activated factor X (fXa). Though membrane-bound fXa catalyzes both of the activating cleavages on the zymogen prothrombin, membrane-bound fXa is inefficient in this process in the absence of activated factor V (fVa). The association of fXa with fVa on a membrane surface in the presence of divalent metal ions results in the formation of the prothrombinase complex (1). The formation of the prothrombinase complex results in a reversal of the activating cleavages of prothrombin accompanied by a 300,000 fold increase in the rate of thrombin formation (2).

Membrane-bound fXa activates prothrombin through initial activating cleavage occurring at Arg^{271} resulting in the enzymatic inactive intermediate prethrombin 2 (residues 272-579) and fragment 1-2 (residues 1-271). Subsequent cleavage of
prethrombin 2 at Arg\textsuperscript{320} produces the active enzyme thrombin. The incorporation of fVa into the prothrombinase complex directs catalysis by fXa in a manner resulting in a reversal of the order of the activating prothrombin cleavages (3). The prothrombinase complex initial cleavage of prothrombin occurs at Arg\textsuperscript{320} generating the meizothrombin intermediate (4). Meizothrombin is an enzymatic intermediate that is quickly consumed by prothrombinase by cleavage at Arg\textsuperscript{271} to generate thrombin. Figure 1 shows the two pathways to thrombin generation as catalyzed by membrane-bound fXa and the prothrombinase complex. The molecular details on fVa’s involvement in prothrombinase are not well understood.

The zymogen factor V (fV) is composed of three homologous A domains, a B domain and two homologous C domains that are arranged in an A1-A2-B-A3-C1-C2 fashion (5). The zymogen fV is composed of 2196 amino acids and has a relative molecular weight of 330,000. Proteolytic activation of fV occurs through three sequential thrombin catalyzed cleavages occurring after residues Arg\textsuperscript{709}, Arg\textsuperscript{1018} and Arg\textsuperscript{1545} resulting in an amino-terminally derived heavy chain composed of residues 1-709 associated through Ca\textsuperscript{2+} to the carboxyl-terminally derived light chain composed of residues 1546-2196 (6,7). The activated cofactor, fVa, is involved in recognition of all of the components of prothrombinase. It is well established that both the heavy and light chains of fVa interact with fXa and that the light chain of fVa is involved in interacting with activated membrane surfaces (8-10). It has also been demonstrated on several occasions that the carboxyl-terminal region of the heavy chain is involved in substrate recognition by directly interacting with prothrombin (11-14).
The carboxyl-terminal region of the heavy chain of fVa has a highly acidic region that shares homology with hirudin, a known anticoagulant molecule that inhibits prothrombin activation by directly interacting with prothrombin (15). We have recently shown that a synthetic pentapeptide from this region with the sequence DYDYQ (representative of residues 695-699) strongly inhibits thrombin generation by prothrombinase (16). This peptide has also been shown to directly inhibit thrombin mediated activation of fV (17). A recent recombinant study deleted the carboxyl terminal region of the heavy chain of fV and showed the cofactor had decreased specific activity in clotting assays (~80%), while activity based assays suggested an increased rate of thrombin production (~120%) (18).

3.3 EXPERIMENTAL PROCEDURES

_Materials, reagents and proteins_ – L-α-phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL) and prepared as previously described (19) followed by quantification by a phosphorus assay as previously described (20). The chromogenic substrate Spectrozyme-TH, normal reference plasma and fII deficient plasma were purchased from America Diagnostica, Inc (Greenwich, CT). Human α-thrombin, prothrombin, prethrombin 1, prethrombin 2, fragment 1-2, fragment 1, fragment 2, and the fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (Dapa), were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The monoclonal antibody αhFV1 coupled to Sepharose, and the two monoclonal antibodies against factor V (against the heavy and the light chains respectively, αHFV_HC#17 and αHFV_LC#9) were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington,
VT). Human fXa was purchased from Enzyme Research Laboratories (South Bend, IN). Diisopropyl fluorophosphates (DFP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), Trizma base, Coomassie Blue R-250, Hirudin 54-65 (sulfated hirudin, Hir\textsuperscript{54-65} (SO\textsubscript{3})), hirudin 54-65 (Hir\textsuperscript{54-65}, non-sulfated hirudin) and factor V-deficient plasma were purchased from Sigma. Polyethylene glycol (Mr 8000) was purchased from J. T. Baker (Danvers MA). The chemiluminescent reagent ECL\textsuperscript{+} and heparin-Sepharose were from Amersham Biosciences. The thromboplastin reagent (recomboplatin) used in the clotting assays was purchased from Beckman (Fullerton, CA). The pentapeptide DYDYQ (D5Q) and its sulfated version (D5Q1,2) were custom synthesized by New England Peptide Inc. (Gardner, MA) and by American Peptide Company (Sunnyvale, CA). Human fV and fVa were purified and concentrated using methodologies previously described employing the monoclonal antibody αhFV\textsuperscript{#1} coupled to sepharose and heparin-sepharose (21-23). The recombinant prothrombin molecules, rP2-II and rMZ-II, were prepared and purified as previously described (24,25).

**Procedures for Working with Synthetic Peptides** – All peptides were custom made by New England Peptide and stored in a desiccator at -20\textdegree C for future use. Samples weighing between 3 – 5mg of peptides were diluted in 1ml of water and thoroughly mixed followed by centrifugation at 14,000RPM for 5 minutes to remove any particles not in solution. Aliquots of every peptide preparation were frozen for sequence analysis to confirm the concentration and composition of the peptides, and all peptides solutions were used within a week or stored at -20\textdegree C for future use. The concentration of the peptides was calculated based on the weight (mg) of peptide divided by the volume of water (ml) dissolved corrected for the percent peptide concentration provided by New
England Peptide. All calculated concentrations were confirmed through amino-terminal sequence analysis of every peptide solution.

Measuring D5Q Inhibition of Prothrombin Activation – Activity assays measuring the inhibitory potential of D5Q were monitored through cleavage of a chromogenic substrate specific for thrombin. Incubation of prothrombin with increasing concentrations of D5Q or an equivalent volume of buffer was performed for 10 minutes before addition into reaction mixtures. Since D5Q inhibition has been shown to act on the substrate, reactions were performed in the presence and absence of fVa to assay the effects the interaction of prothrombin with the peptide has on prothrombinase and fXa catalyzed reactions independently. In both cases reaction mixtures contained 20μM PCPS and 10μM DAPA in a HEPES buffer containing 5mM Ca$^{2+}$. Results are presented as percent control with respect to the control reactions performed in the absence of D5Q. All reactions were performed in triplicate and the graphing program Prisma 2.01 was used for interpreting data and generating graphs of percent inhibition against D5Q concentration.

SDS Page Analysis of D5Q Inhibition on Prothrombin Activation. – The effects D5Q inhibition has on prothrombin activation was visualized and monitored through SDS PAGE of both prothrombinase and fXa catalyzed reactions. Titration of prothrombinase and fXa catalyzed reactions with increasing concentrations of D5Q demonstrated the concentration dependence of D5Q inhibition and indicated which pathway to prothrombin activation was used or inhibited. Measurements of prothrombin consumption were performed through densitometry scanning using U-SCAN-IT software. In all cases prothrombin was incubated with increasing concentrations of D5Q for 10 minutes at ambient temperature before addition into reaction mixtures containing 20μM
PCPS, 10μM DAPA and 10nM fVa when present. Aliquots of the reaction mixture before the addition of fXa were removed and placed in two fold volume of 0.2M acetic acid for a sample before the reaction was initiated. Reactions were initiated by the addition of fXa and additional time points were removed along the course of the reaction and placed into 0.2M acetic acid to stop the reaction. A centrifugation was used to concentrate all samples followed by reconstituted in 0.1M Trizma buffer, 1% BME and 1% SDS at pH 6.8. SDS PAGE was stained with coomassie blue and densitometry scanning was performed to measure the rates of prothrombin consumption throughout both series of titrations. Comparison of the rates obtained from inhibited reactions to control reactions performed in the absence of D5Q were performed.

_D5Q Inhibition With Respect to Individual Prothrombin Cleavages_ – The effects of D5Q inhibition on the individual prothrombin activating cleavages were determined independently of each other using the recombinant prothrombin molecules rMZ-II and rP2-II which are only capable of being cleaved at R³²⁰ and R²⁷¹ respectively. SDS PAGE monitored both prothrombinase and fXa catalyzed reactions titrated with D5Q using either rMZ-II or rP2-II as substrate for the reactions. This allowed us to measure the inhibitory effect of D5Q on both prothrombin activating cleavages independently. Incubation of the respective prothrombin mutants with D5Q was performed at ambient temperature for 10 minutes before addition into the reaction mixtures. Aliquots of 5μg were removed from reaction mixture before and at specific time intervals after the addition of fXa and placed in two fold volume of 0.2M acetic acid followed by concentration in a centrifugation for preparation of SDS PAGE. Stained gels were subjected to
densitometry scanning to measure the rate of rMZ-II or rP2-II consumption and compared to the rates of the uninhibited control reactions performed at the same time.

*Plasma-Derived Prothrombin Fragments* - Since the activation of prothrombin consists of more than one activating cleavage, the physiological substrates of prothrombinase include all of the normally occurring prothrombin derivatives. The prothrombin fragments used throughout this series of experiments were purchased from HTI and consist of prothrombin (residues 1-579), prethrombin 1 (residues 156-579), prethrombin 2 (residues 272-579), fragment 1·2 (residues 1-271), fragment 1 (residues 1-155) and fragment 2 (residues 156-271). All proteins were dialyzed in trizma buffered saline solution composed of 20mM trizma at pH 7.40 containing 5mM CaCl$_2$. Their concentrations were determined using the extinction coefficients ($\varepsilon_{1\%280nm}$) and molecular weights as follows; prothrombin 13.8 and 72,000, prethrombin 1 17.8 and 50,500, prethrombin 2 17.3 and 37,000, fragment 1·2 11.2 and 34,500 and thrombin 18.3 and 36,700. Protein aliquots were frozen after dialysis for future use. Reactions using the substrate prethrombin 1 were performed in the presence and the absence of fragment 1 and reactions using the substrate prethrombin 2 were performed in the presence and absence of fragment 1, fragment 2 and fragment 1·2. The substrate concentration for all reactions was at least twice that of their reported Km to ensure maximum velocity of the uninhibited enzyme and specific reaction conditions are given in the figure legends.

*Activity Assays for Determining Thrombin Production* – Measurements of the rate of activation of prothrombin and prothrombin derived fragments were performed in a discontinuous assay monitored through cleavage of a chromogenic substrate specific for thrombin (Spectrozime TH, America Diagnostica). Reactions mixtures were prepared in
a HEPES buffered saline (20mM HEPES) solution containing 5mM CaCl₂ and initiated by the addition of fXa. Several time points from the first two minutes of the reaction were removed and immediately quenched in a HEPES buffered saline solution containing EDTA to stop the reaction. All reactions were compared to a thrombin standard for quantitative purposes. Initial velocities were determined using Prism graphing software to generate graphs of OD vs. time (seconds) followed by comparison of the slopes of the reactions to a thrombin standard for quantification of reactions. Results using peptides were always compared to reactions performed without peptide and percent inhibition was determined by comparison of inhibited reactions to the control reactions.

**SDS PAGE of Reactions** – In all cases the substrate used in any reaction was incubated in the presence of D5Q for ten minutes at ambient temperature before addition into reaction mixtures. Reactions catalyzed by prothrombinase or fXa alone were initiated by the addition of fXa. Aliquots of 4-6μg of protein from the reaction mixture were removed at various time points determined empirically dependent on the substrate used (i.e. prothrombin, prethrombin 1 or prethrombin 2) and placed in a two-fold volume of 0.2 M acetic acid to stop the reaction. Samples were then concentrated using a centrivap attached to a cold trap and suspended in a loading buffer containing 1% SDS and 1% BME followed by preparation for SDS PAGE. Control reactions performed in the absence of peptides were performed simultaneously with reactions performed in the presence of peptide as a means of ensuring any differences in reaction rates or pathways were due to the presence of the peptides being used. Differences in cleavage patterns were determined according to molecular weights of products and confirmed through amino terminal sequencing. Differences in the rates of cleavage were determined by
densitometry scanning of SDS-PAGE followed by comparison to control reactions performed in the absence of peptide.

3.4 RESULTS

Previous studies demonstrated that the pentapeptide D5Q was a competitive inhibitor of prothrombinase with respect to prothrombin and that inhibition occurred by substrate depletion. The current study was performed to ascertain the effect D5Q has on the pathway to thrombin generation by prothrombinase. Figure 2 shows the titration of a prothrombinase catalyzed reaction with D5Q. The titration demonstrates that inhibition is concentration dependent and shows 50% inhibition occurs at 7.5µM D5Q under the conditions employed. As the concentration of D5Q was increased complete inhibition never occurred though approached 100% inhibition at 15µM D5Q.

To directly assay the effect of the peptide on the pathway to thrombin generation in a prothrombinase catalyzed reaction SDS PAGE was performed. This allowed the determination of the intermediates involved. The results presented in figure 3 show the effect D5Q has on the pathway to prothrombin activation. Panel A shows a control reaction performed in the absence of peptide and demonstrates the normal pathway to thrombin production by prothrombinase. Prothrombin is rapidly consumed with initial activating cleavage occurring at Arg320 producing meizothrombin, as evident by the appearance of fragment 1·2A. The subsequent cleavage at Arg271 quickly consumes the transient meizothrombin intermediate and generates thrombin and fragment 1·2. All of the prothrombin present within the reaction mixture is consumed within ten minutes resulting in complete conversion of the prothrombin to thrombin. Panel B shows a prothrombinase catalyzed activation of prothrombin in the presence of a saturating
**Figure 3.1** – The activation of prothrombin. **Pathway I** - Membrane-bound fXa catalyzes initial cleavage at Arg^{271} producing the inactive intermediate prethrombin 2 and fragment 1-2. Prethrombin 2 is then sequentially cleaved at Arg^{320} to produce thrombin and fragment 1-2. **Pathway II** – In the presence of fully assembled prothrombinase complex the rate of thrombin generation is greatly enhanced and the order of the activating cleavages on prothrombin are reversed. Prothrombinase initially cleaves prothrombin at Arg^{320} generating the active enzymatic intermediate, meizothrombin. Meizothrombin is then cleaved at Arg^{271} to produce thrombin and fragment 1-2.
Figure 3.2 – DYDYQ titration of prothrombinase catalyzed activation of prothrombin. Reaction mixtures composed of 5nM fVa, 0.5nM fXa, 50μM DAPA and 10μM PCPS assembled in a HEPES buffered saline solution containing 5mM CaCl$_2$ were started by the addition 1.4μM prothrombin that was incubated with increasing concentration of DYDYQ for ten minutes. All reactions were performed in triplicate and compared to control reactions that were performed in the absence of DYDYQ. Error bars are representative of standard deviance and Prisma Graphing software was used in generating the graph.
Figure 3.3 – SDS PAGE analysis of prothrombin activation. Reactions performed in the absence and the presence of DYDYQ were subjected to SDS PAGE analysis to determine the effect DYDYQ had on the pathway to thrombin generation. Panel A shows a prothrombinase catalyzed reaction demonstrating the optimal rate of the reaction with initial cleavage occurring at Arg^{320} as evident by the presence of F1·2A. Panel B shows a prothrombinase catalyzed reaction performed in the presence of 20 μM DYDYQ and demonstrates initial cleavage occurring at Arg^{271} as evident by the presence of prethrombin 2. Aliquots from both reaction mixtures were removed at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 300, 360, 600, 1200, 1800 and 3600 seconds after the addition of fXa. The prothrombin derived fragments are labeled: II, prothrombin (residues 1-579); F1·2A, fragment 1·2 A chain (residues 1-320); F1·2, fragment 1·2 (residues 1-271); P2, prethrombin 2 (residues 271-579); B, B chain of thrombin (residues 320-579).
concentration of D5Q (20µM). In the presence of 20µM D5Q prothrombin consumption by prothrombinase is greatly delayed with the initial activating cleavage occurring at Arg^{271} as evident by the occurrence of prethrombin 2 and fragment 1·2. The subsequent cleavage occurring at Arg^{320} produces thrombin at a greatly delayed rate. Prothrombin was still present within the reaction mixture after an hour of incubation.

To determine the concentration dependence of the peptide on the switch in pathways and the concentration at which meizothrombin formation is completely inhibited a titration of D5Q on a prothrombinase catalyzed reaction was monitored through SDS PAGE. Figure 4 shows the effects of increasing concentrations of D5Q on prothrombinase activation of prothrombin. Panels A – D demonstrate the disappearance of fragment 1·2A, representative of meizothrombin, corresponding to an increased production of prethrombin 2 as seen in panels C – F. As the concentration of D5Q is increased from 2µM to 4.2µM the preferred pathway to thrombin formation by prothrombinase switches from the meizothrombin intermediate to the prethrombin 2 intermediate, because the initial activating cleavage of prothrombin by prothrombinase switches from Arg^{320} to Arg^{271} and consequently the rate at which prothrombin is consumed is greatly inhibited. Densitometry scanning of SDS PAGE presented in figure 4 show the rate decrease of prothrombin consumption in the presence of varied concentrations of D5Q. A 41-fold decrease in the rate of prothrombin consumption is observed as the concentration of the peptide is raised to 20µM.

To assay the peptide’s effect of the peptide on both prothrombin activating cleavages independently of each other, two recombinant prothrombin mutants were employed which were only capable of one activating cleavage. The prothrombin mutants
Figure 3.4 – SDS PAGE analysis of DYDYQ titration of prothrombinase catalyzed reaction. Plasma derived prothrombin was incubated with increasing concentrations of DYDYQ as indicated in the figure. The time points and the prothrombin derived fragments are the same as indicated in figure legend 3. The concentrations of DYDYQ used throughout this experiment were: Panel A, prothrombinase in the presence of 0 µM DYDYQ; panel B, prothrombinase in the presence of 0.2 µM DYDYQ; panel C, prothrombinase in the presence of 2 µM DYDYQ; panel D, prothrombinase in the presence of 4.2 µM DYDYQ; panel E, prothrombinase in the presence of 8.5 µM DYDYQ; panel F, prothrombinase in the presence of 16.5 µM DYDYQ.
Table 3.1

<table>
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<th>Fold Decrease</th>
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<td>20</td>
<td>0.2 ± 0.2</td>
<td>41</td>
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**Table 3.1 – Scanning densitometry.** The rates of prothrombin consumption in the presence of increased DYDYQ concentrations were generated through densitometry of the gels presented in figures 3 and 4. The fold decrease in the rate of prothrombin consumption was generated as a ratio of the prothrombinase catalyzed reaction performed in the absence of DYDYQ to the prothrombinase catalyzed reaction in the presence of DYDYQ.
rMZ-II (R^{155} \rightarrow A, R^{271} \rightarrow A and R^{284} \rightarrow A) and rP2-II (R^{155} \rightarrow A, R^{284} \rightarrow A and R^{320} \rightarrow A) were used in prothrombinase catalyzed reactions performed in the presence and absence of D5Q to assay the peptide’s effect on the individual activating cleavages independently of each other. Figure 5 presents SDS PAGE analysis of prothrombinase catalyzed reactions performed on rMZ-II (panel A) and rP2-II (panel B). Panel A shows the reaction performed in the absence of D5Q in lanes 1 – 9 and the reaction performed in the presence of D5Q, lanes 10 – 18, demonstrating the inhibition of the initial cleavage at Arg^{320}. Panel B shows the prothrombinase catalyzed activation of rP2-II in the absence (lanes 1 – 9) and the presence of D5Q (lanes 10 – 18) and demonstrates that D5Q has little effect on the activating cleavage of prothrombin at Arg^{271}. Densitometry scanning of SDS PAGE presented in figure 5 and SDS PAGE not shown are presented in table 2 and demonstrate the selective inhibition of D5Q on the activating cleavage at Arg^{320}, when the reaction is catalyzed by prothrombinase. Although at high D5Q concentrations inhibition of initial activating cleavage at Arg^{271} is observed.

To assay the effect of the interaction of D5Q with prothrombin has on the fXa catalyzed activation of prothrombin activity assays monitored through the cleavage of a chromogenic substrate were performed on a D5Q titration. Figure 6 shows the titration of D5Q on the activity of a fXa catalyzed activation of prothrombin 50% inhibition occurred at a 12μM D5Q concentration. However concentrations of D5Q as high as 48μM were incapable of completely inhibiting thrombin formation by fXa. SDS PAGE analysis of the lipid-bound fXa catalyzed activation of prothrombin demonstrated a greatly accelerated rate of prothrombin consumption, but showed a delayed generation of thrombin. Figure 7 demonstrates the rapid consumption of prothrombin with the
Figure 3.5 - Analysis of prothrombinase catalyzed activation of recombinant prothrombin molecules in the absence and presence of DYDYQ. Reactions were performed using the recombinant prothrombin molecules rMZ-II and rP2-II which are only capable of being cleaved at Arg^{320} and Arg^{271} respectively. Lanes 1-9 and 10-18 are aliquots that were removed before the addition of fXa and 1, 4, 6, 10, 20, 30, 45 and 60 minutes after the addition of fXa. The table was generated through densitometry scanning and gives the rate of prothrombin consumption in the absence and the presence of DYDYQ for both of the respective recombinant prothrombin molecules.

* The rates that are presented are moles consumed \( \cdot \text{s}^{-1} \cdot \text{mole fXa}^{-1} \)

** NS, not significant consumption.
Figure 3.6 – DYDYQ titration against membrane-bound fXa catalyzed activation of prothrombin. Prothrombin (1.4μM) was incubated with increased concentrations of DYDYQ for ten minutes before addition into a reaction mixture containing fXa. The initial velocity of the reactions were measured and compared to reactions performed in the absence of DYDYQ. All measurements were performed in triplicate using different protein and peptide preparations and error bars are representative of standard deviation.
Figure 3.7 – SDS PAGE analysis of membrane-bound fXa activation of prothrombin in the absence and the presence of DYDYQ. Plasma-derived prothrombin was incubated with DYDYQ or an equivalent volume of buffer for ten minutes before addition into a reaction mixture containing 2.5nM fXa. Panel A is the reaction that was performed in the absence of DYDYQ and panel B is the reaction that was performed in the presence of 24μM DYDYQ. Lanes 1-19 are aliquots that were removed before the initiation of the reaction and 0.5, 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150 and 180 minutes after the reaction was started. The prothrombin fragments are the same as described in figure 3 with the additional fragments being: P1, prethrombin 1 (residues 156 – 579) and F1, fragment 1 (residues 1 – 155).
accumulation of the prethrombin 2 intermediate, evident of initial activating cleavage occurring at Arg$^{271}$. However the production of thrombin is greatly delayed as evident by the delayed appearance of the B-chain of thrombin, meaning the subsequent cleavage of the prethrombin 2/fragment 1-2 at Arg$^{320}$ is strongly inhibited.

To evaluate the effects D5Q has on the individual activating cleavages independently in a fXa catalyzed reaction the recombinant prothrombin mutants rMZ-II and rP2-II were once again employed. Figure 8 shows SDS PAGE analysis of the fXa catalyzed activation of the recombinant prothrombin molecules in the absence and the presence of D5Q. Panel A shows the cleavage of the rMZ-II prothrombin mutant by lipid-bound fXa and demonstrates that the presence of D5Q (lanes 10-18) has little effect on the activating cleavage at Arg$^{320}$ when compared to the control reaction performed in the absence of peptide (lanes 1-9). Panel B shows the consumption of the rP2-II prothrombin mutant by lipid-bound fXa (lanes 1-9) and demonstrates a greatly accelerated rate of the cleavage at Arg$^{271}$ in the presence of 20μM D5Q (lanes 10-18). Densitometry scanning of the SDS PAGE presented in figures 7 and 8 and additional SDS PAGE of reactions performed in the presence of different D5Q concentrations not shown are presented in table 3. A D5Q concentration of 48μM accelerated the rate of plasma derived prothrombin consumption by lipid-bound fXa by 50-fold, while a lower concentration of D5Q (24μM) accelerated the rate of rP2-II consumption by lipid-bound fXa 70-fold. Table 3 also demonstrates that D5Q has little effect on the rate of proteolysis at Arg$^{320}$ as evident by the similar rate of rMZ-II consumption by lipid-bound fXa in the absence or presence of D5Q.
Figure 3.8

Figure 3.8 – Analysis of the activation of the recombinant prothrombin molecules by membrane-bound fXa. Membrane-bound fXa was used in SDS PAGE monitored reactions with the two prothrombin mutants rMZ-II and rP2-II as substrates used in the reactions performed in the presence and absence of DYDYQ. Lanes 1-9 and 10-18 are aliquots of the reaction mixture that were pulled out before initiation of the reaction and 5, 15, 30, 60, 90, 120, 150 and 180 minutes after the addition of fXa into the reaction mixture. The prothrombin derived fragments are the same as explained in figure legend 3.
Table 3.2

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<tr>
<th>Peptide Concentration (µM)</th>
<th>Plasma-derived Prothrombin</th>
<th>rMZ-II</th>
<th>rP2-II</th>
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<td>0</td>
<td>0.06 ± 0.03*</td>
<td>0.08 ± 0.022</td>
<td>0.014 ± 0.005</td>
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<tr>
<td>20</td>
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<td>0.06 ± 0.03</td>
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<td>48</td>
<td>3 ± 0.7</td>
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</table>

Table 3.2 – Rates of prothrombin consumption by membrane-bound fXa. Densitometry scanning of the SDS PAGE shown in figures 7 and 8 were performed to measure the rates of consumption of prothrombin and recombinant prothrombin mutants rMZ-II and rP2-II in reactions performed in the absence and presence of increasing concentrations of DYDYQ.

* Rates of prothrombin consumption are measured as moles consumed · s⁻¹ · mole fXa⁻¹.
Since the carboxyl-terminal portion of the heavy chain of fVa shares homology with the hirudin derived anti-coagulant peptide molecules, we next investigated the similarity of inhibition between D5Q and the hirudin derived peptides. It has been previously determined that the hirudin peptides’ interaction with thrombin enhances the ability of thrombin to cleave thrombin specific chromogenic substrates. To investigate the possibility that the interaction of D5Q has a similar effect on thrombin’s ability to cleave chromogenic substrates, D5Q and the hirudin peptides were incubated in the presence of thrombin before the addition of the chromogenic substrate S-2338. Figure 9 show that the interaction of the sulfated and non-sulfated versions of the hirudin peptides increases the rate of cleavage of S-2338, while the interaction of D5Q with thrombin does not enhance the observed rate. This indicates that the hirudin peptides ability to inhibit thrombin formation in activity assays monitored through cleavage of a chromogenic substrate are misleading, since a dual effect is observed.

To assay the ability of the hirudin peptides on the pathway to thrombin formation SDS PAGE of prothrombinase catalyzed activation of prothrombin were performed. Figure 10 shows the results generated when prothrombin was incubated in the absence and presence of 100μM of the sulfated and non-sulfated versions of the hirudin peptides (panels B and C). Panel A shows the uninhibited reaction and demonstrates the optimal rate of thrombin generation by a prothrombinase catalyzed reaction. Panel B shows the reaction under the same conditions, but in the presence of 100μM hirS54-65(SO3−) and demonstrates approximately 50% inhibition, while panel D shows the same reaction as monitored through the cleavage of a chromogenic substrate and implies an increased rate of thrombin generation due to the increased activity of thrombin to cleave S-2238 in the
Figure 3.9 – Direct effect of peptides on thrombin’s cleavage of Spectrozyme TH. Thrombin (50nM) was incubated in the presence of DYDYQ, Hir$_{54-65}$(SO$_3^-$), and Hir$_{54-65}$ for ten minutes before addition of the chromogenic substrate, Spectrozyme TH. After five minutes incubation the optical density of the samples were read at 405nm. All samples were performed in triplicate and error bars are representative of standard deviation.
Figure 3.10 – SDS PAGE analysis of Hir$^{54-65}$(SO$_3^-$) and Hir$^{54-65}$ inhibition of prothrombinase catalyzed activation of prothrombin. Panel A is a control reaction performed in the absence of peptide. Panel B is a reaction performed in the presence 100μM Hir$^{54-65}$(SO$_3^-$). Panel C is a reaction that was performed in the presence of 100μM Hir$^{54-65}$. Lanes 1-19 are representative of aliquots removed from the respective reaction mixtures before the addition of fXa and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 300, 360, 600, 1200, 1800 and 3600 seconds after the addition of fXa. Panel D is a measurement of the initial rates of the reactions shown in panels A-C that was measured through the cleavage of a chromogenic substrate specific for thrombin and compared to a thrombin standard for quantification. Error bars are representative of standard deviation and graph was generated using Prisma Graphing software.
Table 3.3

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<th>Prothrombinase</th>
<th>Prothrombin Consumption (moles consumed · s⁻¹ · mole fXa-1)</th>
<th>Prothrombin Consumption (Percent of Control)</th>
<th>Thrombin Formation (nMIIa Formed · min⁻¹ · nMfXa-1)</th>
<th>Thrombin Activity (Percent Control)</th>
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<td>Control (no peptide)</td>
<td>26.1 ± 1.2</td>
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<td>1770.2 ± 227</td>
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<td>Hir54-65(SO3⁻)</td>
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**Table 3.3 – Comparison of densitometry scanning and activity based assays.** SDS PAGE from figure 10 were subjected to densitometry scanning to determine the rate of prothrombin consumption. The rate of thrombin generation was determined through activity based assays monitoring thrombin generation through cleavage of a chromogenic substrate specific for thrombin. Comparison of the two methods of determining thrombin generation demonstrates the misleading nature of monitoring inhibition by the hirudin peptides using a chromogenic substrate specific for thrombin.
presence of 100μM Hir$^{54-65}$($SO_3^-$). Panel C shows the reaction performed in the presence of 100μM Hir$^{54-65}$ and demonstrates a greatly inhibited generation of thrombin, however initial cleavage still occurs at Arg$^{320}$ as evident by the generation of fragment 1-2A. Panel D shows the rate of thrombin generation in the absence and presence of the hirudin peptides and D5Q as determined by an activity assay monitored through the cleavage of the chromogenic substrate S-2338, demonstrating the discrepancy of the inhibitory potential of the hirudin peptides when monitored in this fashion. Densitometry scanning of the SDS PAGE presented in figure 10 was performed to generate the rates of prothrombin consumption presented in table 3, establishing the discrepancy between the two ways of monitoring the inhibitory potential of the respective peptides.

Since the inhibition of the hirudin peptides on the generation of thrombin by prothrombinase has previously been shown to be optimal in the absence of a membrane surface, D5Q was next titrated against a fXa/fVa complex generated in the absence of PCPS. Figure 11 shows a similar inhibition response to D5Q concentration as compared to a titration of D5Q in the presence of a fully assembled prothrombinase complex assembled on a PCPS surface. This demonstrates a contrast between the inhibition of D5Q when compared to the hirudin peptides because the presence of a membrane surface has little influence on the inhibitory potential of D5Q on the fXa/fVa complex.

As a means of monitoring the effect that Hir$^{54-65}$($SO_3^-$) and D5Q has on the pathway to thrombin generation, reactions performed in the absence and presence of the respective peptides and monitored through SDS PAGE. Figure 12 consists of time courses of prothrombin activating reactions performed in the absence of a lipid bilayer catalyzed by fXa (panel A), a fXa/fVa complex (panel B), a fXa/fVa complex in the
Figure 3.11 – DYDYQ titration of a fVa/fXa complex in the absence of PCPS. To assay the dependency of a membrane surface on the inhibitory potential of DYDYQ a complex of fVa/fXa was titrated with an increasing concentration of DYDYQ. Prothrombin (500nM) was incubated in the presence of increasing concentrations of DYDYQ for ten minutes before addition into a reaction mixture containing 10nM fXa, 5nM fVa and 3µM DAPA. Initial rates of thrombin generation were measured and reactions performed in the presence of DYDYQ were compared to reactions performed in the absence of peptide as a means of determining the percent inhibition due to the concentration of DYDYQ present. All reactions were performed in triplicate and error bars are representative of standard deviation.
**Figure 3.12 – SDS PAGE analysis of fVa/fXa complex in the presence of DYDYQ and Hir^{54-65}(SO_3^-).** To monitor the effect of the peptides on the pathway to prothrombin activation in the absence of PCPS SDS PAGE was used to monitor reactions. Panel A is a control reaction catalyzed by fXa, in the absence of fVa. Panel B is a control reaction performed in the presence of a fXa/fVa complex. Panel C is a reaction catalyzed by a fXa/fVa complex performed in the presence of DYDYQ. Panel D is a reaction catalyzed by a fXa/fVa complex performed in the presence of Hir^{54-65}(SO_3^-). Lanes 1-19 are aliquots that were removed from the reaction before the addition of fXa and 0.5, 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150 and 180 minutes after the addition of fXa. The prothrombin derived fragments are the same as identified in figure 3.
presence of 92μM D5Q (panel C) and a fXa/fVa complex in the presence of 100μM Hir^{54-65}(SO_{3}^{-}). Panel A shows the ability of fXa to activate prothrombin in the absence of PCPS, thrombin is slowly generated through initial cleavage occurring at Arg^{271} as evident by the generation of the prethrombin 2 intermediate. Panel B shows a greatly accelerated generation of thrombin due to the addition of fVα into the reaction mixture, however in the absence of the PCPS surface the initial activating prothrombin cleavage occurs at Arg^{271}. The addition of Hir^{54-65}(SO_{3}^{-}) (panel C) and D5Q (panel D) slows the generation of thrombin by the fXa/fVa complex at a rate similar to that observed by the fXa catalyzed reaction (panel A).

Prethrombin 1 is a naturally occurring derivative of prothrombin that is generated through proteolysis at Arg^{155} by thrombin or fXa and results in the generation of fragment 1 (prothrombin residues 1 – 155) and prethrombin 1 (prothrombin residues 156 – 579). Activity assays measuring thrombin generation by prothrombinase in the absence and presence of Hir^{54-65}(SO_{3}^{-}) and D5Q were performed using prethrombin 1 in the absence and presence of fragment 1. Figure 13 shows the percent activity of these reactions and demonstrates the inability of D5Q to inhibit prothrombinase catalyzed activation of prethrombin 1, while Hir^{54-65}(SO_{3}^{-}) inhibited prethrombin 1 activation both in the absence and presence of fragment 1. The dissimilar inhibition of reactions performed with prethrombin 1 as a substrate, again suggest dissimilar interaction of the two peptides with prothrombin.

To monitor the pathways to thrombin production from the prethrombin 1 substrate in the absence and presence of fragment one and the effects of Hir^{54-65}(SO_{3}^{-}) and the D5Q peptides SDS PAGE was performed. Figure 14 show reactions performed using
Figure 3.13 – Activity assays measuring prothrombinase catalyzed activation of prethrombin 1 in the absence and presence of fragment 1. The prothrombinase catalyzed activation of prethrombin 1 was monitored through the chromogenic substrate specific for thrombin, Spectrozyme TH. Reactions performed in the presence of the DYDYQ and Hir^{54-65}(SO_3^-) were compared to control reactions performed in the absence of these peptides to determine the percent inhibition. Panel A shows the prothrombinase catalyzed activation of prethrombin 1 in the absence and the presence of DYDYQ and Hir^{54-65}(SO_3^-). Panel B shows the prothrombinase catalyzed activation of prethrombin 1/fragment 1 in the absence and the presence of DYDYQ and Hir^{54-65}(SO_3^-). All reactions were performed in triplicate and error bars are representative of standard deviation.
Figure 3.14

**Pre1 alone**

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**Figure 3.14** – SDS PAGE analysis of prothrombinase catalyzed activation of **prethrombin 1**. Panels A and B are control reactions monitoring the activation of prethrombin 1 catalyzed by membrane-bound fXa (10nM) in the absence (panel A) and presence (panel B) of fragment 1. Panels C and D are prothrombinase (1nM fXa and 15nM fVa) catalyzed activation of prethrombin 1 in the absence (panel C) and presence (panel D) of fragment 1. Panels E and F are the same conditions as panels C and D, but in the presence of 92μM DYDYQ. Panels G and H are the same conditions as panels C and D, but in the presence of 100μM Hir54-65(SO3−). Lanes 1-19 are aliquots from the various reaction mixtures that were removed before the addition of fXa and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 30, 45, 60, 90 and 120 minutes after the addition of fXa. The prothrombin derived fragments are the same as identified in figures 3 and 7 with F2-A being fragment 2 covalently attached to the A chain of thrombin (residues 156 – 320).
prethrombin as a substrate in the absence and the presence of fragment 1. Panels A and B are control reactions catalyzed by fXa and demonstrate the insufficient generation of thrombin by lipid-bound fXa in the absence of fVa. Panels C and D are prothrombinase catalyzed reactions and show that in the absence of fragment 1 (panel C) both prethrombin 1 activating cleavages occur at similar rates. When fragment 1 is added into the reaction mixture (panel D) the initial cleavage occurs at Arg\(^{320}\), as evident by the occurrence of fragment 2A, with the sequential cleavage at Arg\(^{271}\) occurring at an accelerated rate. Though the consumption of prethrombin 1 by prothrombinase is slower in the presence of fragment 1, the generation of thrombin is faster when fragment 1 is present, because the subsequent cleavage is greatly accelerated. Panels E and F are reactions performed in the presence of 92\(\mu\)M D5Q and demonstrate that D5Q does not inhibit prothrombinase catalyzed activation of prethrombin 1 in the absence (panel E) or the presence of fragment 1 (Panel F). Hir\(^{54-65}(SO_3^-)\) inhibited both the prothrombinase catalyzed activation of prethrombin 1 in the absence (panel G) and presence of fragment 1 (panel H) as indicated by the activity assay presented in figure 13 and the SDS PAGE presented in figure 14.

Densitometry scanning of the SDS PAGE presented in figure 14 was performed to determine the relative rates of prethrombin 1 consumption in the absence and presence of fragment 1 (Table 4) as well as the relative rate of generation of the B-chain of thrombin (figure 15). Table 4 shows that the rate of prethrombin 1 consumption by prothrombinase is 16 ± 2.9 moles consumed \(\cdot\) s\(^{-1}\) \cdot mole fXa\(^{-1}\), while the rate of consumption of prethrombin 1 in the presence of fragment 1 is slower, being 11.1 ± 2.5 moles consumed \(\cdot\) s\(^{-1}\) \cdot mole fXa\(^{-1}\). However, when comparing the generation of the B-chain of
### Table 3.4

<table>
<thead>
<tr>
<th>Enzyme/Inhibitor</th>
<th>Prethrombin 1 (moles consumed · s⁻¹ · mole fXa-1)</th>
<th>Prethrombin 1 + Fragment 1 (moles consumed · s⁻¹ · mole fXa-1)</th>
</tr>
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<tbody>
<tr>
<td>fXa alone (Control)</td>
<td>0.8 ± 0.6</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Prothrombinase (control)</td>
<td>16 ± 2.9</td>
<td>11.1 ± 2.5</td>
</tr>
<tr>
<td>Prothrombinase + DYDYQ</td>
<td>17.8 ± 2.6</td>
<td>11.4 ± 0.55</td>
</tr>
<tr>
<td>Prothrombinase + Hir54-65(SO₃⁻)</td>
<td>5.2 ± 3.5</td>
<td>NS*</td>
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**Table 3.4** – Densitometry scanning of SDS PAGE measuring the consumption of prethrombin 1. Densitometry scanning was performed on the SDS PAGE shown in figure 14 and the rate of prethrombin 1 consumption determined.

*NS, not significant consumption. The concentration of prethrombin 1 varied from 15μM at time 0 to ~12μM following 2 hr incubation with prothrombinase in the presence of Hir^{54-65}(SO₃⁻).
Figure 3.15 – Generation of the B chain of thrombin in the absence and presence of fragment 1. Densitometry scanning of the prothrombinase catalyzed controls presented in figure 14 was performed to measure the generation of the B chain of thrombin. The results are presented as density of B-chain band (arbitrary units * 10^3) verses time (minute) for the prothrombinase catalyzed activation of prethrombin 1 in the absence and the presence of fragment 1.
thrombin as shown in figure 15, it is demonstrated that prethrombin 1 is activated approximately 3-fold faster in the presence of fragment 1, with reversal of the initial cleavage occurring at Arg\(^{320}\) as evident by the generation of fragment 2A.

We next looked at the activation of prethrombin 2 (prothrombin residues 271 – 579) in the absence and presence of fragment 1·2 (prothrombin residues 1 – 270) and fragments derived from fragment 1·2 and the inhibitory potential of the peptides Hir\(^{54-65}\)(SO\(_3^–\)) and D5Q on this substrate. Results from activity assays employing prethrombin 2 (panel A), prethrombin 2 in the presence of fragment 1·2 (panel B), prethrombin 2 in the presence of fragment 1 (panel C), prethrombin 2 in the presence of fragment 2 (panel D), and prethrombin 2 in the presence of both fragment 1 and fragment 2 (panel E) as substrates in a prothrombinase catalyzed reactions are presented in figure 16. Once again we observed a dissimilar inhibition pattern between the Hir\(^{54-65}\)(SO\(_3^–\)) and D5Q peptides. D5Q only inhibited the prothrombinase catalyzed reactions employing prethrombin 2 in the presence of fragment 1·2, Fragment 1 or fragment 1 in addition to fragment 2 (panels B, C and D respectively) as substrates demonstrating the necessity of the presence of fragment 1 for D5Q inhibition in the activation of prethrombin 2. While Hir\(^{54-65}\)(SO\(_3^–\)) displayed inhibition in reactions using the substrates prethrombin 2, prethrombin 2 in the presence of fragment 1, prethrombin 2 in the presence of fragment 2 and prethrombin 2 in the presence of both fragment 1 and fragment 2 (panels A, C, D and E respectively).

SDS PAGE was employed to visualize the prothrombinase catalyzed activation of prethrombin 2 in the presence of fragment 1·2 and prethrombin 2 in the presence of both fragment 1 and fragment 2 separately. Figure 17 shows these reactions performed in the absence and in the presence of both the Hir\(^{54-65}\)(SO\(_3^–\)) and D5Q peptides. Panels A-C
**Figure 3.16 – Prethrombin 2 activity assays.** Panel A shows the activity of a prothrombinase catalyzed activation of prethrombin 2 in the absence and presence of DYDYQ and Hir$_{54-65}$(SO$_3^-$). Panel B shows the activity of a prothrombinase catalyzed activation of prethrombin 2/fragment 1:2 in the absence and the presence of DYDYQ and Hir$_{54-65}$(SO$_3^-$). Panel C shows the activity of a prothrombinase catalyzed activation of prethrombin 2/fragment 1/fragment 2 in the absence and the presence of Hir$_{54-65}$(SO$_3^-$). Panel D shows the activity of a prothrombinase catalyzed activation of prethrombin 2/fragment 1 in the absence and presence of DYDYQ and Hir$_{54-65}$(SO$_3^-$). Panel E shows the activity of a prothrombinase catalyzed activation of prethrombin 2/fragment 2 in the absence and presence of DYDYQ and Hir$_{54-65}$(SO$_3^-$). All reactions were performed in triplicate and error bars are representative of standard deviation.
Figure 3.17

<table>
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<tr>
<th>Pre2/F1•2</th>
<th>Pre2/F1/F2</th>
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<tr>
<td><img src="Prothrombinase" alt="Image A" /></td>
<td><img src="Prothrombinase" alt="Image D" /></td>
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<td><img src="Prothrombinase+DYDYQ" alt="Image B" /></td>
<td><img src="Prothrombinase" alt="Image E" /></td>
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<tr>
<td><img src="Prothrombinase+Hir%5E%7B54-65%7D(SO_3%5E-)" alt="Image C" /></td>
<td><img src="Prothrombinase" alt="Image F" /></td>
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Figure 3.17 – SDS PAGE analysis of prethrombin 2 activation in the presence of fragment 1-2. Fragment 1-2 was used before and after cleavage by fXa (to produce fragment 1 and fragment 2 non-covalently attached) in reactions monitoring the prothrombinase catalyzed activation of prethrombin 2 in the presence and absence of the respective peptides DYDYQ and Hir$^{54-65}$(SO$_3$). Panels A-C and D-F are prothrombinase catalyzed activations of prethrombin 2 in the presence of fragment 1-2 and in the presence of fragment 1 and fragment 2 respectively. Panels B and E are reactions performed in the presence of 76µM DYDYQ and panels C and F are performed in the presence of 100µM Hir$^{54-65}$(SO$_3$). Panels A-C had aliquots removed from the reaction mixture before addition of fXa and 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 30, 45, 60 and 90 minutes after the addition of fXa. Panels D-F had aliquots removed from the reaction mixture before addition of fXa, fragment 1 and fragment 2 and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 300, 360, 600, 1200, 1800 and 3600 seconds after the addition of the fXa, fragment 1 and fragment 2 mixture. The prothrombin derived fragments are the same as identified in figure 3 and 7 with F2 being fragment 2 (residues 156-270).
show the results of the reaction using prethrombin 2 in the presence of fragment 1-2 as the substrate. Panel A shows the control reaction performed in the absence of any inhibitors and demonstrates the optimal rate of prethrombin 2 activation when fragment 1-2 is present. As indicated by the activity assay presented in figure 16 when 76μM D5Q is present (panel B) the consumption of prethrombin 2/fragment 1-2 and the generation of the B-chain of thrombin are delayed, while the presence of 100μM Hir^{54-65}(SO_3^-) (panel C) had no effect on either the consumption of prethrombin 2/fragment 1-2 or the generation of the B-chain of thrombin. Panels D-F demonstrate that 76μM D5Q (panel E) inhibits prethrombin 2 activation in the presence of cleaved fragment 1-2 (reaction mixture contained both fragment 1 and fragment 2) at a rate similar to that of prethrombin 2/fragment 1-2 (panel E), while 100μM Hir^{54-65}(SO_3^-) strongly inhibited thrombin formation from prethrombin 2 in the presence of both fragment 1 and fragment 2 it showed little inhibition in the activation of prethrombin 2/fragment 1-2. The ability of the Hir^{54-65}(SO_3^-) peptide to inhibit the activation of prethrombin 2 was restored upon cleavage of fragment 1-2.

To directly assay the nonexclusive interaction of the two peptides we next studied the prethrombin 2 activation in the presence of fragment 1-2 in an activity based assay. Since the activation of prethrombin 2/fragment 1-2 by prothrombinase was inhibited by D5Q, but unaffected by Hir^{54-65}(SO_3^-), if both peptides were interacting at the same site on the prethrombin 2/fragment 1-2 the Hir^{54-65}(SO_3^-) would interfere with D5Q ability to inhibit this reaction. Increasing concentrations of Hir^{54-65}(SO_3^-) were incubated in the presence of prethrombin 2/fragment 1-2 followed by titration with D5Q. Figure 18 shows
Figure 3.18 – DYDYQ titration of prethrombin 2 activation in the presence of fragment 1-2 and Hir\textsuperscript{54-65}(SO\textsubscript{3}\textsuperscript{−}). Prethrombin 2 was incubated in the presence of fragment 1-2 with increasing concentration of Hir\textsuperscript{54-65}(SO\textsubscript{3}\textsuperscript{−}) and then this ternary complex was titrated with increasing concentrations of DYDYQ. Results were standardized to a thrombin standard and graphed nMIIa · min\textsuperscript{−1} · nMfXa\textsuperscript{−1} verse the concentration of DYDYQ.
that the inhibitory potential of D5Q was not affected by the increased concentrations of the Hir$^{54-65}$(SO$_3^-$) peptide suggesting a nonexclusive interaction of the two peptides.

3.5 DISCUSSION

A recent study stated that the carboxyl-terminal portion of the heavy chain of fVa was not involved in recognition of prothrombin, however the study clearly showed that the removal of this portion of fVa resulted in a 50% loss in the specific activity of the cofactor which is consistent with several previous studies which demonstrated the involvement of the carboxyl-terminal portion of the heavy chain of fVa in the recognition of prothrombin. It has also been suggested that the recognition of prothrombin by fXa is altered in the presence of fVa due to a prothrombin binding site present on fVa. We have previous shown that the pentapeptide D5Q inhibited prothrombinase in a competitive fashion by substrate depletion. In addition we demonstrated that D5Q did not interfere in the highly specific fVa/fXa interaction. The present study has explored the mode of inhibition of D5Q on prothrombinase catalyzed activation of all of the physiological relevant substrates derived from prothrombin.

The present study demonstrated that the interaction of D5Q with prothrombin resulted in the specific inhibition of cleavage at Arg$^{320}$ by prothrombinase. Prethrombin 2 in the presence of fragment 1-2 is the naturally occurring intermediate of a membrane-bound fXa catalyzed activation of prothrombin (pathway 1 in figure 1) and consequently is a substrate that would naturally occur as a response to an injury, since fXa is generated before fVa. Therefore prothrombinase would naturally recognize the prethrombin 2/fragment 1-2 intermediate, since the generation of prothrombinase occurs after the generation of fXa and consequently after some prothrombin has been converted to the
prethrombin 2/fragment 1·2 intermediate. Interestingly, D5Q was incapable of inhibiting other naturally occurring derivatives of prothrombin.

Surprisingly, the interaction of D5Q with prothrombin greatly accelerated the ability of lipid-bound fXa to cleave the zymogen prothrombin at Arg$^{271}$ to produce the intermediate prethrombin 2/fragment 1·2. The subsequent activating cleavage at Arg$^{320}$ by lipid-bound fXa was greatly inhibited due to the interaction of the intermediate with D5Q resulting in a greatly decreased generation of thrombin by fXa. The interaction of D5Q with prothrombin or the prethrombin 2/fragment 1·2 intermediates resulted in a greatly decreased rate of thrombin generation by both prothrombinase and fXa in the absence of fVa. However D5Q had little inhibitory effect on the activation of other prothrombin derived fragments or on the activation of prethrombin 2 in the absence of fragment 1. The prothrombinase catalyzed activation of the intermediates prethrombin 1 or active-site blocked meizothrombin (data not presented) were unaffected by the presence of D5Q.

Since the carboxyl-terminal region of the heavy chain of fVa shares homology with the hirudin derived peptides, we initially postulated that D5Q probably interacted with prothrombin in a fashion similar to the interaction of Hir$^{54-65}(SO_3^-)$ with prothrombin. However the present study demonstrates that D5Q and Hir$^{54-65}(SO_3^-)$ have dissimilar inhibition patterns and suggests that the interaction of D5Q with prothrombin and the derivatives of prothrombin occur in a manner independent of the interaction of Hir$^{54-65}(SO_3^-)$ with prothrombin or the prothrombin derivatives. Further studies directly identifying the specific binding site of D5Q on prothrombin and its derivatives need to be performed to establish this, though presently all experimental data imply a distinct
binding site for D5Q. Finally it is interesting to note that the ability of Hir$^{54-65}$(SO$_3^-$) to inhibit the activation of the naturally occurring derivatives of prothrombin demonstrate almost an exact opposite inhibition pattern as D5Q. While both Hir$^{54-65}$(SO$_3^-$) and D5Q display strong inhibition on prothrombin, Hir$^{54-65}$(SO$_3^-$) does not reverse the cleavage pattern observed. Hir$^{54-65}$(SO$_3^-$) strongly inhibit the activation of prethrombin 1, and prethrombin 2 in the absence of fragment 1-2, while D5Q had little effect in these reactions. In contrast D5Q strongly inhibited the prothrombinase catalyzed activation of prethrombin 2 in the presence of fragment 1-2 while Hir$^{54-65}$(SO$_3^-$) showed little ability to inhibit this reaction.

In conclusion, the present study has demonstrated that a synthetic pentapeptide derived from the carboxyl-terminal portion of the heavy chain of fVa, strongly inhibits the activation of prothrombin by both prothrombinase and lipid-bound fXa by selectively inhibiting the activating cleavage that occurs at Arg$^{320}$. While D5Q demonstrated little ability to inhibit the activation of most prothrombin derived intermediates, it did strongly inhibit the most commonly generated intermediate, prethrombin 2/fragment 1-2. While the sequence D5Q occurs within a region of fVa that shares homology with the well-established anticoagulant peptide molecule derived from hirudin, the interaction between prothrombin with D5Q or Hir$^{54-65}$(SO$_3^-$) are probably different and result in different inhibition pattern when the two molecules (i.e. D5Q and Hir$^{54-65}$(SO$_3^-$)) are compared.
3.6 REFERENCES


CHAPTER IV

THE AMINO-TERMINAL PORTION OF THE LIGHT CHAIN OF FACTOR Va INTERACTS WITH FACTOR Xa IN THE PROTHROMBINASE COMPLEX

4.1 ABSTRACT

Prothrombinase assembly occurs when activated factor V (fVa) interacts with activated factor X (fXa) on a phospholipid bilayer in the presence of divalent metal ions. Previous studies have shown that the A3 domain from the light chain of fVa contains an interactive site for fXa which is necessary for proper prothrombinase assembly and function. To determine which amino acids present within the A3 domain of the light chain of fVa are involved in recognition of fXa we have used overlapping synthetic peptides representative of residues 1546-1612 of the A3 domain to screen their ability to inhibit prothrombinase activity in a fXa dependent manner. The peptide representative of the 13 amino acids spanning residues 1546 through 1558 strongly inhibited prothrombinase activity demonstrating an IC\textsubscript{50} of 50\textmu M. To investigate the individual amino acids involved in recognition of fXa overlapping synthetic pentapeptides from this region were
used in further flurometeric studies. Peptides which included amino acids representative of residues 1553-1558 of the A3 domain were capable of inhibited prothrombinase activity. To further investigate the hypothesis that this region is important in prothrombinase function a recombinant fV mutant was generated deleting residues 1549 - 1558 (fVa_{dS13A}). The fVa_{dS13A} mutant demonstrated a greatly decreased specific activity in clotting based assays. Prothrombinase assembled with fVa_{dS13A} showed a 5 fold increase in the apparent Kd value for the fVa/fXa interaction and a 50% reduction in the overall rate of the enzyme. The present study defines a potential fXa interactive site present within residues 1546-1558 of the A3 domain of fVa.

4.2 INTRODUCTION

The timely repair to vascular injury is dependent on the efficient activation of thrombin by the enzymatic complex prothrombinase. Prothrombinase is composed of activated fX, associated with activated fV in the presence of calcium ions on a phospholipid bilayer (1). The enzymatic component, fXa, is capable of activating the zymogen prothrombin through two sequential proteolytic cleavages occurring after residues Arg^{271} and Arg^{320} (2). The inclusion of fVa in the prothrombinase complex reverses the order of these two activating cleavages and accelerates the generation of thrombin by five orders of magnitude, thus the formation of the prothrombinase complex is necessary for the timely activation of prothrombin (3-5).

The assembly of the prothrombinase complex is dependent on the activation of both clotting factors X and V. Activation of fX occurs through a single proteolytic cleavage catalyzed by either the intrinsic or the extrinsic Xase complexes (6). The catalytic activity of fXa is not enhanced by the zymogen fV until it has been converted to
its active form. The activation of fV is catalyzed by either fXa or thrombin and occurs through three sequential cleavages occurring after residues 709, 1018 and 1545 (7). The activation of fVa results in the release of an interconnecting B-domain and a fully functional cofactor molecule composed of by an amino-terminally derived heavy chain non-covalently associated to a carboxyl-terminally derived light chain (8). The heavy chain of the fVa is composed of by residues 1-709 and is involved in the recognition of both fXa and prothrombin, while the light chain is composed of by residues 1546-2196 and is involved in recognition of both fXa and the membrane surface (8-13).

The A3 domain from the light chain of fVa has been implicated many times in binding fXa. Activity assays performed in the presence of a monoclonal antibody recognizing an epitope in this region of the light chain showed a decrease interaction between fVa and fXa (8). A decrease in the ability of fVa to interact with fXa was also demonstrated by mutagenic studies involving site-directed glycosylation of Thr residues 1683 (14). Another study used the enzymes cathepsin G and elastase to activate fV to a fVa-like species, both resulted in a cofactor molecule with a truncated heavy chain and an elongated light chain having a 15 and 8 fold decrease affinity for fXa respectively. Further treatment of these molecules with thrombin restored a normal light chain and consequently the affinity for fXa (15). Site directed mutagenesis at Arg^{1545} showed similar results demonstrating the necessity of a fully formed light chain as a prerequisite for an optimal interaction between fVa and fXa (16). Results obtained using an enzyme from the snake venom of *Naja nigricollis nigricollis* to activate fV generating a fVa-like molecule composed of a heavy chain of Mr 100,000kDa and a light chain with a Mr of 80,000kDa displayed a 20 fold increase in the Kd for the fVa/fXa interaction. Further
cleavage of this molecule with thrombin produced a normal light chain and restored the
affinity of fVa for fXa (17). Taken together all of the above experiments show a strong
dependency of the fVa/fXa association on proper exposure of the A3 domain of the light
chain of fVa.

4.3 EXPERIMENTAL PROCEDURES

Materials, reagents and proteins. L-\(\alpha\)-phosphatidylserine (PS) and L-\(\alpha\)-
phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL) and
prepared as previously described (18) followed by quantification by assaying for
phosphorus. The chromogenic substrate Spectrozyme-TH was purchased from America
Diagnostica, Inc (Greenwich, CT). Human \(\alpha\)-thrombin, prothrombin, human fXa and the
fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1.5-pentanediyl)amide (Dapa)
were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The
monoclonal antibody \(\alpha\)hFV1 coupled to Sepharose, and the two monoclonal antibodies
against factor V (against the heavy and the light chains respectively, \(\alpha\)HFV\(_{HC}\)#17 and
\(\alpha\)HFV\(_{LC}\)#9) were provided by Dr. Kenneth G. Mann (Department of Biochemistry,
University of Vermont, Burlington, VT). Diisopropyl fluorophosphates (DFP), O-
phenylenediamine dihydrochloride (OPD), 4-(2-hydroxyethyl)-1-
piperazineethanesulfonic acid (HEPES), Trizma, Coomassie Blue R-250 and factor V-
deficient plasma were purchased from Sigma. Polyethylene glycol (Mr 8000) was
purchased from J. T. Baker (Danvers MA). The secondary anti-mouse and anti-sheep IgG
coupled to peroxidase were purchased from Southern Biotechnology Associates Inc.
(Birmingham, AL). The chemiluminescent reagent ECL\(^+\) was purchased from Amersham
Biosciences. The thromboplastin reagent (recomboplastin) used in clotting assays was
purchased from Beckman (Fullerton, CA). The cDNA encoding fV was purchased from American Type Tissue Collection (ATCC 40515 pMT2-V, Manassas, VA). All restriction Enzymes were purchased from New England Biolabs (Beverly, MA). Human fV and fVa were purified and concentrated using methodologies previously described employing the monoclonal antibody αhFV#1 coupled to sepharose and heparin-sepharose respectively (19-21).

The recombinant fV light chain mutant used throughout this series of experiments was generated using mutagenic primers. The fV deletion mutant (rfVdS13A) was designed to remove the amino acids represented by the S13A peptide (The deleted amino acids were residues 1549-1558) and was generated using the primers 5’GCAGCATGGTACCTCCGCAGCAACAATGAAGAAATATCCTGGGATTATTCA G-3’ and 5’-CTGAATAATCCCAGGATATTTCTTCATTGTTGCTGC-GGAGGTACCATGCTGC-3’. These mutagenic primers were used in PCR employing pMT2-fV plasmid as a template and the product was subjected to DpnI digestion before transformation into chemically competent bacteria. Colonies were selected and purified plasmids were screened for the presence of the desired mutation by DNA sequencing.

Purification and Determination of Concentration of rfV Mutants – The recombinant proteins were generated as previously described (22). Cultured VP-SFM medium of WT and rfVa dS13A concentrated using a vivaflow 50 connected to a masterflex L/S to a final volume of approximately 10ml followed by washing with a trizma (20mM trizma base) buffered saline solution with 5mM Ca^{2+} at pH 7.40. Purification was performed on 1 ml columns of αHFV-1 antibody coupled to sepharose and eluted using 2M NH_{3}Cl at a pH 7.40 and concentration by centrifugation on YM-30 centricons
followed by three consecutive washes with trizma buffered saline solution in the presence of 5mM CaCl$_2$. Aliquots of purified protein were stored at -80°C for future use. Protein concentration were determined using spectrophotometry and an extension coefficient of 0.96 ($\varepsilon_1^{1\%}_{280nm}$) followed by ELISA (as previously described) to independently confirm the concentration (22). Protein purity was determined by coomassie and silver staining of SDS-PAGE.

*Activation of Recombinant Molecules* – Throughout the present study activation of all recombinant fV molecules was accomplished by incubation of the purified molecules with thrombin at a 1/100 (enzyme to substrate) ratio for ten minutes at 37°C. Addition of 2mM DFP, a specific serine protease inhibitor, stopped the reaction, followed by a 30 minute incubation on ice. Previous experiments showed that these conditions assure complete activation of the cofactor with minimal thrombin degradation to the activated molecule. However in all cases western blots using monospecific antibodies that recognize the heavy and light chains respectively was performed to assure minimal degradation of the activated protein.

*Determining the Kinetic Parameters Governing Mutant Molecules* – Assays measuring the kinetic parameters consisted of prothrombinase assembled in the presence of prothrombin and measured the appearance of the product thrombin by the secondary event of cleavage of a chromogenic substrate. This is a discontinuous assay measuring the initial velocity of the reaction through monitoring several time points within the first two minute of the reaction. The determination of the affinity of fVa for fXa was measured directly through titrating a limiting fXa concentration with increasing concentrations of fVa to determine the apparent K$_d$ value for the fXa/fVa interaction.
Assays were performed in 96 well plates and measured the initial velocities of product formation from the different concentrations of the titrant (fVa). Time points from each of the different reactions were stopped by addition into a buffer containing EDTA. Prism graphing software was used to interpret the data and generate $k_d$ values for the titrations. In all cases mutants were compared to wild type protein and measurements were done in triplicate with different protein purifications. The obtained $k_d$ values were used to determine proper ratios of fVa mutants to fXa to ensure similar concentrations of the assembled prothrombinase complex to be used in the prothrombin titrations. Prothrombin titrations were performed to determine the apparent $k_m$ values for prothrombinase assembled with rfVa$^{dS13A}$. Prothrombin titrations were performed in 96 well plates and reactions consisted of a limiting fXa concentration in the presence of saturating fVa concentrations as determined by $k_d$ values obtained from the fVa titrations. Prism graphing software was used to determine measurements of the initial velocity of the varied prothrombin concentrations and to generate a secondary graph measuring maximum velocity and $k_m$ of the various reactions.

*Effects of Mutant Molecules in Prothrombin Activation.* – Since prothrombin activation consists of two ordered sequential cleavages resulting in intermediates with enzymatic activity, meizothrombin, and without enzymatic activity, prethrombin 2, deficiency in assays measuring thrombin formation monitored through a chromogenic substrate may not show the reason for the deficiency. Monitoring prothrombin consumption using SDS PAGE allows determination of cleavage patterns. Prothrombinase assembled with rfVa$^{dS13A}$ were used in prothrombin activating reactions subjected to SDS-PAGE followed by coomassie blue staining. Aliquots of reaction
mixtures were placed in 0.2M acetic acid to stop the reaction followed by preparation for SDS-PAGE as previously described (23).

**Clotting Times Measurements** – One stage and two stage measurements of time to clot formation were performed with identical concentrations of all the fVα molecules used throughout this study. One-stage clotting assay were performed by the addition of the procofactor (fV) into fV deficient plasma, while two stage clotting assays were performed with the activated cofactor (fVα) being added to fV-deficient plasma. In both cases coagulation was initiated by the addition of thromboplastin reagent and the time to clot formation measured. Recombinant molecules unable to accelerate fibrin clot formation in fV-deficient plasma to the same degree as wild type fVα are considered to be functionally defective. All purified protein preparations were assayed in triplicate in this manner.

**Fluorescence Measurements of Thrombin Generation** – Thrombin formation was analyzed using the fluorescent inhibitor DAPA on a PerkinElmer LS-50B Luminescence spectrophotometer. Reactions were performed in the presence and absence of the various peptides by incubating a final concentration of 10nM fXa with the various concentrations of the different peptides for ten minutes at ambient temperature before addition into reactions mixture composed of 4nM fVα, 20μM PCPS, 350nM prothrombin in a HEPES buffered saline solution. Reactions were monitored using an excitation wavelength at 280nm and an emission wavelength of 520nm. All reactions performed in the presence of peptides were compared to reactions performed in the absence of peptides to measure the percent inhibition.
4.4 RESULTS

Since previous studies demonstrated the possibility that the interaction of fXa with the light chain of fVa involved the amino-terminal region of the A3 domain (8,14-17), initial studies were performed using overlapping synthetic decapeptides representative of the A3 domain sequence to screen their ability to inhibit prothrombinase activity. Figure 1 shows a schematic of the synthetic peptides used throughout this study with reference to the sequence they represent and their chosen nomenclature. Fluoremetric analysis of thrombin generation by prothrombinase in the presence of the various peptides is presented in figure 2 and shows only two of the screened peptides significantly inhibited thrombin generation. Both S13A and LCD3, peptides representative of residues 1546-1558 and 1569-1578 respectively, inhibited thrombin generation by prothrombinase in a manner similar to the activity of fXa alone, implying a possible diminished interaction between fXa and fVa. S13A was the stronger of the two inhibitory peptides and is representative of the amino-terminal region of the light chain of fVa, a region conserved through evolution as shown in figure 3. All of the other peptides from this region had little to no inhibitory effect on the activation of prothrombin by prothrombinase.

Since S13A showed the strongest inhibition in the initial screening of this region, we next designed a series of overlapping pentapeptides from the sequence of S13A. Figure 4 is a schematic showing the next series of peptides used throughout this study with their sequence compared to that of S13A and their assigned nomenclature on the right of their sequence. A follow up fluoremetric study presented in figure 5 demonstrated only the latter three of these five pentapeptides had any inhibitory
Figure 4.1 – Synthetic peptides generated for screening light chain’s ability to associate with fXa. Figure 1 shows a schematic of the synthetic peptides used throughout this study. The corresponding sequence of the light chain of fVa is presented at the top of the figure along with the corresponding residue numbers. The nomenclature used throughout the study for these peptides are presented at the left-side of the schematic, while the actual sequences are shown under the sequence from the light chain of fVa they represent.
Figure 4.2 – The synthetic peptides abilities to inhibit prothrombinase. The synthetic peptides introduced in figure 1 were incubated with 10nM fXa before addition into a reaction mixture composed of 4nM fVa, 20μM PCPS, 10μM Dapa and 350nM prothrombin. Reactions were monitored through fluorescence as described in the experimental procedures section. Figure 2 shows the control reactions catalyzed by the prothrombinase complex and lipid-bound fXa followed by reactions catalyzed by prothrombinase assembled in the presence of the 100μM of respective peptides. Reactions performed in the presence of the various peptides were compared to reactions performed in the absence of peptide as a means of calculating percent control.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMAN</td>
<td>SNN GNR RN YY IAA</td>
</tr>
<tr>
<td>BOVINE</td>
<td>SNT GNR KY YY IAA</td>
</tr>
<tr>
<td>MOUSE</td>
<td>GHG GHK KF YY IAA</td>
</tr>
<tr>
<td>PORCINE</td>
<td>SNN GNR RN YY IAA</td>
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</tbody>
</table>

**Figure 4.3** – *A comparison of the amino-terminal sequence of the light chain of fVα from different species.* The sequence of the region represented by the S13A peptide from bovine, mouse and porcine is compared to the human sequence and demonstrates strong homology.
**Figure 4.4**

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S13A</td>
<td><strong>SNNGNRRNYYIAA</strong></td>
</tr>
<tr>
<td>S7R</td>
<td><strong>SNNGNRR</strong></td>
</tr>
<tr>
<td>N6A</td>
<td><strong>NYYIAA</strong></td>
</tr>
<tr>
<td>N6A (FF)</td>
<td><strong>NFFIAA</strong></td>
</tr>
<tr>
<td>S5N</td>
<td><strong>SNNGN</strong></td>
</tr>
<tr>
<td>N5R</td>
<td><strong>NGNRR</strong></td>
</tr>
<tr>
<td>N5Y</td>
<td><strong>NRRNY</strong></td>
</tr>
<tr>
<td>R5I</td>
<td><strong>RNYYI</strong></td>
</tr>
<tr>
<td>Y5A</td>
<td><strong>YYIAA</strong></td>
</tr>
</tbody>
</table>

**Figure 4.4 – Synthetic peptides used in determining the binding site present within the light chain of fVa for fXa.** Schematic showing the peptides used for the determination of the binding site present within the light chain of fVa for fXa. S13A is a synthetic peptide which strongly inhibits thrombin generation in a prothrombinase catalyzed reaction that is representative of the amino-terminal region of the light chain of fVa (residues 1546 – 1558). All other peptides used are shorter derivatives of S13A and are aligned under the sequence for S13A.
Figure 4.5 – Inhibition study using overlapping pentapeptides representative of the amino-terminal region of the light chain of fVa. Overlapping pentapeptides representative of amino acid residues 1546 – 1558 of the light chain of fVa were synthesized to screen their ability to inhibit thrombin activation by prothrombinase. Reaction mixtures consisting of 4nM fVa, 700nM DAPA, 10μM PCPS and 350 nM prothrombin were initiated with 10nM fXa (final concentration in the reaction) that was incubated for 10 minutes in the presence of 150μM of the respective peptides as indicated in the figure. Initial velocities were measured throughout the first 30 seconds of the reaction and compared to a standard reaction performed in the absence of peptide to determine the percent inhibition. All reactions were performed in triplicate and error bars are representative of standard deviation.
ability on thrombin generation by prothrombinase. The three pentapeptides N5Y, R5I and Y5A all showed the ability to inhibit thrombin generation with Y5A being the strongest inhibitor of the series of overlapping S13A derived peptides. Y5A demonstrated an IC$_{50}$ of 150μM and shows 100% conservation of the compared sequences presented in figure 3.

Since peptide inhibition of prothrombinase only occurred when amino acids representative of residues 1553-1558 were used and no inhibition was observed with peptide sequences representative of amino acids 1546-1552, two additional peptides were generated from the S13A sequence. As shown in figure 4 the two peptides representative of amino acids 1546-1552 and 1553-1558 were S7R and N6A respectively. When S7R and N6A were titrated into a prothrombinase catalyzed activation of prothrombin and monitored by fluoremeter, N6A displayed a similar inhibition pattern as compared to S13A. This suggested that the amino acid sequence from 1553-1558 was involved in the observed inhibition, so this sequence was then altered as a control for the specificity of this sequence. The charge of the pentapeptide was weakened by the removal of the charges from two amino acids, two tyrosine residues were conservatively substituted to phenylalanines as depicted in figure 4. The N6A(FF) peptide lost the inhibitory effect demonstrated by the unaltered sequence of the N6A peptide as shown in figure 6 (opened triangles).

Since all of the peptide studies suggested that the amino-terminal region of the light chain of fVa was involved in the recognition of fXa, we next studied the possibility that this region of fVa was involved in fXa recognition through recombinant methods. A recombinant molecule deleting the region represented by the S13A peptide was generated
Figure 4.6 – Titration of S13A, S7R, N6A(WT) and N6A(FF) in a prothrombinase catalyzed activation of prothrombin. Increasing concentrations of the synthetic peptides S13A, S7R, N6A(WT) and N6A(FF) were incubated in the presence of 10nM fXa for 10 minutes before addition into a reaction mixture composed of 4nM fVa, 700nM DAPA, 10μM PCPS, and 350nM prothrombin. Reactions were monitored through the fluorescence properties of DAPA and compared to standards performed in the absence of peptides to determine the percent inhibition. All reactions were performed in triplicate and error bars are representative of standard deviation.
as described in the material and method section. The recombinant fVa molecule missing residues 1546-1558 (rfVa_{dS13A}) was activated by incubation with thrombin and subjected to SDS PAGE followed by Western blotting. Figure 7 shows a Western blot using monoclonal antibodies which recognizing the heavy and light chains of rfVa_{dS13A} and rfVa_{WT}. Upon activation both the rfVa_{dS13A} and rfVa_{WT} molecules generated the heavy and light chains of the active cofactor, however the rfVa_{dS13A} molecule always produced a fragment present at 220,000kDa that persisted even after extended incubation with thrombin.

We next studied the ability of prothrombinase assembled with the rfVa_{dS13A} molecule to activate prothrombin and monitored the reaction through SDS PAGE as presented in figure 8. When reactions catalyzed by prothrombinase assembled with the rfVa_{dS13A} (panel B) are compared to reactions catalyzed by prothrombinase assembled with plasma derived fVa (panel A) an approximately 50% reduction in the ability to activate prothrombin is observed. When the impaired function of prothrombinase assembled with the rfVa_{dS13A} molecule is directly compared to the inhibition of the S13A peptide on prothrombinase assembled with fVa (panel C) similar rates are observed. Densitometry scanning of the SDS PAGE presented in figure 8 was performed to produce the rates of prothrombin consumption presented in table 1. As shown, the rate of prothrombin consumption in the presence of saturating concentrations of S13A, 4.5 moles consumed·s^{-1}·mole of fXa^{-1}, was comparable to the rate of prothrombin consumption by prothrombinase assembled with the recombinant fVa_{dS13A} molecule, 5.7 moles consumed·s^{-1}·mole of fXa^{-1}. 
Figure 4.7 – Western blot of recombinant molecules. The monoclonal antibodies $\alpha_{hV^9}$ and $\alpha_{hV^917}$ were used to detect both the heavy and light chains of the rfVa$^{WT}$ and rfVa$^{dS13A}$ molecules. The positions of both the heavy and light chains of fVa are indicated along with a partial activation fragment present in the rfVa$^{dS13A}$ sample.
Figure 4.8

rfVa^{WT} control

rfVa^dS13A

Plasma Va +S13A
Figure 4.8 – SDS PAGE analysis of prothrombinase catalyzed activation of prothrombin in the absence and presence of S13A and prothrombinase assembled with rfV\textsubscript{a}\textsuperscript{dS13A}. Panels A-C are prothrombinase catalyzed reactions that were performed under the following conditions: 10nM fV\textsubscript{a} species (panel A used rfV\textsubscript{a}, panel B used rfV\textsubscript{a}\textsuperscript{dS13A} and panel C used plasma derived fV\textsubscript{a} in the presence of 100\textmu M S13A), 0.5nM fX\textsubscript{a}, 10\textmu M DAPA, 20\textmu M PCPS and 1.4\textmu M prothrombin. Reactions were initiated by the addition of fX\textsubscript{a} and aliquots were removed for SDS PAGE before the addition of fX\textsubscript{a} and 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 300, 360, 600, 1200, 1800 and 2400 seconds after the addition of fX\textsubscript{a}. 
Table 4.1

<table>
<thead>
<tr>
<th></th>
<th>Moles consumed·s⁻¹·mole of fXa⁻¹</th>
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<tbody>
<tr>
<td>WT</td>
<td>12.6</td>
</tr>
<tr>
<td>fVa^{dS13A}</td>
<td>5.7</td>
</tr>
<tr>
<td>fVa + S13A</td>
<td>4.5</td>
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Table 4.1 – Densitometry scanning of SDS PAGE presented in figure 8. Densitometry scans for the three SDS PAGE presented in figure 8 were performed to determine the rate of prothrombin consumption by prothrombinase assembled with the rfVa^{dS13A} mutant and prothrombinase in the presence of 100μM S13A. Scanning of the control reaction of prothrombinase assembled with rfVa^{WT} was also performed as a control. The rates shown are moles of prothrombin consumed per second per mole of fXa present in the reaction.
To explore the possibility that the inhibition pattern observed in figure 8 is due to a decrease affinity of rfVa$^{dS13A}$ for fXa, a limiting concentration of fXa was titrated against an increasing concentration of the rfVa$^{dS13A}$ mutant. Figure 9 shows the graph generated when the rfVa molecules were titrated against 15pM fXa. The titrations were carried out to 20nM rfVa concentrations, however the graph only shows the titrations to 10nM. As shown, both titrations were carried out until increasing the concentration of the different recombinant cofactor molecules no longer significantly increased the rate of thrombin production over the previous concentrations implying a maximum rate of thrombin production had been obtained. The determined apparent Kd values obtained from the fVa titrations are presented in table 2. The rfVa$^{dS13A}$ apparent Kd value of 1.915 is 5.5 times higher than the rfVa$^{WT}$ apparent Kd value suggesting a greatly weakened interaction between the rfVa$^{dS13A}$ and fXa when compared to the rfVa$^{WT}$ affinity for fXa.

The rfVa$^{dS13A}$ apparent Kd value was used to determine the saturating concentration of rfVa$^{dS13A}$ used in the prothrombin titration presented in figure 10. A concentration of 20nM rfVa$^{dS13A}$ was used to ensure that more than 95% of the fXa in solution was associated with the rfVa$^{dS13A}$ molecule for the prothrombin titration. This allowed the determination of apparent Km and Kcat values for prothrombinase assembled with the rfVa$^{dS13A}$, because the concentration of the enzymatic complex formed rather than the concentration of the constituents of the enzymatic complex were measured. Figure 10 presents the graphs generated from the prothrombin titration of prothrombinase assembled with the rfVa$^{WT}$ and the rfVa$^{dS13A}$ cofactor molecules respectively. Initial rates of thrombin formation over the various prothrombin concentrations used in the reactions were graphed to determine the maximum velocity of the respective enzymes. Table 2
Figure 4.9 – fVa titration with prothrombinase assembled with rfVa\textsuperscript{WT} and rfVa\textsuperscript{dS13A}. The determination of the k\textsubscript{d} values for the rfVa\textsuperscript{dS13A} was performed through titrating increasing concentrations of rfVa\textsuperscript{dS13A} into a prothrombinase catalyzed activation of prothrombin. Reaction conditions used were: 0.015nM fXa, 10\textmu M DAPA, 20\textmu M PCPS and 1.4\textmu M prothrombin. Titrations were performed in triplicate using different protein preparations and compared to titrations performed using rfVa\textsuperscript{WT}. Error bars are representative of standard deviation. The empirically derived k\textsubscript{d} values are presented in table 2.
Figure 4.10 – Prothrombin titrations performed in the presence of fully saturated prothrombinase assembled with rfVaWT and rfVadS13A. The determination of the kinetic parameters governing rfVadS13A was performed through titrating fully complexed prothrombinase assembled with rfVadS13A with increasing concentrations of prothrombin. Reactions conditions for the titrations were: 0.5pM fXa, 10μM DAPA, 20μM PCPS and 20nM rfVadS13A for the prothrombinase assembled with rfVadS13A and 10nM rfVawt for the prothrombinase assembled with the wild type cofactor. Initial velocities were monitored through the chromogenic substrate S-2238 and compared to a thrombin standard for quantification. The empirically derived kinetic values are presented in table 2.
Table 4.2 – The apparent kinetic constants as derived from the titrations presented in figures 9-10. The titrations presented in figures 9 and 10 were analyzed using Prisma 2.01 Graphing software to generate the kinetic values presented in table 2.

<table>
<thead>
<tr>
<th></th>
<th>Kd (nM)</th>
<th>Km (µM)</th>
<th>Kcat (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rfVa^{WT}</td>
<td>0.3500</td>
<td>0.2681</td>
<td>2002</td>
</tr>
<tr>
<td>rfVa^{dS13A}</td>
<td>1.915</td>
<td>0.3724</td>
<td>1136</td>
</tr>
</tbody>
</table>
shows the apparent Km and Kcat values generated from the prothrombin titration presented in figure 10. The apparent Km value was not significantly altered when prothrombinase was assembled with the rfVa\textsuperscript{dS13A} as compared to prothrombinase assembled with rfVa\textsuperscript{WT}, as evident by the respective values; 0.3724\(\mu\)M and 0.2681\(\mu\)M. However the Kcat of the reaction for the prothrombinase assembled with rfVa\textsuperscript{dS13A} was 50% reduced, demonstrating that the weakened interaction of the rfVa\textsuperscript{dS13A}/fXa complex resulted in a defective enzymatic complex which could not be corrected for by increasing the concentration of the rfVa\textsuperscript{dS13A} molecule.

4.5 DISCUSSION

The present study strongly suggests, through synthetic peptide analysis and recombinant mutagenesis, that amino acid residues 1546-1558 from the light chain of fVa are crucial for fXa recognition and thus prothrombinase assembly. The synthetic peptide S13A, representative of residues 1546-1558 strongly inhibited prothrombinase ability to generate thrombin. The use of overlapping synthetic peptides from this region demonstrated that the amino acid sequence representative of residues 1553-1558 were the main contributors of the inhibition of S13A on prothrombinase. This was confirmed by the fact that N6A had the same inhibition profile as S13A. Further, when the charged residues present within this region were exchanged for amino acids lacking charge (i.e. tyrosine residues exchanged for phenylalanine residues), in essence removal of the charge, the ability of N6A to inhibit prothrombinase function was lost, suggesting the involvement of the two tyrosine residues present at positions 1554 and 1555. While all of the peptide studies generated consistent results, the major weakness of the peptide portion of the present study is the specificity of peptides and whether the observed inhibition is
due to the weakened interaction of the highly specific fVa/fXa association as opposed to a nonspecific inhibition.

To ascertain whether the indicated region from the peptide study was involved in the fVa mediated assembly of the prothrombinase complex a recombinant study employing a fV mutant deleting the indicated region was undertaken. The rfVa\textsuperscript{dS13A} mutant was directly assayed for its ability to recognize fXa and the measurements of the interaction for the rfVa\textsuperscript{dS13A} with fXa demonstrated a greatly weakened association between the two proteins when compared to the rfVa\textsuperscript{WT}/fXa interaction. This strongly suggests that the loss of the ability of the rfVa\textsuperscript{dS13A} molecule to recognize fXa with the same affinity as rfVa\textsuperscript{WT} is due to the loss of a major binding site present within the deleted region. When fXa is fully saturated with the rfVa\textsuperscript{dS13A} mutant a reduced K\textsubscript{cat} is observed, this is consistent with a previously observed phenomenon using recombinant fVa mutants with a diminished ability to recognize fXa (23). As previously shown, the ability of fVa to optimally promote catalysis through fXa is dependent on the proper association between several points of contact between the two proteins and the lowered K\textsubscript{cat} generated from prothrombinase assembled with the rfVa\textsuperscript{dS13A} mutant is consistent with this previously observed phenomenon (23).

A major concern for the recombinant portion of this study is the accuracy of the determined concentration of the rfVa\textsuperscript{dS13A} molecule. If the determined concentration of the rfVa\textsuperscript{dS13A} mutant is elevated over the true concentration, then the determined K\textsubscript{D} would also be elevated and the generated results would be skewed. However, this would not account for the decreased ability of the rfVa\textsuperscript{dS13A} cofactor to promote the generation of thrombin when assembled into the prothrombinase complex, since the apparent K\textsubscript{D}
values enabled the determination of the concentration of the cofactor molecule needed to fully saturate fXa. Another concern with the recombinant portion of this study is a concern that any recombinant study has being the possibility that the induced mutation had a detrimental effect on the confirmation of the protein. Though rfVa^{dS13A} generated a light chain upon thrombin incubation comparable to that of the rfVa^{WT}, a persistent activation fragment around 220,000kDa persisted and appeared to be resistant to thrombin cleavage. However, even though detectable concentrations of the light chain were always generated, the function of the rfVa^{dS13A} cofactor molecule could not be restored by increasing the concentration, suggesting that the decreased cofactor activity observed in the rfVa^{dS13A} was due to the loss of an interactive site for fXa.

Since the characterization of the recombinant deletion molecule displayed an impaired function resulting from a weakened interaction with fXa, the hypothesis that a major binding site is present within the deleted portion of rfVa^{dS13A} is greatly strengthened. Coupled with the fact that both the peptide study and the recombinant study suggested the same region of the light chain of fVa was involved in the recognition of fXa also strongly supports the hypothesis. The amino-terminal portion of the light chain of fVa has an extremely conserved sequence across species with the sequence of the 13 amino acids represented by S13A showing strong conservation. The high degree of conservation of this region suggests an important function present within this region and coupled with previous observations of the light chains involvement in the recognition of fXa, further strengthens the hypothesis that this region is involved in recognition of fXa.

In conclusion, the present study strongly suggests that the amino-terminal portion of the light chain of fVa is involved in the association with fXa and that the loss of this
interactive site results in a decreased ability of prothrombinase to generate thrombin in an efficient manner. Further, the involvement of amino acid residues 1546-1558 in the recognition of fXa is a prerequisite for the association of fVa with fXa and consequently these residues are necessary for proper prothrombinase assembly.
4.6 REFERENCES


CHAPTER V

OVERALL CONCLUSIONS

5.1 CONCLUSION

The assembly of prothrombinase is crucial for the function of prothrombinase; the two events are interconnected and cannot be separated from each other. The contribution of fVa to the assembly/function of the prothrombinase complex is essential; since it is involved in recognizing all of the components of prothrombinase. Hence fVa can be considered the driving force behind prothrombinase complex formation and function. The interaction of fVa with all of the components of prothrombinase is the accelerating event in the activation of prothrombin which results in the rapid generation of thrombin through formation of the enzymatic intermediate, meizothrombin (1,2). Prothrombinase consists of an assembly of the two activated clotting factors (i.e., fXa and fVa) in the presence of divalent metal ions on an activated platelet surface (3). The association between fVa and fXa has been shown to involve two distal portions of fVa, involving residues present within the A2 domain of the heavy chain and A3 domain of the light chain (4).
Previous studies have demonstrated that the association between fVa and fXa is partially mediated through the region encompassing residues 307-348 present within the heavy chain of fVa (5). Presently we have shown that this interaction between the A2 domain of the heavy chain of fVa and fXa is mediated through four distinct amino acid residues; Asp\textsuperscript{323}, Tyr\textsuperscript{324}, Asp\textsuperscript{330} and Val\textsuperscript{331}. Recombinant technologies were used to induce site-specific mutations present within these residues to assay their contribution to the activity of prothrombinase. The data demonstrated that the removal of either binding sites present on fVa (i.e., \textsuperscript{323}DY\textsuperscript{324} or \textsuperscript{330}DV\textsuperscript{331}) for fXa resulted in a decreased $K_D$ for the interaction between the two proteins with little effect on the $K_{cat}$ for prothrombinase assembled with fVa mutated at either of the two binding sites. When the induced mutations for both of the discrete binding sites present within the heavy chain of fVa were combined into the same recombinant protein, a normal $K_{cat}$ value could not be restored when assembled into prothrombinase. This demonstrates that the optimal interaction between fVa and fXa is a prerequisite for the catalytic efficiency normally displayed by prothrombinase (6,7).

It has been demonstrated on several occasions that the interaction of fVa with fXa involves the light chain of the activated cofactor (8-11). Screening overlapping synthetic peptides representative of sequences present within the light chain of fVa for their ability to inhibit the prothrombinase catalyzed activation of prothrombin demonstrated that the peptide representative of the first thirteen amino acids present at the beginning of the light chain of fVa was the strongest inhibitor of prothrombinase. The recombinant follow up study demonstrated that the removal of this region from the light chain of fVa resulted in a diminished association with fXa and when the deletion protein was assembled into
prothrombinase, the enzymatic complex displayed a 50% reduction in its ability to activate prothrombin. Demonstrating again that for prothrombinase to achieve optimal catalytic efficiency the unhindered interaction between fVa and fXa is necessary.

Prothrombinase recognizes prothrombin in a fashion different than membrane-bound fXa. Several studies have demonstrated that the carboxyl-terminal portion of the heavy chain of fVa is involved in the recognition of prothrombin by prothrombinase (12-15). We have previously shown that residues \(^{695}DYDYQ^{699}\) from the heavy chain of fVa are crucial for optimal prothrombinase activity and have also demonstrated that a synthetic peptide representative of these residues strongly inhibited thrombin generation by prothrombinase in a manner consistent with substrate depletion (16). Presently we have explored the ability of lipid-bound fXa and prothrombinase to activate prothrombin and derivatives of prothrombin in the presence of the pentapeptide D5Q. We have seen the association of D5Q with prothrombin causes a switch in the pathway to thrombin generation by prothrombinase and greatly delays thrombin generation by lipid-bound fXa by inhibiting the activation of the prethrombin 2/fragment 1-2 intermediate (17). In both cases the interaction of D5Q with prothrombin specifically inhibited the activating cleavage at Arg\(^{320}\) and resulted in a greatly delayed generation of thrombin. Interestingly, D5Q was incapable of significantly inhibiting the activation of any of the other prothrombin derived substrates tested throughout this study suggesting the selective nature of the observed inhibition (18).

The optimal catalytic efficiency of prothrombinase can only be realized when fVa is properly associated with fXa. This is because the accelerated enzymatic capability of fXa is dependent on the association with fVa through residues present in both the heavy
and light chains of fVa. We have shown that the association between fVa and fXa involve residues $^{323}DY^{324}$ and $^{330}DV^{331}$ from the A2 domain of the heavy chain of fVa and also involves amino acids present within residues 1546 through 1558 of the A3 domain (6). While the residues $^{323}DY^{324}$ and $^{330}DV^{331}$ and the region from residues 1546 through 1558 are important for proper assembly of prothrombinase, the carboxyl terminal portion of the heavy chain of fVa is necessary for prothrombinase to properly recognize prothrombin (16). It has been demonstrated that a loss of the carboxyl terminal portion of the heavy chain of fVa results in a cofactor with reduced activity when assembled into prothrombinase (19). Further, a pentapeptide from this region, DYDYQ, greatly inhibits the activation of prothrombin by prothrombinase in a fashion consistent with substrate depletion, suggesting that the sequence $^{695}DYDYQ^{699}$ directly interacts with the substrate, prothrombin (16).

Since all of the above mentioned regions are crucial for prothrombinase assembly and consequently prothrombinase function, they represent possible targets for inhibiting prothrombinase. Prothrombinase function may be stopped through interrupting its assembly, since prothrombinase assembly is a prerequisite for its proper function. Through defining major points of interaction between the components of prothrombinase novel therapeutic targets are defined that can specifically interfere with the assembly of prothrombinase. Therefore, inhibitory synthetic peptides derived from amino acid sequences present within fVa, not only illuminate the molecular functions of prothrombinase but may represent a backbone on which to design potential therapeutic agents which will specifically target prothrombinase.
5.2 REFERENCES


