

INVESTIGATION OF THE INTERACTION OF MULTIMERIN 1 WITH  
COMPONENTS OF PROTHROMBINASE, *IN VITRO*

By

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**TITLE:** Investigation of the interaction of multimerin 1 with components of prothrombinase, *in vitro*

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## ABSTRACT

Prothrombinase is an enzymatic complex that accelerates the conversion of prothrombin to thrombin for efficient blood clot formation at sites of vessel injury. Prothrombinase consists of the enzyme factor Xa and its cofactor factor Va, assembled on a phosphatidyl serine-containing membrane, in the presence of calcium. Multimerin 1 (MMRN1) is a polymeric, factor V/Va-, prothrombin-, and phosphatidyl serine-binding protein that is stored in platelet and endothelial cell secretion granules. When released, MMRN1 binds to their cell surface and to the extracellular matrix. Unlike plasma factor Va, platelet factor Va is stored complexed to MMRN1 in platelet  $\alpha$ -granules, and is resistant to inactivation by activated protein C (APC). Previous studies revealed that exogenous MMRN1 inhibits thrombin generation in plasma. My thesis investigated the interaction of MMRN1 with components of prothrombinase in order to elucidate the molecular mechanisms by which MMRN1 modulates coagulation.

ELISA binding assays that used prothrombin derivatives revealed that the prothrombin gamma-carboxyglutamic acid and kringle domains have potential MMRN1 binding sites. Thrombin generation assays that used purified proteins and/or phospholipid vesicles revealed that MMRN1 inhibits thrombin generation in the presence and absence of factor Va, but not in the absence of phospholipid vesicles. These findings suggest that MMRN1 inhibits phospholipid-dependent thrombin generation through its interactions with factor Va and prothrombin.

MMRN1 did not affect APC-mediated loss of factor Va, which suggest other post-translational modifications in platelet factor Va account for its increased APC resistance. In thrombin generation assays with plasma, immobilized MMRN1 captured factor V/Va of human and mouse origin for subsequent thrombin generation, consistent with preservation of function that could be important for localizing factor V/Va for prothrombinase assembly. Collectively, these findings suggest that MMRN1 has a dual role in coagulation: as an inhibitor in fluid phase, and as a promoter when immobilized.

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## LIST OF SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used within this thesis include:

$\alpha$	alpha
$\beta$	beta
$\gamma$	gamma
ADP	adenosine diphosphate
APC	activated protein C
bp	base pairs
BSA	bovine serum albumin
CAT	calibrated automated thrombogram
cDNA	complementary DNA
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMILIN	protein family, originally named after an elastin microfibril interface located protein
EGF	epidermal growth factor
FII	prothrombin
FIIa	thrombin
FBS	fetal bovine serum
Gla	gamma-carboxyglutamic acid
GP	glycoprotein
HEK	human embryonic kidney
HRP	horseradish peroxidase

kb	kilobases
$K_D$	equilibrium dissociation constant
kDa	kilodaltons
MMRN1	human multimerin 1
mRNA	messenger ribonucleic acid
$M_r$	molecular weight
NEM	N-ethylmaleimide
OD	optical density
PAR	protease-activated receptor
PC	phosphatidyl choline
PS	phosphatidyl serine
RGD	arginine-glycine-aspartic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TAFI	thrombin-activatable fibrinolysis inhibitor
VWF	von Willebrand factor

## CHAPTER 1

### INTRODUCTION

#### 1.1 OVERVIEW ON HEMOSTASIS

Hemostasis is the arrest of bleeding from an injured vessel which requires platelet adhesion and aggregation, and activation of the blood coagulation cascade (1). Hemorrhage occurs when hemostatic plug formation is not adequate to stop bleeding, which can occur in individuals with defects in: platelet adhesion or aggregation; blood coagulation factors; or fibrinolysis (1,2). Thrombosis can occur when there is excess blood clot formation in veins (which causes venous thrombosis and/or pulmonary embolism) or in arteries (which can cause a heart attack, stroke or other arterial occlusions) (1,2). Normal hemostasis is regulated by the functions of endothelial cells, blood platelets, coagulation factors and fibrinolytic proteins. Normal hemostasis involves four interconnected phases: platelet adhesion and aggregation at sites of vessel injury to form the initial platelet plug; blood coagulation to form insoluble fibrin networks that bind to the platelet aggregates and stabilize the hemostatic plug; the downregulation of coagulation, through anticoagulation pathways; and fibrinolysis to dissolve the fibrin-rich blood clot (1,3,4). These processes are tightly regulated and an imbalance can result in bleeding or hypercoagulable conditions that require medical therapies (2).

Platelets (thrombocytes) are small, anucleated blood cells that are necessary for normal hemostasis (5). Platelets are produced from bone marrow megakaryocytes and circulate in the blood, where they play a key role in hemostasis by: adhering to an injured

site and aggregating, undergoing activation with release of proteins from their storage granules, and alterations in membrane lipids to provide a surface for coagulation reactions to take place (4,6). The initial response to vessel injury exposes blood to the subendothelial matrix and to the medial (smooth muscle) and adventitial layers of the vessel wall, where adhesive matrix proteins (such as collagen) and tissue factor are found (1,7,8). The exposed collagen and tissue factor initiate two distinct pathways (discussed below) that lead to platelet activation, and thrombin generation for fibrin formation and further platelet activation, as seen in *in vivo* studies using genetically altered mice to model hemostatic events (9,10).

At sites of vascular damage, the exposed collagen in the subendothelial matrix triggers platelet adhesion and activation, as a first line of defense to impede blood loss (1). Platelet adhesion to collagen is mediated by the interaction of platelet glycoprotein (GP) VI receptor and the integrin  $\alpha_2\beta_1$  with exposed collagen on the vessel wall in areas where shear stress is low (<200 per second) (11,12). It is mediated by interaction of platelet GP Ib $\alpha$  (in GP Ib-V-IX complex) receptor with von Willebrand factor (VWF) bound to collagen in areas where blood flow is at high shear (>600 per second, as in arteries and arterioles) (13,14). VWF is present in the plasma, but does not have the ability to bind GP Ib until VWF is bound to collagen, or exposed to shear (11). VWF can also be released from intracellular stores as high molecular VWF multimers are stored within endothelial Weibel-Palade bodies, with the integral membrane protein P-selectin, and within the electron lucent zone of platelet  $\alpha$ -granules with the adhesive proteins i.e. multimerin 1(15,16). Subsequent platelet-platelet cohesion (platelet aggregation) is

mediated by the interaction of platelet integrin  $\alpha_{IIb}\beta_3$  with VWF and fibrinogen (8).

Although VWF and fibrinogen function as mediators of platelet adhesion and aggregation, mice lacking both VWF and fibrinogen, are able to form thrombi (17). This observation suggests other adhesive ligand(s) (i.e. perhaps multimerin 1) have roles in mediating platelet adhesion and aggregation at sites of vessel injury. Following platelet deposition, cell signalling pathways lead to platelet activation and the exocytosis of intracellular contents (discussed below) including VWF to further arrest platelets and develop the platelet plug (11,18).

The tissue factor pathway of coagulation activation is initiated by disruption to the vascular integrity, whereby plasma coagulation factors come in contact with extravascular tissue factor, which initiates the coagulation cascade (1). This pathway leads to thrombin formation (discussed in Section 1.2-Overview of blood coagulation) and is independent of VWF and GP VI (10). Thrombin cleaves the G protein-coupled receptors, protease activated receptors 1 and 4 (PAR-1 and PAR-4, respectively), expressed on the platelet surface (19), thereby activating platelets. The relative contributions of the tissue factor- and the collagen-mediated pathways to platelet activation is currently unknown (1).

Activated platelets secrete the contents of their dense granules which promote further platelet activation and recruitment (i.e. through ADP and serotonin receptors) and vasoconstriction (i.e. serotonin) to reduce blood loss (20-23). Platelets also release contents of their  $\alpha$ -granules which include adhesive proteins (i.e. VWF and fibrinogen, which are found in platelets and plasma and multimerin 1 which is sequestered in platelets and endothelium), coagulation proteins (including factor V, which is normally

stored in complex with multimerin 1) and growth factors (15,24,25 16,26). Platelets also secrete the contents of their lysosomal granules (i.e. acid hydrolases) (27). During platelet activation, an influx of extracellular calcium leads to the translocation of phospholipid phosphatidyl serine from the inner to external leaflet of the membrane (28). Phosphatidyl serine exposure on the platelet membrane mediates the binding of coagulation factors, thereby enabling the assembly of prothrombinase and tenase enzyme complexes, which are critical for thrombin generation (discussed in Section 1.3-Overview of prothrombinase and its importance to coagulation). While phosphatidyl serine exposure is necessary for the formation of enzyme complexes that promote blood coagulation, its release may not be sufficient to initiate coagulation (29). Studies suggest platelet binding proteins (i.e. multimerin 1) could possibly contribute to the regulation of coagulation factor complex formation on platelets for thrombin generation (30).

The endothelium that lines the interior of blood vessels is also important for regulating hemostasis and thrombosis (31). The endothelial surface has molecules involved in anticoagulation (i.e. thrombomodulin; and heparan sulfate that binds and activates antithrombin) (31). The endothelium also secretes proteins that modulate fibrinolysis (i.e. tissue plasminogen activator, urokinase plasminogen activator, and plasminogen activator inhibitors) (32) the process by which fibrin is solubilized by plasmin (32). In addition, endothelial cells generate and secrete molecules (i.e. prostacyclin, nitric oxide, carbon monoxide) which inhibit nearby platelet activation and aggregation (22,33). Therefore, the endothelium normally provides an antithrombotic environment. The endothelium also regulates vessel wall tone by synthesizing

vasoconstrictors (i.e. endothelins), and vasodilators (i.e. prostacyclin) (34). Injury to the protective barrier of the endothelium alters the normal anti-adhesive and anticoagulant phenotype of the endothelium (31).

The endothelial protein thrombomodulin has a key role in the regulation of coagulation and fibrinolysis (3,32). When thrombomodulin binds thrombin, it makes thrombin an efficient activator of protein C while downregulating the procoagulant properties of thrombin (3,32,35). Activated protein C (APC) downregulates cofactors factor Va and factor VIIIa, which have an important role in accelerating coagulation (3,32,35). Protein C activation is accelerated when protein C is bound to endothelial protein C receptor (36). In addition, if thrombomodulin has bound chondroitin sulfate, it has increasing affinity for thrombin (37). APC activity is inhibited by protein C inhibitor (PCI) but enhanced by its cofactor protein S, which in turn, is inhibited by C4b-binding protein (38,39). In addition, when thrombomodulin binds thrombin, this blocks the procoagulant functions of thrombin (i.e. activation of platelets and factors V, VIII and fibrinogen) (31), without inhibiting thrombin inactivation by antithrombin (31). Furthermore, when thrombomodulin binds thrombin, thrombin is converted into an antifibrinolytic enzyme that activates thrombin activatable fibrinolysis inhibitor (TAFI) (3). In turn, the active form of TAFI, TAFIa, downregulates plasmin generation, and thereby reducing fibrinolysis (3). Therefore, thrombin bound to thrombomodulin provides a link between coagulation and fibrinolysis by regulating the generation of APC and TAFIa, respectively (3,32).

## 1.2 OVERVIEW ON PHASES OF BLOOD COAGULATION

Blood coagulation is required to stabilize the platelet rich hemostatic plugs formed at sites of vessel injury (40). Coagulation proteins interact on activated membrane surfaces that express phosphatidyl serine, in order to assemble into the enzyme complexes that activate coagulation (1,4). These complexes involve a vitamin K-dependent serine protease (reviewed in 41), associated with a cofactor in order to efficiently convert the zymogen substrate into an activated coagulation factor (1,4). The final stage of coagulation generates thrombin, an enzyme that has procoagulant functions, including activating platelets, factors V, VIII and XI to respectively generate factors Va, VIIIa and XIa, and convert soluble fibrinogen into insoluble fibrin. As previously discussed, thrombin also has other functions, including activating protein C and TAFI (3,42). Blood coagulation is thought to occur in three overlapping stages: initiation, amplification, and propagation (Figure 1) (1,4). The next section summarizes additional information on these stages of coagulation.

### 1.2.1 Initiation

The initiation of coagulation involves the extrinsic pathway, which begins when vessel injury exposes blood to cells that express tissue factor outside of blood vessels (4). The plasma protein factor VII, has the ability to autoactivate to its active form, factor VIIa, which interacts with tissue factor and forms a complex termed extrinsic tenase on the activated cellular surface (43). Extrinsic tenase has two principal substrates, factors IX and X, which are vitamin-K dependent proteins (41). The proteolytic activation of these

substrates results in the formation of serine proteases, factors IXa and Xa (44). However, extrinsic tenase has a higher affinity for the substrate factor IX than factor X (44). The factor Xa that remains membrane-bound is protected from inactivation by plasma protease inhibitors (i.e. tissue factor pathway inhibitor; and antithrombin) (4). In contrast, factor IXa can dissociate from tissue factor-bearing cells and bind to nearby platelets (4). Factor V, in its active form, factor Va, functions as the cofactor for factor Xa to form prothrombinase (discussed under Section 1.3-Overview of prothrombinase and its importance to coagulation), which converts a small amount of prothrombin to thrombin (45). Factor Va comes from one of two sources: platelets and plasma. When platelets adhere to collagen at sites of vessel injury, they become activated and release factor V that is in a partially activated form, complexed to multimerin 1(46,47). In addition, the factor V present in plasma can be activated by factor Xa (48). The physical and functional differences between plasma and platelet factor V will be discussed under Section 1.4-Overview of factor Va and its importance to coagulation. Therefore, during the initiation phase, the extrinsic pathway occurs on tissue factor-bearing cells to generate a small amount of thrombin.

### **1.2.2 Amplification**

The small amount of thrombin generated by tissue factor-bearing cells function to amplify the procoagulant response (4). Thrombin binds to its receptor GP Ib on the platelet surface and triggers a signalling mechanism resulting in platelet activation (49). Thrombin bound to the platelet surface interacts with substrates including: cleavage of PAR-1, a key process in platelet activation (19); activation of factor VIII present in

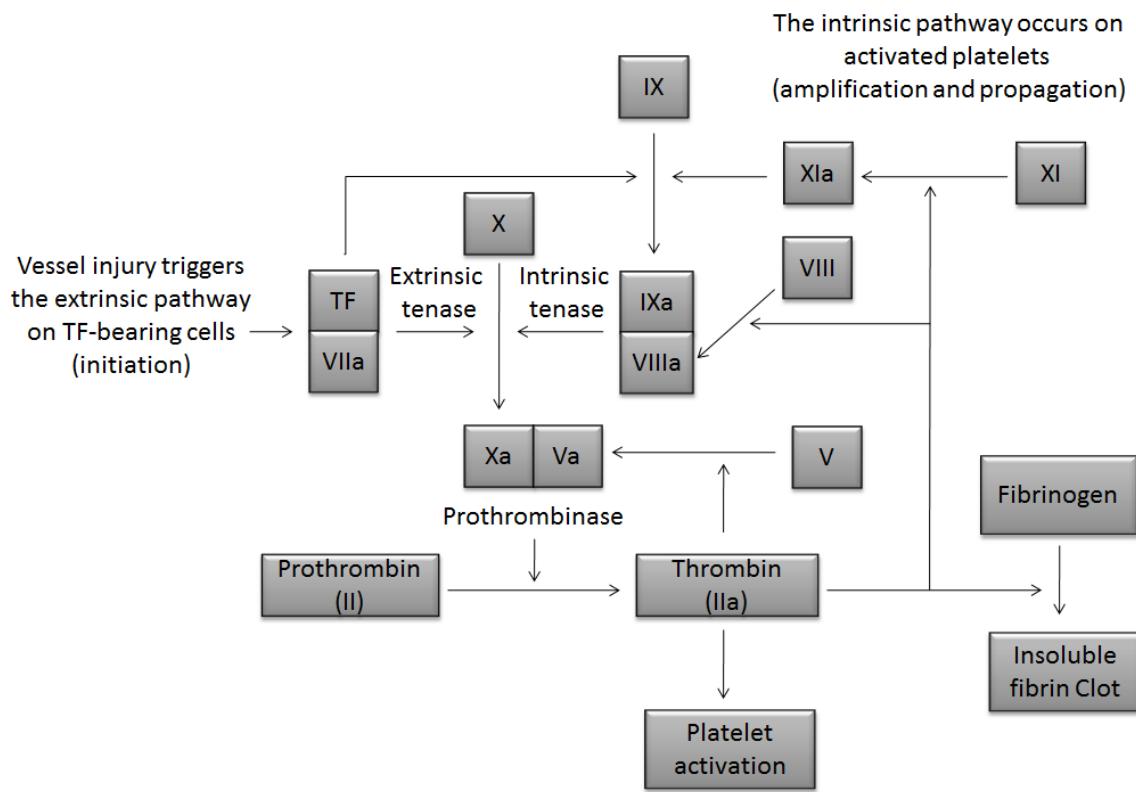
plasma, resulting in its dissociation from VWF (50); activation of factor V (51); and activation of factor XI present in plasma (52). These steps are required for subsequent large-scale thrombin generation on the platelet surface (4).

### **1.2.3 Propagation phase**

The propagation phase involves the intrinsic pathway, which occurs on activated platelets. Factor IXa generated during the initiation phase binds to platelet-bound factor VIIIa, to form a complex termed intrinsic tenase, that is important to efficiently generate essential levels of factor Xa (1). Additional factor IXa is generated from platelet-bound factor XIa (52). The importance of intrinsic tenase is illustrated in hemophilia A (factor VIII deficiency) and B (factor IX deficiency), which result in bleeding tendencies (53). Factor Xa binds to platelet-bound factor Va (or factor Va bound to another cellular surface i.e. microparticles 54) to form prothrombinase which enhances the rate of thrombin generation by 300 000-fold more compared with that in the absence of factor Va (55,56). Therefore, the intrinsic tenase and prothrombinase complexes on activated platelet surfaces lead to a burst of thrombin generation required to clot fibrinogen (1,45).

The high levels of thrombin formed during the propagation phase have several functions in addition to clotting fibrinogen. Thrombin incorporates into the clot at a rapid rate and participates in remodelling the clot structure from a loosely packed to a more tightly packed and rigid structure (4). Moreover, thrombin stabilizes the clot by: activating TAFI, which inhibits the fibrinolytic enzyme plasmin (3,57); and it also activates factor XIII, which forms bonds between fibrin strands within the clot (58,59).

Although the human platelet surface has two PAR receptors that are activated by thrombin, PAR-1 and PAR-4, and both contribute to full platelet activation, PAR-4 requires high levels of thrombin (60). Therefore, during propagation, thrombin levels can activate PAR-4 and ensure full platelet activation and secretion of granule contents. The excess active thrombin that stays bound to the fibrin clot, is protected from inhibition from antithrombin, and may provide a backup for procoagulant response if the clot were to be disrupted (4).



**Figure 1. Current model of blood coagulation.** At sites of vessel injury, plasma and platelet clotting factors interact to form enzyme complexes on activated membranes that convert zymogens to active serine proteases, which culminate in thrombin generation (40). Thrombin has multiple roles in coagulation including the conversion of fibrinogen to fibrin to stabilize the hemostatic plug (40). Coagulation is divided into three phases: initiation, amplification, and propagation. Initiation begins when vessel injury exposes blood to tissue factor (TF)-bearing cells that bind circulating factor VIIa (activated clotting factors are denoted with the suffix a) to form extrinsic tenase (43). Extrinsic tenase proteolytically activates two substrates: factors IX and X (40). Membrane-bound factor Xa inefficiently activates prothrombin to thrombin, in the absence of factor Va

(56,61). Amplification begins when the small amounts of thrombin activate factors V and VIII (19). Propagation begins when factor IXa binds platelet-bound factor VIIIa to form intrinsic tenase to efficiently generate factor Xa (40). Factor Xa binds platelet-bound factor Va to form the prothrombinase complex which enhances the rate of thrombin generation 300 000-fold more than in the absence of factor Va (55,56). In addition, thrombin activates factor XI which generates factor IXa for further intrinsic tenase formation (52). Overall, the tenase and prothrombinase complexes efficiently generate thrombin levels necessary to catalyze fibrinogen activation to form fibrin clots at a physiologic rate.

### **1.3 OVERVIEW ON PROTHROMBINASE AND ITS IMPORTANCE TO COAGULATION**

Prothrombinase has several important roles in coagulation. First, prothrombinase localizes blood clotting reactions to the site of vessel injury by forming on the surfaces of platelets that have adhered to the injured site (6). Second, prothrombinase generates thrombin that accelerates coagulation (62). Third, prothrombinase controls its own function by protecting its constituents, factors Va and Xa, from inhibitors or inactivators in the plasma. Specifically, factor Xa in prothrombinase is protected from inhibition by antithrombin and the factor Va in prothrombinase is protected from inactivation by APC (factor Va inactivation will be discussed under Section 1.4-Overview of factor Va and its importance to coagulation) (63,64).

Prothrombinase assembly involves membrane-protein and protein-protein interactions. The required catalytic membrane surface for prothrombinase can be supplied by a variety of cell surfaces in blood (i.e. platelets, monocytes, microparticles, endothelial cells) (6,54,65,66). However, the current understanding of prothrombinase properties, assembly and function are based on enzyme kinetic constants (i.e. Michaelis-Menton constant; Km) and binding constants (i.e. equilibrium dissociation constant; K<sub>D</sub>) derived from studies using purified proteins and synthetic membranes (unilamellar phospholipid vesicles) (65). The interaction of factor Va with membrane vesicles is a high-affinity interaction ( $K_D \sim 2.5$  nM) (67), whereas the interaction of factor Xa with membrane vesicles is a relatively lower affinity interaction ( $K_D \sim 110$  nM) (65). In the absence of membrane vesicles, factor Va and factor Xa form a 1:1 complex by a low affinity

interaction ( $K_D \sim 800$  nM) (68). The presence of membrane vesicles effectively enhances the interaction of factor Va and factor Xa ( $K_D \sim 1$  nM) (65,69), indicating the importance of membrane-protein and protein-protein interactions for prothrombinase assembly and function at sites of vascular injury.

## 1.4 OVERVIEW OF ACTIVATED FACTOR V AND ITS IMPORTANCE TO COAGULATION

Factor V is a single chain precursor of the nonenzymatic cofactor protein factor Va (45,70). Single chain factor V has a molecular weight of 330 kilodaltons (kDa) (71,72). Factor V circulates in the plasma at a concentration of 20 nM (72). Factor V is also located in platelet  $\alpha$ -granules where it is stored complexed to multimerin 1 (26,73). Plasma factor V is synthesized in the liver and in humans, platelet factor V originates from endocytosis of plasma factor V by megakaryocytes (74,75). In mice, platelet factor V is synthesized by megakaryocytes (76). The gene encoding factor V spans 80 kilobases (kb) of DNA, and is located on chromosome 1q21-25 (77). The gene consists of 24 introns and 25 exons (78). The cDNA for factor V is approximately 7 kb and encodes a 2224 amino acid protein that undergoes posttranslational processing (79).

Human factor V is composed of three A domains, a B domain, and two C domains (79). The 105 kDa-heavy chain is composed of two A domains, A1 and A2 (residues 1-303 and 317-656, respectively, which are connected by mostly basic amino acids, residues 304-316). The C-terminal of the heavy chain is composed of acidic amino acids (residues 657-709) (72,79). The 74-kDa light chain is composed of an A3 domain (residues 1546-1877) and two C domains, C1 and C2 (residues 1878-2036, and 2037-2196, respectively) (72,79). Prior to activation, the factor V heavy chain and light chain are interconnected by the B-domain (residues 710-1545) (72,79). The A domains of factor V are homologous to those found in ceruloplasmin and factor VIII (80,81). The C domains are homologous to those found in the slime mold protein discoidin and factor

VIII (79,82,83). The B-domain is not as highly conserved as other regions of factor V (70,84). In the most current structural model of factor Va, the triplicate A-domains are localized on top of the two aligned C-domains (85).

Factor V has multiple sites for *N*-linked glycosylation which are important for secretion (86). Many of these glycosylation sites are located within the B-domain (79). Factor V undergoes many additional post-translational modifications that affect factor Va function. Tyrosine sulfation enhances factor V activation by thrombin (discussed below) and factor Va procoagulant activity (87). Factor Va is phosphorylated by platelet casein kinase II at Ser-692 on the heavy chain, which accelerates its inactivation by APC (discussed below) (88-90).

Factor V activation is normally mediated by thrombin which cleaves factor V at three sites: Arg-709, Arg-1018, and Arg-1545 (Figure 2) (91). These cleavages liberate the B-domain as two fragments of 71 kDa and 150 kDa (residues 710-1018 and 1019-1545, respectively) (45), both of which are highly glycosylated (79,92). Factor Va is composed of the heavy chain (A1-A2 domains) and light chain (A3-C1-C2 domains) that are noncovalently associated by a calcium bridge (92). Factor Va cleaved by thrombin at both Arg-709 and Arg-1018 has partial activity and subsequent rapid cleavage at Arg-1545, is required for the generation of factor Va with maximal activity (45,91). Factor V can be activated by membrane-bound factor Xa (45,48). However, at physiologic concentrations, factor Xa does not appear to contribute significantly to plasma factor V activation (61). Other proteases that can partially activate factor V include: platelet

calpain, cathepsin G, neutrophil elastase, and plasmin, which can also inactivate factor Va (61).

Factors V and Va have separate functions in hemostasis. Factor V has anticoagulant roles as a synergistic cofactor with protein S for APC mediated inactivation of factor VIIIa, a function that requires the carboxyl region of the factor V B domain (93). In addition, factor V has a potential role as a cofactor for APC in the inactivation of factor Va (70). Factor Va has procoagulant roles as a cofactor for factor Xa in prothrombinase to accelerate the activation of prothrombin to thrombin by: stabilizing the interaction of factor Xa-prothrombin complex (discussed below) (94) and by influencing the kinetic rate and mechanism of prothrombin activation (55) (discussed under Section 1.5-Overview of prothrombin and its importance to coagulation). Unlike factor Va, factor V does not bind to factor Xa or prothrombin (45). Thus, the active form, factor Va, is necessary for procoagulant functions (45). Recently, it has been shown that factor Va also functions as a cofactor for thrombin in the activation of factor XI of the intrinsic pathway (95).

Factor Va interacts with other components of prothrombinase through interactions with its light and heavy chain domains (Figure 3). Factor Va and its precursor, factor V, bind to activated cellular surfaces through interactions with acidic phospholipids (i.e. phosphatidyl serine), that do not require calcium ions (29,96). Factor Va binding sites for activated membranes have been identified in the light chain domains (A3-C1-C2). The factor Va C2 domain contains the key residues, Trp-2063 and Trp-2064, required for high-affinity binding to activated membranes (97). The homologous factor Va C1 region, which is aligned in an edge-to-edge orientation with the C2 domain, contributes to

membrane binding with the side chains Tyr-1956 and Tyr-1957 (85,98). In contrast, the factor Va A3 domain (located on top of the C-domains along with the heavy chain (A1-A2)), also contains membrane binding sites (85,99). The interaction of factor Va with factor Xa requires calcium and predominately involves binding sites in the heavy chain domains, but additional binding sites have been characterized in the light chain domains (100,101). The interaction of factor Va with prothrombin involves the heavy chain and does not require calcium (102). The acidic region of factor Va, which is located towards the C-terminus region of the heavy chain (residues 697-709), contributes to prothrombin binding and is required for prothrombin incorporation and optimal activation by prothrombinase (103).

Factor Va inactivation is mediated by APC and leads to the downregulation of prothrombinase to limit thrombin generation (Figures 2 and 4) (41,104). APC is a vitamin K-dependent serine protease that cleaves factor Va via proteolysis at three peptide bonds on the heavy chain (104,105). The initial rapid cleavage of factor Va at Arg-506 results in partial loss of factor Va cofactor activity due to impaired interaction with factor Xa (104,105). Subsequent cleavage at Arg-306 results in release of the A2 domain and full factor Va inactivation (104,105). Cleavage at Arg-679 is extremely slow and does not appear to have a significant role in factor Va inactivation (104). A current structural model of factor Va-APC interaction (Figure 4) suggests Arg-506 cleavage is mediated by APC binding to negatively charged regions on factor Va near that cleavage site (106-108). However, cleavage of Arg-306 is mediated by different interactions and may include APC binding sites localized to regions in the light chain of factor Va (107-109).

The importance of Arg-506 cleavage is demonstrated in individuals with the hereditary disorder factor V<sub>Leiden</sub>; these individuals lack the Arg-506 cleavage site due to a sequence change, Arg506Gln, which retards factor Va inactivation by APC and is associated with a mild increased risk for thrombosis (110).

Efficient APC-mediated inactivation of factor Va requires an activated membrane surface and APC cofactor protein S. The membrane promotes but is not required for the initial cleavage of factor Va at Arg-506 (104,105). In contrast, cleavage of factor Va at Arg-306 is dependent on the presence of a membrane surface (104). Protein S is a vitamin-K dependent plasma protein that functions as a cofactor to APC by promoting factor Va cleavage at both Arg-306 and Arg-506 (111). APC inactivates factor Va more rapidly on the surface of endothelial cells than on activated platelets (112). Therefore, the primary role of APC is to prevent thrombosis by down regulating thrombin generation on endothelial cells; and the role of the platelet surface may be to provide a mechanism that protects factor Va from inactivation by APC (112). For example, prothrombinase assembly protects factor Va from inactivation by APC. Factor Xa selectively inhibits APC cleavage of factor Va at Arg-506 (113). Moreover, the incorporation of the substrate prothrombin into prothrombinase impairs APC cleavage of factor Va at both Arg-306 and Arg-506 (63). The release of prothrombin activation products from prothrombinase alleviates the inhibitory effects of prothrombin, and allows factor Va inactivation by APC (63).

The storage of factor V in platelets appears to have a more important role in hemostasis relative to its plasma counterpart because platelets aggregate, provide a

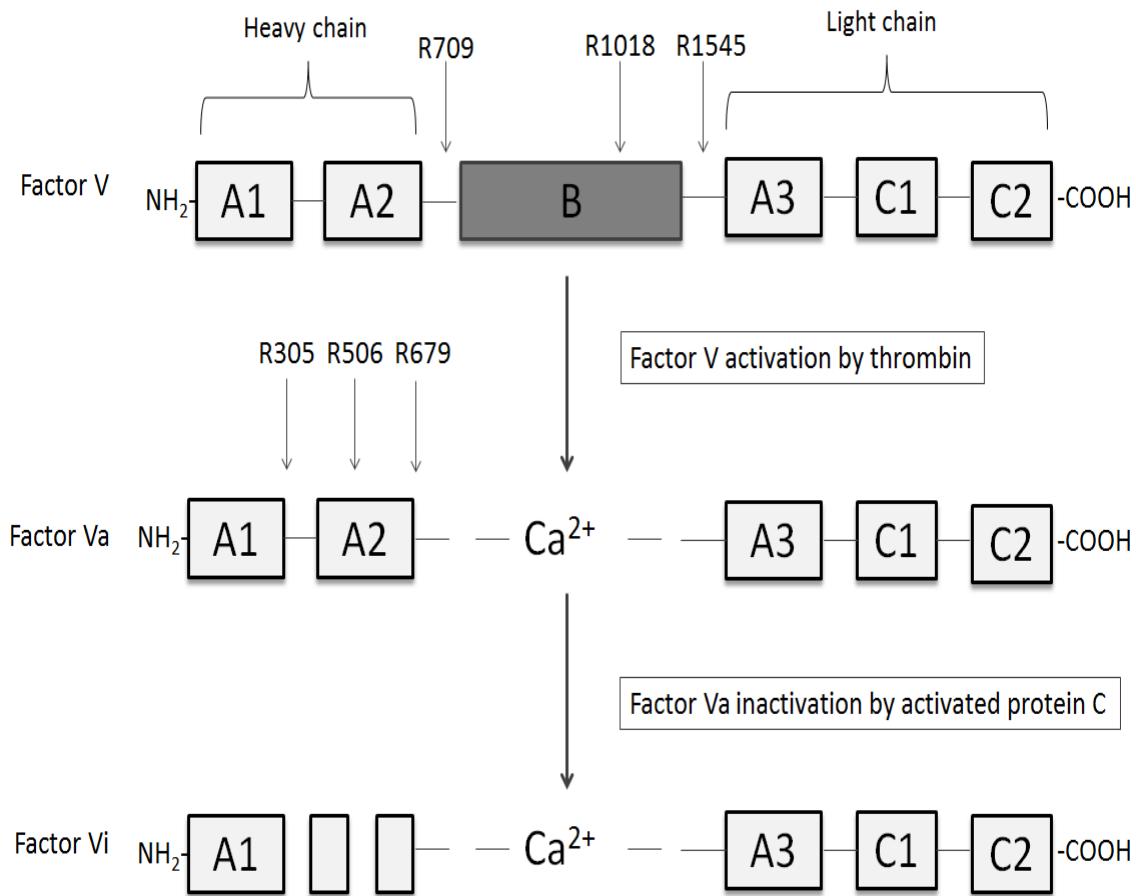
catalytic surface for prothrombinase, and secrete high local concentrations of platelet factor V at sites of vessel injury (47,114). Some studies suggest that platelet factor V is a better predictor than plasma factor V of bleeding tendencies in individuals with severe congenital factor V deficiency, an autosomal recessive disorder characterized by low levels of factor V antigen levels in plasma and platelets (70,115). Congenital factor V deficiency, which is caused by deficiencies or loss-of-function mutations in the gene for factor V (F5), is rare (1 in 6 million) (116). It was recently shown that residual platelet factor V is sufficient to support thrombin generation in individuals with severe congenital factor V deficiency due to underlying gene mutations (plasma factor V clotting activity <1-6%) and mild bleeding symptoms (116). The presence of functional platelet factor V in these individuals suggests that the underlying gene mutations are capable of minimal factor V expression, as factor V knockout mice do not survive (116,117). However, the mechanism that allows for preferential localization of the minimally expressed factor V in platelets, rather than plasma, is unclear (116). The importance of platelet factor V is further illustrated in an individual, with no detectable platelet or plasma factor V activity, who exhibited severe bleeding tendencies (118). In this case, the severe factor V deficiency was due to a frameshift deletion and insertion in the F5 gene (118). Additional disorders with bleeding diathesis have been identified in which there is a deficiency in platelet factor V with normal plasma factor V levels. Quebec platelet disorder (QPD) is an inherited autosomal dominant bleeding disorder, characterized by proteolytic degradation of platelet factor V and its binding protein multimerin 1, despite normal plasma factor V levels. This disorder is caused by increased expression and storage of

urokinase plasminogen activator in platelets resulting in reduced/degraded  $\alpha$ -granule proteins (119,120). Gray platelet syndrome (GPS) is a bleeding disorder, due to NBEAL2 gene mutations, characterized by a deficiency of platelet  $\alpha$ -granules because of a defect in storage resulting in reduced amounts of  $\alpha$ -granule proteins (121-123), including platelet factor V and multimerin 1 (124). Both QPD and GPS have a defect in platelet factor V-dependent prothrombinase activity and thrombin generation (*in vitro*) which may contribute to the associated bleeding diathesis (125,126). As both disorders have deficiencies in multimerin 1, the loss of multimerin 1 may also contribute to the defect in platelet prothrombinase activity (discussed under Section 1.6 –Overview of multimerin 1).

Platelet factor V has unique posttranslational modifications that appear to increase its procoagulant properties relative to plasma factor V (89). Whereas plasma factor V circulates as a single chain protein, platelet factor V is stored in a partially proteolyzed form that expresses significant cofactor activity (89). In addition, plasma factor V is preferably activated by thrombin whereas platelet factor V is more efficiently activated by factor Xa (127). These observations suggest that platelet factor V may initiate prothrombinase assembly and function during the initiation phase of coagulation, followed by further activation by thrombin (127). Platelet factor V activation generates heavy (105 kDa) and light (72/74 kDa) chain fragments which are indistinguishable from those recognized in plasma factor Va (127), except the N-terminal cleavage of platelet factor V occurs at Tyr-1543 rather than Tyr-1545 (89). Plasma and platelet factor Va are proteolyzed by APC at the same sites. However, compared with plasma factor Va, platelet

factor Va is proteolyzed at Arg-506 more slowly resulting in APC resistance (89,128).

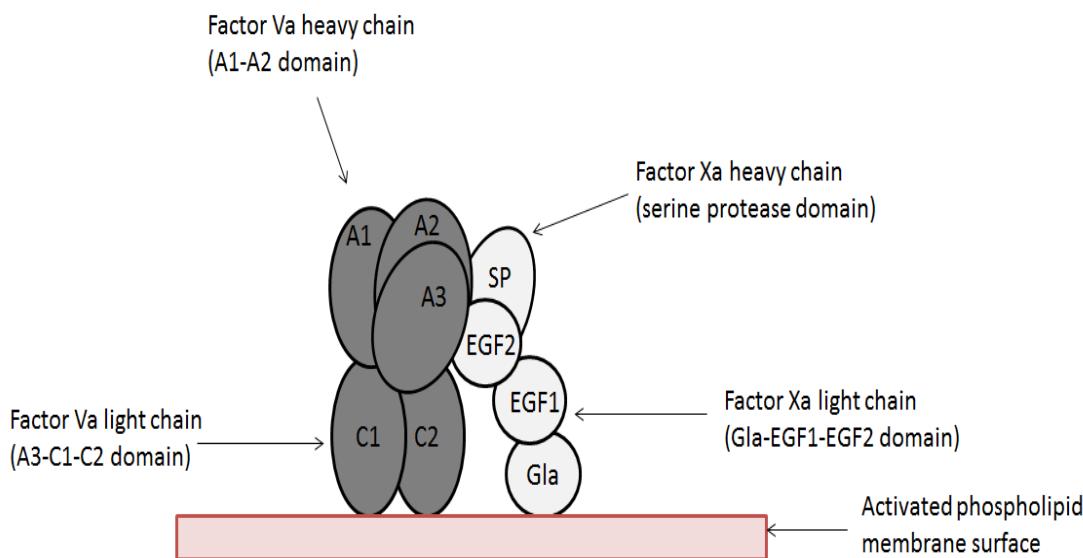
Platelet factor Va resistance to APC may be attributed to its unique features, including: resistance to phosphorylation at Ser-692 by platelet-derived casein kinase II (89); and the presence of an *O*-linked sugar residue at Thr-402 in platelet factor Va that may restrict phosphorylation by casein kinase II (89). It is also possible that the presence of factor V binding proteins in platelets, such as multimerin 1 (which is linked covalently and non-covalently to factor V) (16), contributes to the greater resistance of platelet factor Va to APC (discussed under Section 1.6-Overview of multimerin 1).



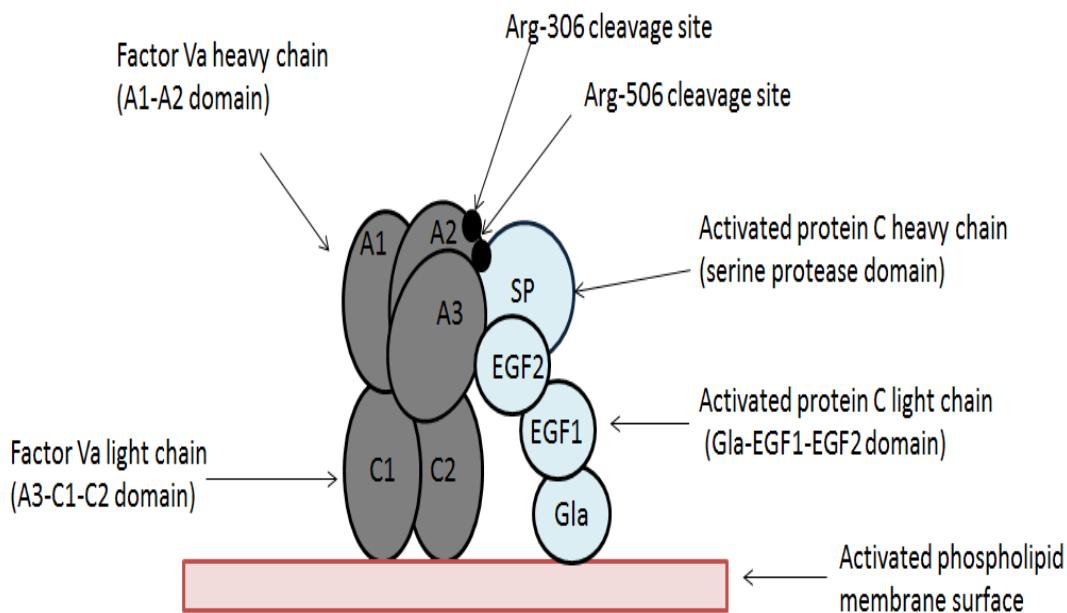
**Figure 2. Schematic of the factor V activation and factor Va inactivation pathway.**

Thrombin catalyzes factor V activation by initial cleavage at Arg (R)-709, followed by cleavage at Arg-1018, liberating the B-domain fragment (residues 710 to 1018) (45,91). Subsequent slower cleavage at Arg-1545 releases another B-domain fragment (residues 1019 to 1545) to generate the active cofactor, factor Va (45,91). Activated protein C and its cofactor protein S in the presence of a membrane surface catalyzes factor Va inactivation by initial cleavage at Arg-506 resulting in reduced factor Va activity

(104,129). Subsequent cleavage at Arg-306 results in full loss of factor Va activity (factor Vi) and dissociation of the A2 domain (104,129). The role of Arg-679 cleavage does not appear to be physiologically relevant (104,129).



**Figure 3. Current model of prothrombinase.** The model on the left in dark gray is factor Va (A1-A2 domains represent the heavy chain; and A3-C1-C2 domains represent the light chain). The model on the right in light gray is factor Xa (serine protease domain, SP, represents the heavy chain; and Gla-EGF1-EGF2 domains represents the light chain). Factors Va and Xa bind to the membrane (red box) via their light chain. The association of factor Va with the membrane enhances the ability of factor Xa to bind to the membrane surface. The A2 domain of factor Va has predominant factor Xa binding sites (130), whereas the EFG2 and SP domain of factor Xa have factor Va binding sites. Adapted from Austin et al. (130).



**Figure 4. Current model of factor Va-APC interaction.** The model on the left in dark gray is factor Va (A1-A2 domains represent the heavy chain; and A3-C1-C2 domains represent the light chain). The model on the right in light blue is activated protein C (APC; serine protease domain, SP, represents the heavy chain, and Gla-EGF1-EGF2 domains represents the light chain) (131). APC binds the regions in the heavy chain of factor Va and rapidly cleaves Arg-506 (black closed circle) (104,129). Subsequent cleavage at Arg-306 (black closed circle), which requires factor Va to be bound to the membrane (red box) via its light chain, liberates the A2 domain and results in full factor Va inactivation (104,129). Adapted from Segers et al (108).

## 1.5 OVERVIEW ON PROTHROMBIN AND ITS IMPORTANCE TO COAGULATION

Prothrombin is the precursor of thrombin. Prothrombin is a vitamin K-dependent, single chain glycoprotein that is synthesized in the liver and secreted into the plasma (42). Prothrombin has a molecular weight of 72 kDa and circulates in the plasma at a concentration of 1.4 µM (132). The prothrombin gene spans 21 kb in size, is located on chromosome 11p11-q12, and contains 13 introns and 14 exons (133). The gene encodes a mature protein of 579 amino acids (134,135). A mutation in the 3' untranslated region of the prothrombin gene (G20210A) has been linked to increased levels of plasma prothrombin and a 3-fold increased risk of venous thrombosis (136,137). Inherited prothrombin deficiencies in humans are rare (1 in 2 million) (42) and mouse embryos that are homozygous prothrombin-deficient do not survive (138).

Prothrombin is composed of four domains (Figure 5). The N-terminal γ-carboxyglutamate (Gla) domain (residues 1 to 40) rich in Gla and aromatic amino acid residues, two central kringle domains (residues 65 to 143 and 170 to 248 , respectively),

and a C-terminal catalytic serine protease domain (residues 321 to 579) (139).

Prothrombin undergoes proteolytic processing by factor Xa (alone or in prothrombinase) and feedback cleavages by thrombin (discussed below) that generates various products that retain specific domains and include: prothrombin fragment 1 (Gla and first kringle domain); prothrombin fragment 2 (second kringle domain), prethrombin 1 (second

kringle and serine protease domain), meizothrombin (all the domains), and prethrombin 2 (serine protease domain) and thrombin (serine protease domain) (140).

The presence of Gla residues is a signature of vitamin K-dependent blood clotting proteins (41). The Gla residues mediate calcium binding which induces a conformation change in these proteins that promotes the membrane-binding required for protein function (41). The Gla domain in prothrombin mediates membrane-binding and is required for prothrombin to be an effective substrate for prothrombinase (141,142).

The kringle domains are folded, contain multiple disulfide bonds, and common to coagulation factors including prothrombin (139,143). The first kringle domain in prothrombin has a potential role in binding factor Va which is important for the interaction of prothrombin with prothrombinase (144). The second kringle domain in prothrombin has several functional characteristics, which include binding to calcium ions and being the key binding site for factor Va (145-147). In addition, studies have demonstrated this interaction to promote factor Va cofactor activity (145,146). However, the effect of this interaction is not fully understood (147). Additionally, fragment 2, which contains only the kringle 2 domain, binds to thrombin ( $K_D \sim 5 \mu\text{M}$ ) (148) and has several potential roles, including: localizing clot formation by inhibiting thrombin inactivation by antithrombin (148), altering the catalytic site of thrombin (149), inhibiting thrombin cleavage of fibrinogen (148), and inhibiting protein C activation by altering its calcium dependence (150).

The serine protease domain is a characteristic property of all coagulation and fibrinolytic enzymes that play key roles in proteolytic zymogen activation in hemostasis, and are part of the chymotrypsin superfamily (reviewed in 151). The serine protease domain in prethrombin 2 and thrombin contains the same sequence (42). Prethrombin 2 is inactive and cleavage at Arg-320 by factor Xa (discussed below) generates the active enzyme thrombin composed of an A chain and B chain linked by a disulfide bridge (55). The B chain has a sodium binding site that induces allosteric activation and appears to modulate whether thrombin functions as a procoagulant protein and cleaves fibrinogen (in the presence of sodium), or as an anticoagulant protein that binds thrombomodulin to cleave protein C (in the absence of sodium) (152), based on changes in substrate specificity. The A chain has a potential role in mediating sodium binding, fibrinogen clotting, and PAR-1 cleavage (for platelet activation) (153). The structural model for prothrombin is currently unknown. However, a recent X-ray crystal structure of prethrombin 1 (kringle 2 and serine protease domain) was solved, which showed that the kringle 2 docking site is on top of the B-chain (154).

Prothrombin activation and function is mediated by specific bond cleavages (Figure 6) (55,140). Prothrombin has four potential cleavage sites (55,140). The activation of prothrombin to thrombin is catalyzed by factor Xa and it involves proteolysis at two sites: Arg-271 and Arg-320. The order of bond cleavage is regulated by factor Va (55). In the presence of factor Va (i.e. when prothrombin is activated by prothrombinase), prothrombin activation proceeds via initial cleavage at Arg-320 to generate meizothrombin (55,140). Similar to thrombin, meizothrombin is an active

product that appears to have procoagulant functions by activating factor V (155) and factor XI (156), as well as anticoagulant function for activating protein C (157). Finally, meizothrombin is cleaved at Arg-271 to yield fragment 1-2 and thrombin (55,140). In the absence of factor Va (or membrane vesicles), prothrombin activation by factor Xa occurs in the reverse order: this generates the inactive intermediate prethrombin 2 (via rapid cleavage at Arg-271) that undergoes slow cleavage at Arg-320 to generate thrombin and fragment 1.2 (55,140). Additional cleavages of prothrombin and its intermediates at residues Arg-155 and Arg-284 may occur by thrombin during the activation process (55,140). The cleavage of fragment 1.2 at Arg-155 causes dissociation of fragment 1 and 2 (55,140). The cleavage of thrombin at Arg-284, removes residues 1 to 13 from the N-terminus resulting in a more stable form of thrombin (55,140).

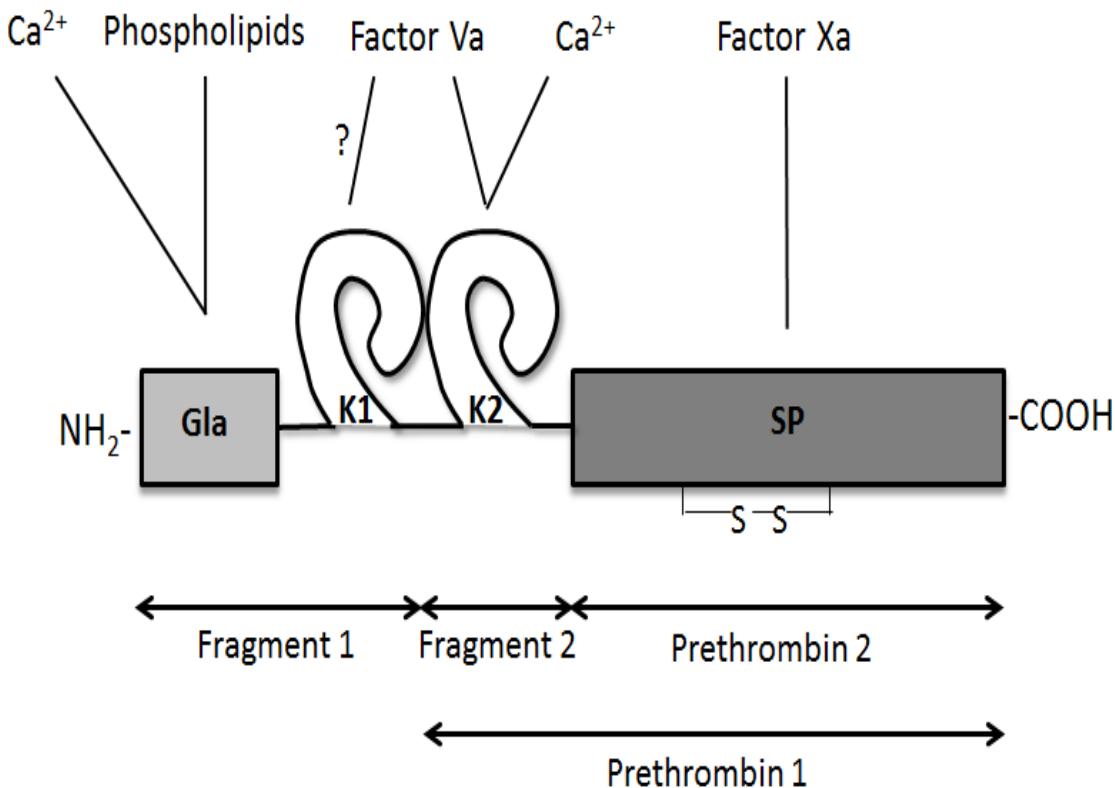
Prothrombin activation is regulated by individual constituents of prothrombinase. In the absence of phospholipids, factor Va, or prothrombinase there is approximately a respective 1000-, 10 000-, and 300-000 fold reduction in the rate of factor Xa-catalyzed prothrombin activation (6,56). Moreover, factor Va regulates the order of bond cleavage in prothrombin as it enhances the catalytic efficiency of Arg-320 cleavage by 20 000-fold which then increases the catalytic efficiency of Arg-271 cleavage by 24-fold (158). This suggests that in the presence of factor Va, Arg-271 in prothrombin is not available for cleavage until Arg-320 is cleaved. In the absence of factor Va, Arg-271 cleavage is 50-fold more effective than cleavage Arg-320 (158). Therefore, the incorporation of factor Va into prothrombinase may be to direct the Arg-320 cleavage site on prothrombin to the active site of factor Xa, and to shift the activation to the meizothrombin pathway

(55,140). Additionally, factor Va may lead to conformational changes in factor Xa exosites (substrate recognition site) involved in enhancing prothrombin recognition (159).

There are currently two proposed models of prothrombinase to describe the time course and concentration profiles of prothrombin and its intermediates upon activation (reviewed in Lee et al 62). The single form theory suggests prothrombin substrates bind to a single form of prothrombinase and undergoes sequential bond cleavage at Arg-320 followed by Arg-271 (160). A “ping pong-like” model suggests that there are two interconverting forms of prothrombinase that are specific for one of two cleavage sites on prothrombin (Arg-271 or Arg-320) (158). Both models can incorporate ratcheting whereby cleavage at one site in prothrombin enhances exposure of the other cleavage site (161); and channelling which involves direct conversion of prothrombin into thrombin, without release of prothrombin intermediate (162).

Inhibitors from plasma and platelets down-regulates prothrombinase activity to ensure that adequate but limited levels of thrombin are produced. The generation of the enzyme factor Xa from extrinsic tenase is inhibited by tissue factor pathway inhibitor (31). In addition, factor Xa is also inhibited by antithrombin (163). The cofactor, factor Va is proteolyzed by APC (104) (discussed in preceding Section 1.4-Overview of factor Va and its role in coagulation). Recently, it was shown that the factor V/Va binding protein multimerin 1 also has an inhibitory effect on prothrombin activation (discussed under Section 1.6-Overview of multimerin 1) (46). In addition, multimerin 1 was also found to bind prothrombin with a high affinity ( $K_D \sim 192$  nM) (unpublished data).

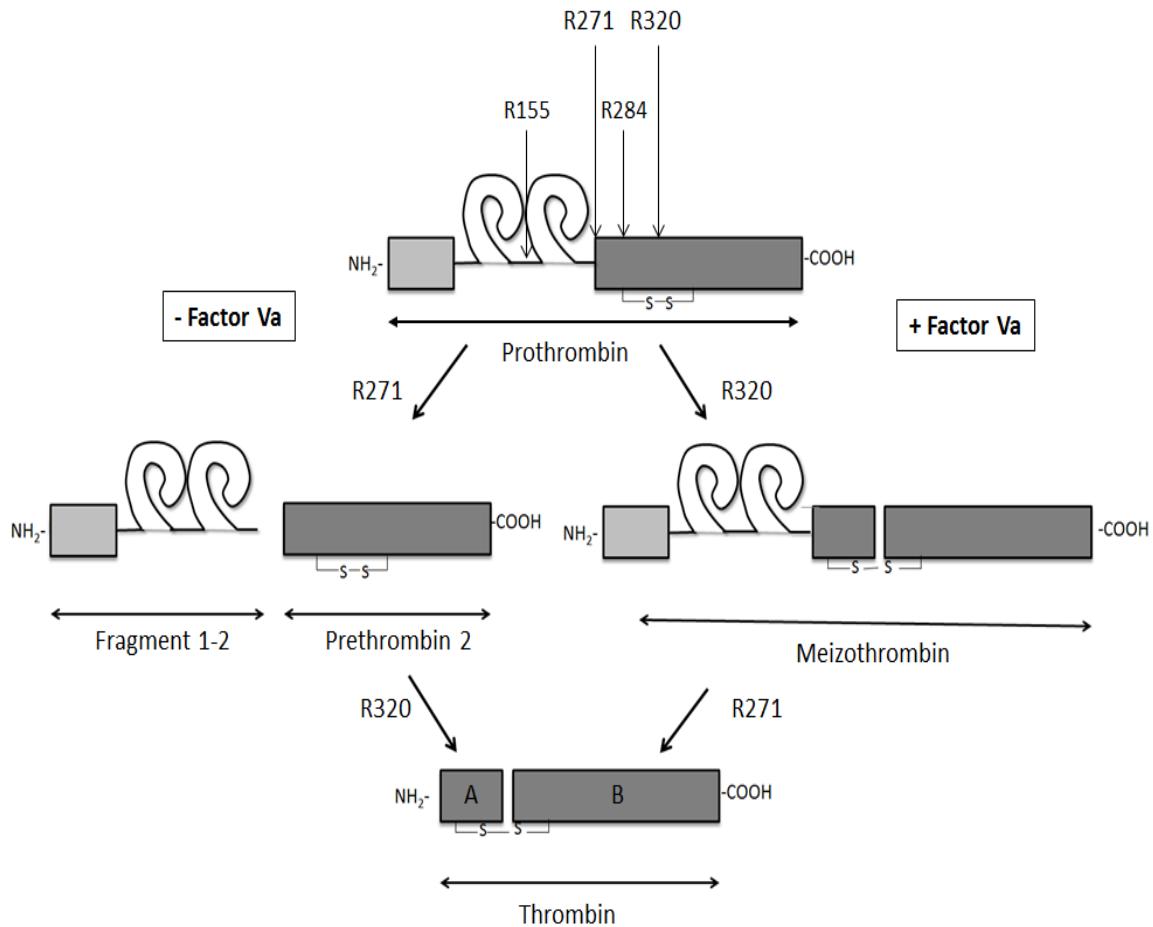
However, the role of multimerin 1 binding to prothrombin, in addition to factor Va, in regulating prothrombin activation is currently unknown.



**Figure 5. Binding sites in prothrombin domains for components in prothrombinase.**

Prothrombin is composed of four domains: the N-terminal  $\gamma$ -carboxyglutamic acid (Gla) domain, two central kringle domains — kringle 1 (K1), and kringle 2 (K2) — and a C-terminal serine protease (SP) domain (154). Fragment 1 is composed of the Gla and K1 domain, fragment 2 is composed of the K2 domain, prethrombin 1 is composed of the K2 and SP domains, and prethrombin 2 is composed of the SP domain. Prothrombin Gla domain has calcium binding sites that mediate phospholipid membrane binding (141,142). The first kringle domain has potential factor Va binding sites (144), whereas the second kringle domain has calcium and key factor Va binding sites (145-147). The

serine protease domain is the site of factor Xa binding and cleavage for prothrombin activation (140).



**Figure 6. Schematic of prothrombin activation pathways.** The activation of prothrombin to thrombin is catalyzed by factor Xa and involves proteolysis at two sites: Arg (R)-271 and Arg-320. In the absence of factor Va, factor Xa catalyzes prothrombin activation by initial cleavage at Arg-271 generating the inactive intermediates fragment 1-2 and prethrombin 2. Subsequent cleavage at Arg-320 results in thrombin formation (A- and B-chain linked by a disulfide bond). In the presence of factor Va, fully assembled prothrombinase (membrane-bound factor Va-factor Xa complex in the presence of calcium), catalyzes prothrombin activation by initial cleavage at Arg-320 generating the

catalytically active intermediate meizothrombin followed by cleavage at Arg-271 to produce thrombin. Thrombin can cleave prothrombin at Arg-155 to form prethrombin 1 and at Arg-284 to form a stable form of thrombin that lacks 13 amino acids in the A chain. Figure is adapted from Bukys et al. (55).

## 1.6 OVERVIEW ON MULTIMERIN 1

Multimerin 1 (short forms: human, MMRN1; mouse, Mmrn1) is a large, soluble “activation protein” sequestered in megakaryocyte, platelet and endothelial cell secretion granules (26,164-169) (reviewed in Jeimy et al. 16). MMRN1 is one of the largest proteins in the human body; it exists as a series of disulfide-linked trimers and large multimers (molecular weights of approximately 400 kDa to several million Daltons (170). MMRN1 designation is a reflection of its unique variably sized structure and its homology to another vascular multimeric protein, multimerin 2 (MMRN2) whose functions are unknown (170-172). MMRN1 and MMRN2 represent a subgroup of the EMILIN family of highly conserved, structurally related proteins (170-172).

In response to vessel injury, MMRN1 is released from secretion granules by activated platelets and endothelium for binding to their cell surfaces, and to extracellular matrix proteins, collagen and VWF (165,173-175). Although MMRN1 shares similarities with VWF, in that they are both large multimeric proteins with adhesive properties, and bind to homologous coagulation cofactors (factors V and VIII, respectively), they are not structurally related (13,26,50,171,174,175).

The human MMRN1 gene is located on chromosome 4 at position q22.1, is 60 kb in size, and contains seven introns and eight exons (16,176). *In vivo*, MMRN1 expression is restricted to megakaryocytes, platelets and the vascular endothelium (164,165,169,171,173). MMRN1 mRNA expression occurs as megakaryocytes and megakaryocytic cell lines differentiate, paralleling the changes in expression of other  $\alpha$ -

granule proteins (164,168,177). The MMRN1 cDNA is 4.7 kb in size and encodes the precursor protein preproMMRN1 (1228 amino acids) (171).

MMRN1 contains the following structural motifs: an Arg-Gly-Asp (RGD) motif, a cysteine rich EMI domain, central coiled-coiled sequences, leucine zipper sequences, epidermal growth factor (EGF)-like domain, and a C-terminal C1q-like globular domain (Figure 7) (16,171). These domains and other regions of MMRN1 are highly conserved which implies preservation of function (16,171). The EMI domain is an EGF-like structure found in other EMILIN proteins. The RGD and EGF domains are unique to MMRN1 in the EMILIN protein family (171,172). The RGD domain supports the adhesion of megakaryocytes, platelets and endothelial cells *ex vivo* (discussed below) while the EGF domain could have roles in mediating protein binding interactions (16,178). The C1q-like domain is found in related trimeric proteins and it could have roles in integrin binding (16). *In silico* analysis of MMRN1 structure predicts that the coiled-coiled domain forms  $\alpha$ -helices and the C1q-like domain forms a globular structure (unpublished). The functional roles of the different regions of MMRN1 in hemostasis are presently unknown.

MMRN1 biosynthesis involves a complex series of processing steps (16). MMRN1 assembles into homopolymers from preproMMRN1 following post-translational modifications that include: proteolytic processing of N-terminus to remove signal peptide to generate proMMRN1 subunits, N-glycosylation, addition of *O*-linked carbohydrates, assembly of disulfide-linked trimers and larger multimers, followed by further processing to remove propolypeptide domains (possibly by endoproteases)

(16,164,165,167,168). MMRN1 also contains motifs for tyrosine sulfation, aspartic acid/asparagine hydroxylation, and phosphorylation (16,171). Endothelial cells and megakaryocytic cell lines synthesize MMRN1 that is constitutively secreted as trimers, while normal megakaryocytes synthesize and store MMRN1 that include higher molecular weight polymers (164,164,165,168). The generation of mature MMRN1 subunits, by proteolytic processing of proMMRN1 subunits ( $M_r \sim 186$  kDa, fully glycosylated) does not disrupt polymer formation, and it generates the smaller MMRN1 subunits found in platelets and endothelial cells (16,164,165,167,168). Platelets are enriched with MMRN1 subunits with an N-terminal cleavage site that is cleaved near the RGD site to yield mature MMRN1 ( $M_r \sim 155$  kDa) (164,170,173). The cleavage at a more N-terminal site generates a larger form of platelet MMRN1 ( $M_r \sim 170$  kDa) (164,166,170,173). In addition, a subpopulation of platelet MMRN1 exists in disulfide linkage to factor V, and has unusual mobility (16,179). The different mobilities of endothelial and platelet MMRN1, after removal of their N-linked carbohydrate, suggest that MMRN1 undergoes cell-type specific proteolytic processing by endoproteases (165).

MMRN1 is sorted to platelet and endothelial cell storage granules for regulated secretion (165,167,168). The distribution of MMRN1 in platelets and endothelium is distinctive. Unlike most  $\alpha$ -granule proteins which are sorted to the central matrix (180), MMRN1 is stored with factor V and VWF in eccentric electron-lucent zones (24,26,164,174). In vascular endothelial cells, MMRN1 is predominantly sorted to round dense core granules distinct from VWF and P-selectin storage in Weibel-Palade bodies (165). As MMRN1 is not detected in plasma and is stored with factor V and VWF for

agonist-induced release (24,26,164,167,168), MMRN1 may have a key role in delivering endothelial and platelet proteins to sites of vessel injury and modulating adhesion and coagulation.

A selective defect or deficiency in MMRN1 has not yet been described in humans and/or mice (16). *Mmrn1* deficiency is not lethal as mice with spontaneous deletions of the genes encoding MMRN1 and  $\alpha$ -synuclein (a protein that inhibits  $\alpha$ -granule protein secretion *in vitro*) are viable (175,181). Platelet MMRN1 deficiencies have been described in human  $\alpha$ -granule disorders, some of which are associated with platelet factor V deficiencies (discussed under the preceding Section 1.4: Overview of factor Va and its importance to coagulation). MMRN1 deficiencies may contribute to impaired clotting and bleeding tendencies associated with these disorders.

MMRN1 is postulated to have roles in platelet adhesion based on its: large size, multimeric structure, adhesive RGD motif, ability to bind to: the extra cellular matrix, VWF and collagen, and the outer surfaces of platelets and endothelium upon release from storage granules at sites of vessel injury (16,164,165). Cell culture studies indicated that MMRN1 has the ability to assemble into extracellular matrix fibres that if deposited in the subendothelium, could have roles in platelet adhesion and thrombus formation (165,167). *In vitro*, MMRN1 supports the adhesion of megakaryocytes and activated platelets by RGD dependent mechanisms via integrin receptors such as  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_3$  (16,174,178). MMRN1 also supports the adhesion of endothelial cells, but by RGD-independent mechanisms, and via unidentified receptors (182). In addition, MMRN1 binds to phosphatidyl serine component of activated platelet membranes *in vitro* (46,183).

MMRN1 adhesion to platelets (and megakaryocytes), require the cells to be activated, and is influenced by shear rates *in vitro* (174). At low shear (150 per second), activated platelets adhere to MMRN1 via the integrins  $\alpha v\beta 3$  and GPIba, by VWF-independent mechanisms (174). At high shear (1 500 per second), activated platelets adhere to MMRN1 via GPIba, and which VWF binds to MMRN1 (174). MMRN1 has also been shown to enhance VWF-dependent platelet adhesion to collagen *in vitro* (174). Therefore, MMRN1 may facilitate platelet adhesion to exposed collagen at sites of vessel injury *in vivo*.

Mice that are deficient in both VWF and fibrinogen, two key mediators of platelet adhesion, can form platelet-rich thrombi (17), which suggest other adhesive ligands, perhaps MMRN1, could support platelet adhesion *in vivo*. In addition, the mice that are double deficient in Mmrn1 and  $\alpha$ -synuclein, have impaired platelet adhesion and aggregation, and thrombus formation which improved with transfusion of exogenous MMRN1 (175). Selective Mmrn1 deficient mice are under development to study Mmrn1 functions.

MMRN1 is a factor V and factor Va binding protein (26,46). The dissociation constant for factor V-MMRN1 and factor Va-MMRN1 binding,  $K_D \sim 2$  and 7 nM respectively, indicate they bind through high affinity interactions (46). *In vivo*, platelets contain MMRN1-factor V complexes in  $\alpha$ -granules derived from endocytosed plasma factor V and megakaryocyte synthesized MMRN1 (26,47,179). The unique structural and functional features of platelet factor V are discussed in the preceding Section 1.4 on: Overview of factor Va and its importance to coagulation, and it is possible that some of

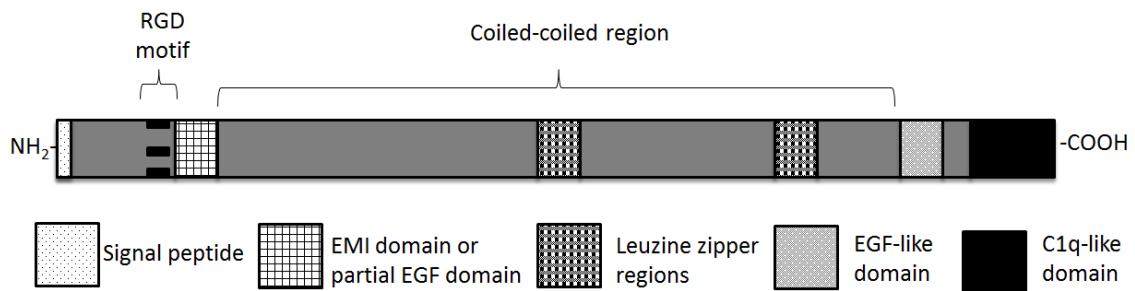
the unique functions of platelet factor V result from its association with MMRN1. In platelets, the majority of MMRN1-factor V complexes are non-covalent interactions whereas 25% contain factor V in disulfide linkage with MMRN1, via factor V B domain (cysteine residue 1085) (26,179). It is not possible to characterize the functional properties of factor V covalently bound to MMRN1 from non-covalent complexes because separation of these forms requires denaturants; also, methods to produce these forms *ex vivo* have not been established (16,179). The concentration of MMRN1 in normal blood is approximately 16 µg per 10<sup>9</sup> platelets, which is in 17 fold molar excess to the amount of factor V in platelets (16,179). Therefore, there is sufficient MMRN1 to bind additional plasma factor V at sites of vessel injury. The MMRN1 binding sites have been localized to the factor V/Va C1 and C2 domains of the light chain, which overlap binding sites for phosphatidyl serine that supports factor Va procoagulant functions (183,184). The activation of factor V by thrombin causes it to dissociate from MMRN1, liberating its light chain binding sites (26,46,179), and suggests a role for MMRN1 to localizing factor V/Va on platelet surface for prothrombinase assembly.

MMRN1 is also a prothrombin binding protein (unpublished data). The dissociation constant for the MMRN1-prothrombin interaction, K<sub>D</sub>~192 nM, (unpublished data) is higher than that for the MMRN1-factor V/Va interactions, but is far lower than the prothrombin concentration of 1.4 µM in plasma (185). These data suggests that plasma prothrombin would likely bind to released MMRN1 at sites of vessel injury. The MMRN1 binding sites on prothrombin are currently unknown. However, MMRN1 does not bind to thrombin (46) which retains the serine protease domain of prothrombin

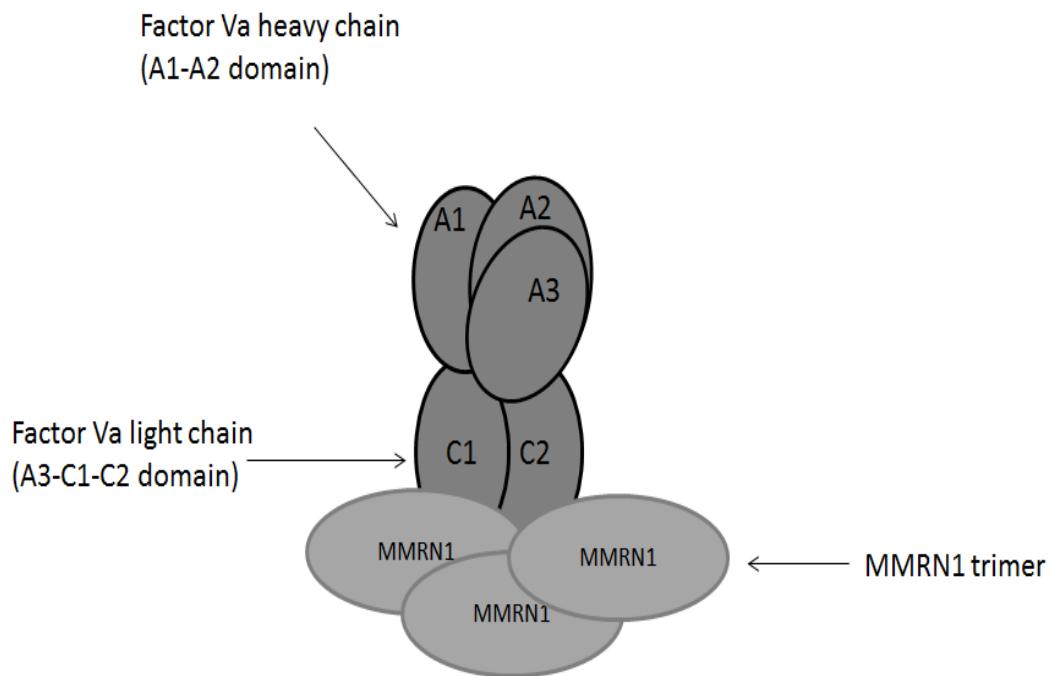
(42) or factor Xa (26), which shares a homologous Gla domain with prothrombin (41).

Therefore, it is possible that MMRN1 binding sites are localized to the kringle domains of prothrombin (proposed functions of prothrombin domains are discussed under the preceding Section 1.5-Overview of prothrombin and its importance to coagulation).

MMRN1 is an inhibitor of factor V activation and factor Va-dependent thrombin generation *in vitro*, whereby exogenous MMRN1 delays thrombin generation in plasma containing platelets, and preformed factor Va in plasma (46). In addition, exogenous MMRN1 delays factor V activation by factor Xa and thrombin in a purified system, and by adding tissue factor to plasma (46). Soluble phosphatidyl serine competitively inhibits both factor V-MMRN1 and factor Va-MMRN1 interactions. This is consistent with the observations that MMRN1 binds to factor V and factor Va in regions that overlap phosphatidyl serine binding sites (46,183,184). Therefore, prior to factor V activation and dissociation from MMRN1, factor Va will not be able to bind to the membrane surface, which is required for it to incorporate into prothrombinase for thrombin generation (45). As MMRN1 also binds to the substrate of prothrombinase, prothrombin (unpublished data), it is possible that the MMRN1-prothrombin interaction is involved in the inhibitory effect of MMRN1 on thrombin generation.



**Figure 7. Schematic of preproMMRN1 domains.** The precursor protein preproMMRN1 contains the following structural motifs (drawing is not to scale): a signal peptide, an RGD motif, a cysteine rich EMI domain, a central coiled-coiled region, leucine zipper regions, EGF-like domain, and a C-terminal C1q-like globular domain. N-terminal cleavage near the RGD site generates mature MMRN1. Figure is adapted from Jeimy et al. (16).



**Figure 8. Model of MMRN1-factor Va interaction.** The model in dark gray is factor Va (A1-A2 domains represent the heavy chain; and A3-C1-C2 domains represent the light chain). The model in light gray shows MMRN1 subunits that have formed a trimer although larger polymers also bind factor V/Va. The MMRN1 binding sites in factor V/Va are localized to residues in the C1 and C2 domains, in regions that overlap with phosphatidyl serine binding residues. Adapted from Jeimy et al. (184).

## 1.7 THESIS STUDY

MMRN1 binds to several components involved in prothrombinase assembly and function including the cofactor factor Va (16,46), the substrate prothrombin (unpublished data) and phosphatidyl serine of activated membrane surfaces *in vitro* (183). MMRN1 inhibits factor Va-dependent thrombin generation in plasma *in vitro* (46). This observation is postulated to be caused by MMRN1 inhibition of factor Va-membrane binding because MMRN1 binding sites on factor Va are in regions that overlap binding sites for phosphatidyl serine (46,183,184). However, as MMRN1 also binds to prothrombin (unpublished data), this interaction could be involved in MMRN1 inhibitory effects on thrombin generation. Furthermore, as platelet factor Va is resistant to proteolysis by APC (due to slow cleavage at Arg-506) (47,89), and MMRN1 is the major platelet factor V/Va binding protein (16,26,46), it is possible that MMRN1 contributes to protecting factor Va from APC proteolysis. However, MMRN1 binding sites are located in the light chain of factor Va (183,184), whereas the APC cleavage sites are located in the heavy chain of factor Va (104,108).

To provide novel information on how MMRN1 modulates blood coagulation, the present studies were undertaken to:

- 1) characterize the region(s) of prothrombin that bind to MMRN1,
- 2) determine the role of this interaction in regulating prothrombinase assembly and function, and
- 3) determine if MMRN1 effects factor Va cleavage by APC.

### **1.7.1 Hypotheses**

MMRN1 effects on thrombin generation are mediated through its interactions with both factor Va and prothrombin. MMRN1 binding sites on prothrombin are localized to regions that are not shared with thrombin, likely the kringle domains of prothrombin.

MMRN1 interaction with factor Va does not affect factor Va proteolysis by APC

### **1.7.2 Objectives**

The following objectives were addressed to test the hypotheses:

1. Elucidate the region(s) of prothrombin involved in binding to MMRN1.
2. Investigate the influence of MMRN1 in purified prothrombinase assays, with and without factor Va or PCPS, and the influence of immobilized MMRN1 on thrombin generation in plasma.
3. Determine the effect of MMRN1 on factor Va proteolysis by APC.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 MATERIALS

Human plasma factor Va, factor Xa, prothrombin, prothrombin fragment 1, prethrombin 1, prethrombin 2,  $\alpha$ -thrombin and fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) (71) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Small unilamellar phosphatidyl choline-phosphatidyl serine vesicles (3:1 ratio; PCPS) vesicles (186) and prothrombin fragment 2 (187) were generously provided by Dr. Jeffrey Weitz (McMaster University, Hamilton, ON). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Oakville, ON). Prionex was from Pentapharm Ltd (Basel, Switzerland).

#### *Sources of antibodies*

The following primary antibodies were used in ELISAs and Western blotting: murine monoclonal anti-MMRN1, JS-1 (epitope is located in residues 961-1139) (171) and RM2 (unknown epitope location), and rabbit polyclonal anti-MMRN1 (170,173); these antibodies were generated in our laboratory. Polyclonal sheep anti-prothrombin was purchased from Affinity Biologicals (Ancaster, ON). Murine monoclonal anti-factor V AHV5146 (epitope is located in heavy chain; residues 307-506) (63,101) was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Secondary antibodies used in the assays included: horseradish peroxidase (HRP)-conjugated anti-murine IgG, anti-

rabbit IgG, and anti-sheep IgG, which were purchased from Jackson ImmunoResearch Laboratories, Inc. (Mississauga, ON).

*Sources of supplies for molecular work*

ELISAs for protein analysis were performed using 96-well, clear, half area polystyrene test plates from Corning Life Sciences (Concord, MA). The normal pooled platelet lysate (contains  $10^9$  platelets/mL; 1 unit of MMRN1/mL) that was prepared as previously described in 179) was acquired from our laboratory stocks. ELISAs for protein binding assays were performed using 96-well, clear, 0.4 ml well MaxiSorp polystyrene test plates from Thermo Scientific (Rockford, IL). The substrate used in ELISAs was Colorburst<sup>TM</sup>Blue liquid 3, 5, 3', 5', tetramethylbenzidine (TMB) from Neogen Corporation (Lexington, KY). For SDS PAGE, ammonium persulfate, N, N, N', N'-tetramethylethane-1,2-diamine (TEMED) and pre-stained protein standards were purchased from Bio-Rad Laboratories (Hercules, CA). For Western blotting, chemiluminescent detection HRP substrate was purchased from Millipore (Billerica, MA), Amersham Hyperfilm X-ray film was purchased from GE Healthcare (Buckinghamshire, UK) and nitrocellulose transfer membrane was from Pall Corporation (Mississauga, ON). Thrombin generation assays with purified proteins were performed using 96-well, black, round bottom, test plates from Corning Life Sciences (Concord, MA). Thrombin generation assays with plasma were performed using 96-well, clear, round bottom, test plates from Corning Life Sciences (Corncord, MA).

*Sources of equipment*

The microplate reader (ELx808) and software (KC4, version 3.4) used for ELISAs were from Bio Tek Instruments (Winooski, VT). The Fluroskan Ascent fluorometer for thrombin generation assays with plasma was from Thermo Scientific (Rockford, IL). The SpectraMax M3 fluorescent plate reader and software (Softmax Pro) used for thrombin generation assays with purified proteins were from Molecular Devices (Sunnyvale, CA). The GS-800 calibrated densitometer used for densitometry analyses was from Bio-Rad Laboratories (Hercules, CA).

## 2.2 METHODS

### 2.2.1 Preparation of recombinant MMRN1

Recombinant MMRN1 was affinity purified from conditioned media of previously generated, stably transfected human embryonic kidney (HEK) 293 cells (167,173,188). In brief, the transfected HEK293 cells were grown in alpha Minimum Essential Media ( $\alpha$ -MEM) containing 10% fetal bovine serum, 10 mmol/L HEPES, 100 U/ml penicillin G, and 10  $\mu$ g/ml streptomycin (Gibco Invitrogen, Burlington, ON). The conditioned harvest media was collected after the cells have reached confluence, at which point it was centrifuged (2500 x g) to remove all cells and cellular debris, and supplemented with protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM N-ethyl maleimide (NEM; Sigma Aldrich, Oakville, ON); and 5 mM EDTA (before storage at -80 degrees Celsius).

To purify MMRN1, the conditioned media containing recombinant MMRN1 was incubated (overnight at 4 degrees Celsius) with the MMRN1 antibody JS-1, that had been pre-conjugated to CNBr-activated Sepharose 4B capture beads (1.14 g conjugated to 5 mg JS-1; GE Healthcare, Baie d'Urfe, QC). The capture bead slurry (200 ml) was poured into columns and the beads were then washed with a 20 times volume of phosphate buffered saline (PBS) containing 0.5 M NaCl, 5 mM EDTA, 5 mM NEM, and 0.25 mM PMSF. MMRN1 bound to the capture beads was then eluted with the addition of 3.5 M MgCl<sub>2</sub> (20 ml). The eluent was collected, concentrated and desalting by centrifugation in PBS or HEPES buffered saline (HBS; final: 20 mM HEPES-NaOH, 150 mM NaCl, pH

7.4) using centrifugal filter units (Amicon Ultra-15; Millipore, Billerica, MA) to a final volume of approximately 100 µl.

### **2.2.2 Protein analysis**

#### *Quantification of MMRN1 by ELISA*

Affinity purified recombinant MMRN1 was quantitated similar to methods previously described (173,188), using a sandwich ELISA specific for MMRN1. In brief, a test plate was coated with monoclonal anti-MMRN1 (JS-1; final: 10 µg/ml) in coating buffer (14.2 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub> and 3 mM NaN<sub>3</sub>, pH 9.6) for 1 hour at room temperature (RT). Plates were then blocked with PBS containing 0.05% Tween 20 and 2% BSA (PBST-BSA), overnight at 4 degrees Celsius. Serial dilutions of MMRN1 samples (1/500 to 1/64 000) were then added to the wells, in duplicate. To generate the standard curve, dilutions of pooled platelet lysate (contains 1 unit of MMRN1 antigen in 10<sup>9</sup> pooled platelet lysate/mL; equivalent to 18.5 µg recombinant MMRN1/mL) (179) were incubated alongside the MMRN1 samples (final MMRN1 concentrations: 0 to 20 milliunits/mL) for 1 hour at RT. To quantify the captured MMRN1, wells were incubated with rabbit polyclonal anti-MMRN1 (1:1000 dilution) for 1 hour at RT, followed by incubation with secondary goat anti-rabbit IgG antibody (1: 1000 dilution) for 1 hour at RT. The wells were washed with PBST (100 µl) three times between incubations. The ELISA plate was developed using a minute incubation with TMB substrate followed by addition of 1 M H<sub>2</sub>SO<sub>4</sub> to stop the reaction. The absorbance was read at 450 nm. All antibodies and proteins were added to wells in a volume of 50 µl, except BSA which was

added in a volume of 100 µl. The absorbance of the wells with recombinant MMRN1 samples was compared with that in wells with the normal pooled platelet lysate standard to determine the concentration of the recombinant MMRN1 sample. The molar concentrations of MMRN1 in subunits were calculated using an average recombinant MMRN1 subunit size of 186 kDa (183).

*Quantification of prothrombin and prothrombin activation products by protein assay*

Prothrombin and prothrombin activation products for use in ELISA binding assays were quantified by a bicinchoninic acid (BCA) assay according to the manufacturer's protocol (BCA Protein Assay Kit, Pierce, Rockford, IL). In brief, 25 µl of BSA standard (final: 25-2000 µg/ml) and 25 µl of diluted protein samples were incubated in separate wells in a microtiter test plate with 200 µl of working reagent (30 minutes, at 37 degrees Celsius), which contains an alkaline medium that causes proteins to reduce Cu<sup>2+</sup> to Cu<sup>1+</sup>. The reduced cuprous cation is chelated by two molecules of BCA which leads to the generation of a purple reaction product for colorimetric detection (189,190). The absorbance of the test plate was read at 560 nm on a plate reader at RT. The absorbance of the wells with diluted protein samples from stock solutions were compared to the absorbance of the BSA standard tested at known concentrations, to determine the concentration of the protein stock preparations. The assays were conducted once in duplicate.

*Evaluation of proteins by Western blotting and silver staining*

The mobilities and purity of MMRN1 preparations were assessed by Western blotting and silver staining after separation by reduced SDS-PAGE (170,173,191). The mobility and purity of prothrombin and activation products were assessed by either Western blotting or silver staining after separation by reduced SDS-PAGE. To prepare the proteins for Western blotting analysis (described below), normal pooled platelet lysate (as standard for MMRN1 mobility), recombinant MMRN1 samples (final: 25 and 50 mU), and prothrombin and activation product samples (final: 10 ng) were diluted with reducing 2X Laemmli sample buffer (10 % 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.12 M Tris, 0.004% bromophenol blue, pH 6.8) (192), and heated (90 degrees Celsius for 5 minutes). To prepare proteins for visualization by silver staining (described below), MMRN1 (final: 150 mU), prothrombin (final: 30 µg) and prothrombin activation product (final: 30 µg) samples were diluted in reducing 2X Laemmli sample buffer, and heated (90 degrees Celsius for 5 minutes), prior to being loaded onto the SDS-PAGE gel.

Western blotting of MMRN1 was performed as previously described (170,173). In brief, the proteins separated on 6% reduced SDS PAGE gels were transferred onto nitrocellulose membranes, which were then blocked with 5% milk powder in PBST. The membranes were incubated with monoclonal anti-MMRN1 (JS-1; final: 2 µg/ml) in block, for 1 hour at RT followed by incubation with HRP-conjugated anti-murine IgG antibody (final: 0.03 µg/ml) in block for 1 hour at RT. Three consecutive washes (5-10 minutes) with PBST were done between incubation steps. The membrane was developed

with chemiluminescent HRP substrate and exposed to an X-ray film for detection of immunoreactive bands. Western blotting of prothrombin and prothrombin activation products was conducted by the same protocol, except the detecting antibodies were sheep polyclonal prothrombin antibody (1:25 000 dilution) and donkey anti-sheep IgG antibody (1:25 000 dilution).

Silver staining of MMRN1, prothrombin and prothrombin activation products, was performed by a modified protocol (193) to visualize proteins. In brief, the reduced SDS PAGE gels were incubated in 250 ml of fix/stop solution (10% acetic acid and 90% deionized water) for 30 minutes followed by a 2 minute wash with distilled water. The gel was incubated in staining solution (375 µl of 37% formaldehyde, 250 mg of silver nitrate, and 250 ml of distilled water) for 30 minutes followed by a 10 second wash with deionized water. The gels were then incubated in developer solution (37% formaldehyde, 10 mg/ml sodium thiosulfate, 7.5 g sodium carbonate, 250 ml deionized water) for 5 minutes or until bands were visible. To stop the reaction, the fix/stop solution was added for 5 minutes, followed by a final wash with distilled water.

### **2.2.3 Binding assays**

The following binding assays testing the interaction of MMRN1-prothrombin and MMRN1-prothrombin activation products were performed by using a modified standard ELISA protocol (46,183,184). The test plates were developed with TMB substrate for 1 minute and the reaction was stopped with 1 M H<sub>2</sub>SO<sub>4</sub>. The absorbance of each well was

read at 450 nm. All antibodies and proteins were added to wells in a volume of 100 µl and all washes used a volume of 200 µl.

*MMRN1-prothrombin and MMRN1-prothrombin activation product binding assays*

Immobilized MMRN1 was tested for prothrombin binding at different prothrombin concentrations to determine if prothrombin binding to MMRN1 showed saturable binding (ELISA saturation curve). In separate experiments, prothrombin activation product binding to immobilized MMRN1, and MMRN1 binding to immobilized prothrombin activation products, was also tested. Prior to conducting the assay with immobilized MMRN1, a sandwich ELISA for detecting prothrombin antigen (protocol and Paired-Antibodies Kit from Cedarlane Laboratories, Burlington, ON) was conducted to verify that each prothrombin activation product that was captured by the sheep polyclonal prothrombin antibody could be detected by the HRP-conjugated polyclonal antibody against prothrombin (supplied from Paired-Antibodies Kit). In this ELISA, the test plate was developed with O-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, Oakville, ON) for 10 minutes, the reaction was stopped with 2.5 M H<sub>2</sub>SO<sub>4</sub>, and the plate was read at 490 nm.

MMRN1, prothrombin, or BSA (control for background binding) were coated to the test plate in coating buffer at a final concentration of 10 µg/ml, washed with PBST, and blocked (PBST-BSA) overnight at 4 degrees Celsius. MMRN1 was added to prothrombin- and prothrombin activation product-coated wells at a concentration (final: 50 mU/ml) determined by Ms. Jenny Yip to be below saturation in assays with

immobilized prothrombin (determined by ELISA saturation curve). Serial dilutions of prothrombin (final: 0-140 nM) were added to MMRN1-coated wells, and incubated for 1 hour at RT. In the separate experiment, prothrombin and prothrombin activation products were added to selected wells at a concentration determined from results of ELISA saturation curve. All testing was done using six consecutive washes between incubations to reduce non-specific binding.

To detect bound MMRN1, wells were incubated with the murine monoclonal anti-MMRN1 antibody (RM2; final: 2 µg/ml), followed by incubation with donkey anti-murine IgG (final: 1 µg/ml). To detect bound prothrombin or activation product, wells were incubated with a sheep polyclonal prothrombin antibody (1:1000 dilution) followed by incubation with a donkey anti-sheep antibody (1:5000 dilution). The absorbance measured for prothrombin and prothrombin activation products binding to immobilized MMRN1, and the absorbance measured for MMRN1 binding to immobilized prothrombin and prothrombin activation products were compared to the absorbance measured for background binding to BSA-coated wells. In addition, the absorbance measured for MMRN1 binding to immobilized prothrombin activation products were compared to the absorbance measured for MMRN1 binding to immobilized prothrombin, following correction for background binding to BSA.

## 2.2.4 Thrombin generation assays

### 2.2.4.1 Thrombin generation assays with purified proteins

To investigate the mechanism by which MMRN1 inhibits thrombin generation by prothrombinase, continuous thrombin generation assays were conducted in the presence or absence of factor Va or PCPS vesicles. Initial rates (kinetic measurement; linear appearance of product with time) of thrombin generation as described in (94,194,195), were measured in the presence or absence of MMRN1. Measuring initial rates is advantageous because it minimizes complicating factors, such as depletion of substrate, and progressive inactivation of the enzyme (i.e. factor Xa). All thrombin generation assays were performed using Assay Buffer containing HEPES (20 mM), CaCl<sub>2</sub> (final: 5 mM), 0.001% Tween-20, and 0.1% Prionex. The 96-well test plates (black, round bottom) were pre-treated with 2% BSA in coating buffer and washed with HBS with 0.1% Tween-20 (HBST) prior to conducting the assays to minimize protein adsorption.

Measurements of prothrombin activation were performed using the fluorescent thrombin inhibitor DAPA because: DAPA prevents feedback cleavage of prothrombin by activation products (i.e. meizothrombin and thrombin (140,196); and DAPA provides a useful technique to monitor the progressive activation of prothrombin since DAPA displays differential fluorescence enhancement properties when complexed to prothrombin activation products (i.e. prethrombin 2, meizothrombin and thrombin). The fluorescence intensity of DAPA was measured over time using a fluorescence plate reader (SpectraMax M3, Molecular Devices, CA). The excitation wavelength was 280 nm, the

emission wavelength was 545 nm, the cut off filter was 515 nm, and the temperature was set to 37 degrees Celsius. The software (Molecular Devices SoftMax Pro) generates plots of relative fluorescence intensity units (RFU) versus time (seconds) to evaluate substrate (prothrombin) activation over time. The meizothrombin-DAPA signal has greater fluorescence intensity than the DAPA-thrombin signal, but will only represent a minor fraction in assays with prothrombinase, since prothrombin activation proceeds to thrombin relatively quickly. The prethrombin 2- DAPA fluorescence intensity is much lower than thrombin-DAPA signal (and meizothrombin-DAPA signal) but may represent a significant fraction of prothrombin activation product in assays with factor Xa as the enzyme (without factor Va or PCPS), since prothrombin activation proceeds relatively slowly. As a control, the effect of MMRN1 on DAPA fluorescence was examined, by mixing 50 µl of thrombin (final: 3 µM) in Assay Buffer, with 50 µl of DAPA (final: 4 µM) with and without MMRN1 (final: 150 nM), and measuring the fluorescence intensity

To determine the initial rate of thrombin generation in molar terms, the plots of RFU versus time were truncated where RFU value increased linearly with time. The RFU value as a function of thrombin concentration (RFU/nM thrombin) was determined in a preliminary experiment: 50 µl of known thrombin concentrations (final: 0-1.5 µM) was mixed with 50 µl of DAPA (final: 2 or 4 µM) in Assay Buffer, and the fluorescence intensity was measured. Following correction for background DAPA fluorescence, without added thrombin, the measured RFU values were plotted versus thrombin concentration to generate a thrombin standard curve, to determine the relationship of RFU value per nM thrombin.

*Effect of MMRN1 on prothrombin activation by prothrombinase*

To examine the effect of MMRN1-factor Va interaction on prothrombin activation by prothrombinase in a purified system, the concentrations of factor Va were chosen to be near saturating for binding to factor Xa, whereby approximately 96 % of factor Va was predicted to bind factor Xa, based on binding affinities in the presence of membrane ( $K_D \sim 1$  nM) (45).

Reactions were initiated by rapidly mixing 50  $\mu$ l of Assay Buffer containing the substrate prothrombin (final: 1  $\mu$ M) and DAPA (final: 2  $\mu$ M) with 50  $\mu$ l of Assay Buffer containing prothrombinase (final concentrations: factor Xa, 10 nM; factor Va, 0.125 nM; PCPS, 50  $\mu$ M) that had been preincubated (15 minutes, RT) with a concentration of MMRN1 (final: 150 nM) predicted to bind 12% of the prothrombin and 96 % of the factor Va, based on binding affinities. As a control, BSA (final: 150 nM) was added to reaction without MMRN1. Two independent experiments were done, each with BSA (in duplicate) and MMRN1 (in single or duplicate).

As most MMRN1 preparations (average stock concentration: 25 U/ml) were purified and collected in PBS buffer, the effect of PBS on prothrombin activation by prothrombinase was examined. Reactions were initiated by rapidly mixing 50  $\mu$ l of Assay Buffer containing the substrate prothrombin (final: 1  $\mu$ M) and DAPA (final: 2  $\mu$ M) with a total of 50  $\mu$ l of Assay Buffer containing prothrombinase (final concentrations: factor Xa, 10 nM; factor Va, 0.125 nM; PCPS, 50  $\mu$ M) with and without 40  $\mu$ l of PBS.

*Effect of MMRN1 on prothrombin activation by factor Xa/PCPS vesicles (in the absence of factor Va)*

To examine the effect of MMRN1-prothrombin interaction on prothrombin activation by factor Xa, continuous thrombin generation assays were performed in the absence of factor Va. The concentration of prothrombin was chosen to saturate binding to factor Xa, in the presence of PCPS ( $K_m$  of prothrombin:  $0.25\pm0.01 \mu\text{M}$ ) (197). Reactions were initiated by rapidly mixing 50  $\mu\text{l}$  of Assay Buffer containing the substrate prothrombin (final: 1  $\mu\text{M}$ ) and DAPA (final: 4  $\mu\text{M}$ ) preincubated (15 minutes, RT) with a concentration of MMRN1 (15 minutes, RT; final: 1300 nM) predicted (by binding affinities) to bind 74% of prothrombin, with 50  $\mu\text{l}$  of Assay Buffer containing the enzyme factor Xa (final: 200 nM) and PCPS (final: 50  $\mu\text{M}$ ). As prothrombin activation is very slow in the absence of factor Va, DAPA was added in four-fold excess of prothrombin, to ensure all prothrombin activation signal (in RFU) would be detected over time. As a control, BSA (final: 1300 nM) was added to reaction without MMRN1. Two independent experiments were performed, each with BSA and MMRN1, in duplicate.

*Effect of MMRN1 on prothrombin activation by factor Xa-factor Va complex (in the absence of PCPS)*

To examine the effect of MMRN1-factor Va interaction on prothrombin activation by factor Xa in the absence of PCPS, the concentration of factor Va was chosen to be approximately equimolar (rather than saturating) to factor Xa in order to enhance prothrombin activation, and detect a sufficient fluorescence signal, since the reaction is

relatively slow in the absence of PCPS ( $K_D$  of factor Va-factor Xa interaction is  $\sim 800$  nM) (68). Reactions were initiated by rapidly mixing 50  $\mu$ l of solution containing the substrate prothrombin (final: 1  $\mu$ M) and DAPA (final: 2  $\mu$ M) with 50  $\mu$ l of solution containing the enzyme factor Xa (final: 25 nM) and factor Va (final: 30 nM) that had been preincubated (15 minutes, RT) with concentrations of MMRN1 (final: 150 nM) predicted to bind (by binding affinities) 12% of the prothrombin and 95% of the factor Va added to the reaction. As a control, BSA (final: 150 nM) was added to reaction with no added MMRN1. Two independent experiments were performed, each with a control (in duplicate or triplicate) and added MMRN1 (in duplicate).

#### **2.2.4.2 Thrombin generation assays with plasma**

##### *Study of matrix MMRN1 interaction with human and mouse plasma factor V*

As extracellular matrix proteins have important roles in localizing coagulation cofactors (i.e. VWF localizes factor VIII) (198), and MMRN1 has the ability to assemble into extracellular matrix fibres (165), a modified thrombin generation assay was conducted to test the effect of immobilized MMRN1 on plasma factor V (46,199). The thrombin generation assays were done similar to the calibrated automated thrombogram (CAT) methods described in Hemker et al. (199). The CAT method is recognized as a physiological functional test of the blood coagulation system (199). This method uses a fluorogenic thrombin substrate which increases fluorescence levels when cleaved by thrombin that is formed. In addition, this method continuously compares fluorescence measurements to a simultaneously run calibrator that has thrombin like activity in a

solution of the same plasma wherein no thrombin generation is initiated, in order to monitor thrombin generation over time (illustrated in the Thrombogram) (199). The parameters of the Thrombogram are the lag time (no observable thrombin generation), peak height (thrombin concentration steeply increases), the area under the curve (endogenous thrombin potential, ETP; proportional to amount of thrombin formed), and the time it takes for thrombin concentration to reach the peak (before going down) (199).

To test the effects of immobilized MMRN1 on plasma factor V, MMRN1 (700 mU/ml) or BSA (10 $\mu$ g/ml; control for non-specific binding) were added in coating buffer to the test plate for 1 hour. Before use, the test plate was washed three times with tris buffered saline (TBS; 20 mM Tris, 130 mM NaCl, pH 7.4) containing 0.02% Tween 20 (TBST), and blocked (TBST with 2 % BSA) overnight at 4 degrees Celsius. The test plate was washed three times with TBST followed by incubation with human plasma (1 hour at RT; final: 1000 ng/ml factor V) or block (TBST-BSA; control). The test plate was again washed three times with TBST, followed by the addition of factor V-deficient platelet poor plasma (George King Bio-Medical, Overland Park, NS). Next, 20  $\mu$ l of trigger solution (Thrombinoscope, BV, Maastricht, The Netherlands) containing recombinant tissue factor (final: 5 pM) and phospholipids (PS, PC, PE; final: 4  $\mu$ M) were added to MMRN1- and BSA-coated wells. In separate MMRN1- and BSA-coated wells, 20  $\mu$ l of calibrator solution ( $\alpha$ 2macroglobulin-thrombin) was added. The reaction was initiated by the automated addition of 20  $\mu$ l of FluCa solution containing calcium chloride (final: 16 mM) and fluorogenic substrate Z-Gly-Gly-Arg-AMC (final: 2.5 mM), to each well. ETP was measured using the Thrombinoscope software. As multimerin 1 structural motifs are

conserved in humans and mice (16,171), the same assay was conducted using mouse plasma (from McMaster Central Animal Facility, Hamilton, ON) instead of human plasma, in order to determine whether human multimerin 1 can capture mouse factor V to support thrombin generation.

### **2.2.5 Western blot and densitometry analysis of factor Va cleavage by APC**

#### *Effect of MMRN1 on APC-mediated inactivation of factor Va at Arg-506*

To examine the effect of MMRN1 on APC-mediated inactivation of factor Va at Arg-506 (site of slow cleavage in platelet factor V resulting in APC resistance) (47,89), factor Va inactivation assays were conducted similar to methods described in Tran et al (63). Western blots (12% reduced SDS-PAGE) were performed to visualize APC-mediated cleavage of factor Va in the presence and absence of MMRN1 *in vitro*. To target APC-mediated cleavage at Arg-506, the experiments were conducted in the absence of phospholipid vesicles, which prevents significant cleavage at Arg-306 (47,104). The factor Va inactivation reactions were conducted in eppendorf tubes precoated with BSA (1-2 hour incubation with 2% BSA in coating buffer, washed with HBST, and air-dried) to minimize protein adsorption.

The reactions were initiated by mixing plasma factor Va (final: 5 nM) that had been preincubated with or without MMRN1 (final: 0 and 480 nM; 15 min, RT, at a concentration chosen to bind 98% of the factor Va) in HBS (with 5 mM calcium chloride and 0.1% BSA). Following the addition of APC, aliquots of the mixture were taken at selected time intervals, mixed with 2X reducing Laemmli sample buffer (192) and heated.

In Western blotting, factor Va heavy chain was detected using monoclonal antibody AHV5146 (epitope is between residues 307-506) and HRP-conjugated anti-mouse IgG. The relative optical densities (OD) of the bands were obtained via densitometry analyses using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

### **2.2.6 Data analysis**

Data represented in this thesis are the means  $\pm$  S.E. of three independent experiments, unless otherwise indicated. One-way analysis of variance (ANOVA), followed by Tukey test, was used to compare several variables using GraphPad Prism Software (version 5.04), and to determine if there were significant differences between: MMRN1 binding to immobilized prothrombin and prothrombin activation products above background to BSA-coated wells; and prothrombin and prothrombin activation products binding to immobilized MMRN1 above background binding to BSA-coated wells.

Two-tailed Student's *t* test was used to compare two variables, and was used to determine if there was significant difference between: the means of initial rates of thrombin generation with and without MMRN1 in thrombin generation assays with purified proteins. In factor Va inactivation assays, two-tailed Student's *t* test was also used to determine if there was significant differences between the means of percent loss of factor Va heavy chain in reactions with and without MMRN1; and the means for factor Va cleavage product generation with and without MMRN1. In each statistical comparison, p-values  $<0.05$  were considered to be significant.

The following derived bimolecular equilibrium model (described in 200) was used to calculate the amount of factor Va ( $K_D \sim 7$  nM) or prothrombin ( $K_D \sim 192$  nM) bound to MMRN1:

$$[x:MMRN1] = \frac{K_D + [x_t] + \frac{[MMRN1_t]}{n} \pm \sqrt{(K_D) + [x_t] + \left[\frac{MMRN1_t}{n}\right]^2 - 4[x_t] \frac{[MMRN1_t]}{n}}}{2}$$

where  $x$  represents factor Va or prothrombin,  $[x:MMRN1]$  represents concentration of factor Va- or prothrombin-MMRN1 complex,  $n$  is number of MMRN1 monomers with a binding site for factor Va or prothrombin,  $x_t$  is the total amount of factor Va or prothrombin,  $MMRN1_t/n$  represents saturation of factor Va or prothrombin binding sites, and  $K_D$  is the equilibrium dissociation constant describing the factor Va- or prothrombin-MMRN1 interaction. Calculations were made assuming a 1:1 stoichiometry of factor Va- or prothrombin-MMRN1 interactions.

The percent of factor Va or prothrombin bound to MMRN1 was determined according the following equation:

$$x(\%) = \frac{[x:MMRN1]}{[x_t]} \times 100$$

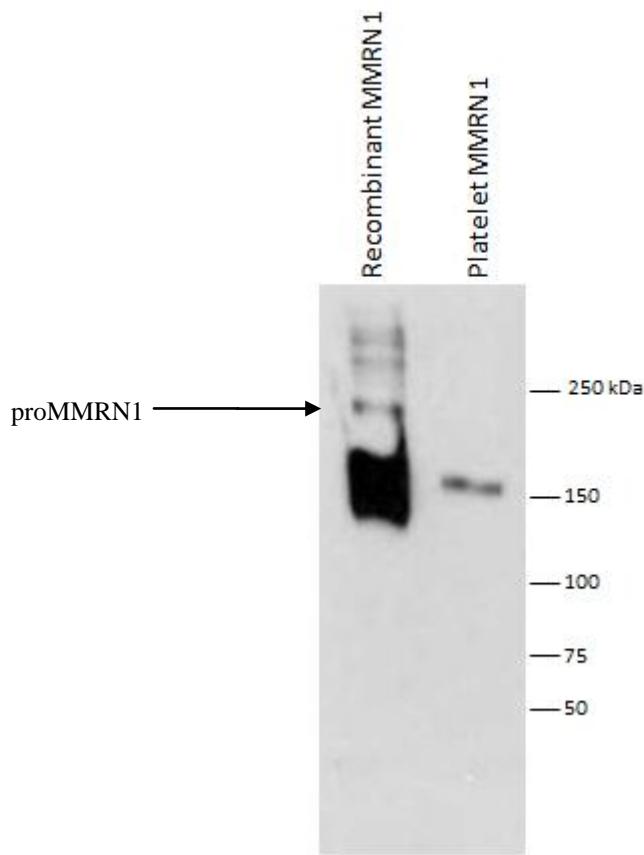
where  $x$  is factor Va or prothrombin,  $[x:MMRN1]$  is the concentration of factor Va- or prothrombin-MMRN1 complex, and  $x_t$  is the total amount of factor Va or prothrombin.

## CHAPTER 3

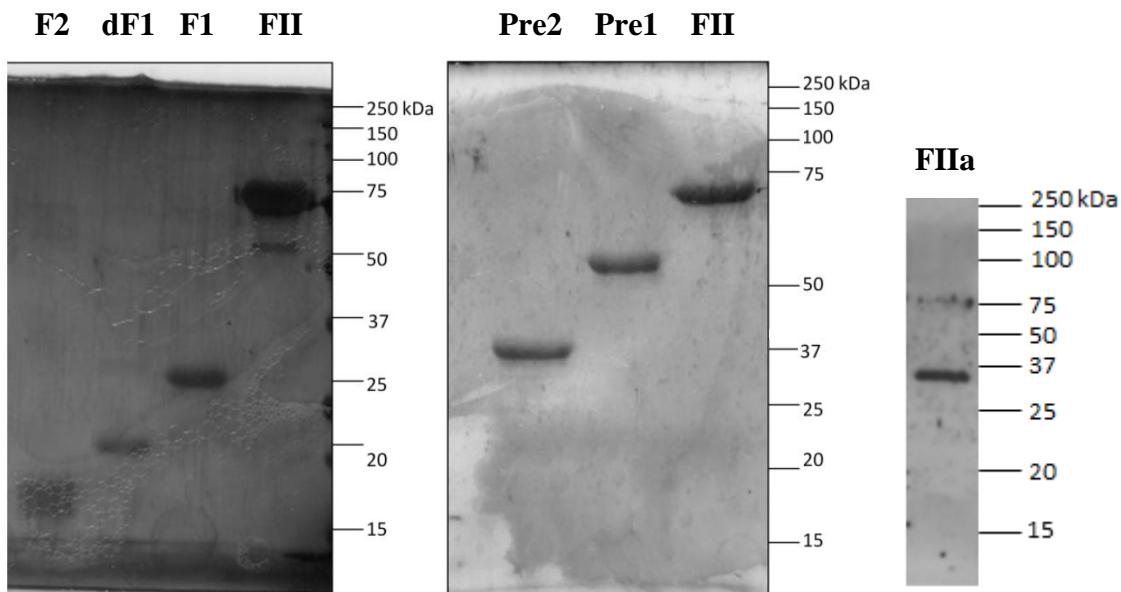
### RESULTS

#### **3.1 Subunit sizes of recombinant MMRN1, prothrombin, and prothrombin activation products**

Recombinant MMRN1, affinity-purified from conditioned media, and purified proteins: prothrombin, fragment 1, fragment 2, prethrombin 1, prethrombin 2 and thrombin were analyzed by reduced SDS-PAGE and Western blotting, and silver staining to verify their expected mobilities, before functional testing. The reduced immunoblot of recombinant MMRN1 (Figure 9) showed a high molecular weight band, consistent with the presence of fully glycosylated proMMRN1 ( $M_r \sim 186$  kDa), and smaller bands of recombinant MMRN1 that had undergone subunit proteolytic processing (165,167). The immunoblot of reduced platelet MMRN1 was used as a standard, and its major subunit migrated with the expected mobility ( $M_r \sim 155$  kDa). The reduced silver stain of prothrombin ( $M_r \sim 72$  kDa), fragment 1 ( $M_r \sim 28$  kDa), fragment 2 ( $M_r \sim 17$  kDa), prethrombin 1 ( $M_r \sim 67$  kDa), and prethrombin 2 ( $M_r \sim 37$  kDa), migrated with the expected mobilities, with the exception of one preparation of fragment 1 that migrated at a lower mobility than expected, likely due to degradation (Figure 10). The reduced silver stain of thrombin (A chain:  $M_r \sim 6$  kDa; B chain:  $M_r \sim 31$  kDa) migrated with the expected mobility. Only proteins that migrated with the expected mobilities were used in ELISA binding assays.



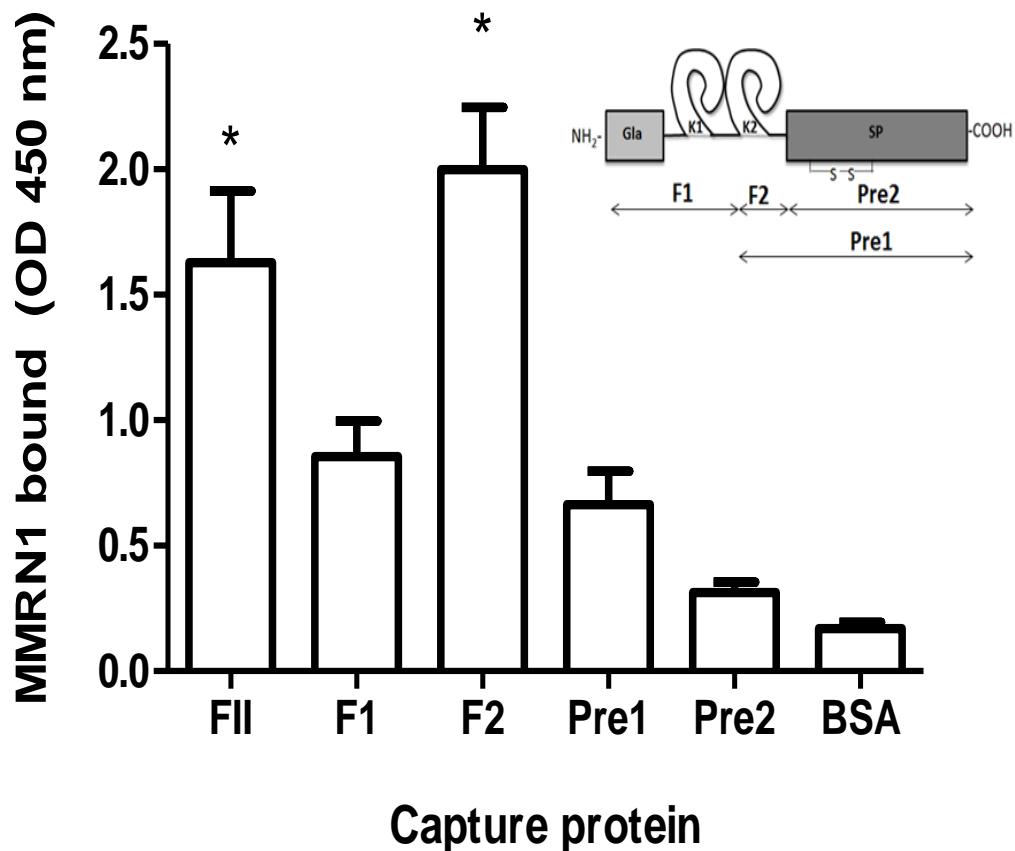
**Figure 9. Western blot analysis of reduced recombinant and platelet MMRN1.** This figure shows a representative Western blot of affinity purified recombinant MMRN1 and platelet MMRN1 from normal pooled platelet lysate, analyzed using 6% reduced SDS-PAGE. The MMRN1 precursor protein proMMRN1 is indicated. The larger bands, above proMMRN1, could reflect nonspecific recognition.



**Figure 10. Representative silver stain and Western blot analysis of prothrombin and prothrombin activation products.** This figure shows a representative reduced silver stain analysis of prothrombin (FII;  $M_r$ : 72 kDa), and its activation products: fragment 1 (F1;  $M_r$ : 28 kDa), fragment 2 (F2;  $M_r$ : 17 kDa), prethrombin 1 (Pre1;  $M_r$ : 67 kDa) and prethrombin 2 (Pre2;  $M_r$ : 37 kDa), on the left and central figure, that were used in experiments except F1 (dF1), that showed degradation. A representative reduced Western blot analysis of thrombin (FIIa B-chain;  $M_r$ : 31 kDa), is shown on the right.

### **3.2 MMRN1 binding to immobilized prothrombin and prothrombin activation products**

To investigate the binding of MMRN1 to immobilized prothrombin and prothrombin activation products, ELISA-based protein binding assays were performed. MMRN1 demonstrated significant binding to prothrombin and fragment 2 above background MMRN1 binding to BSA-coated wells ( $p<0.05$ ) (Figure 11). This data suggested that kringle 2 domain, found in fragment 2, has a MMRN1 binding site. In contrast, MMRN1 did not demonstrate significant binding to prethrombin 1, which also contains the kringle 2 domain (in addition to the serine protease domain)



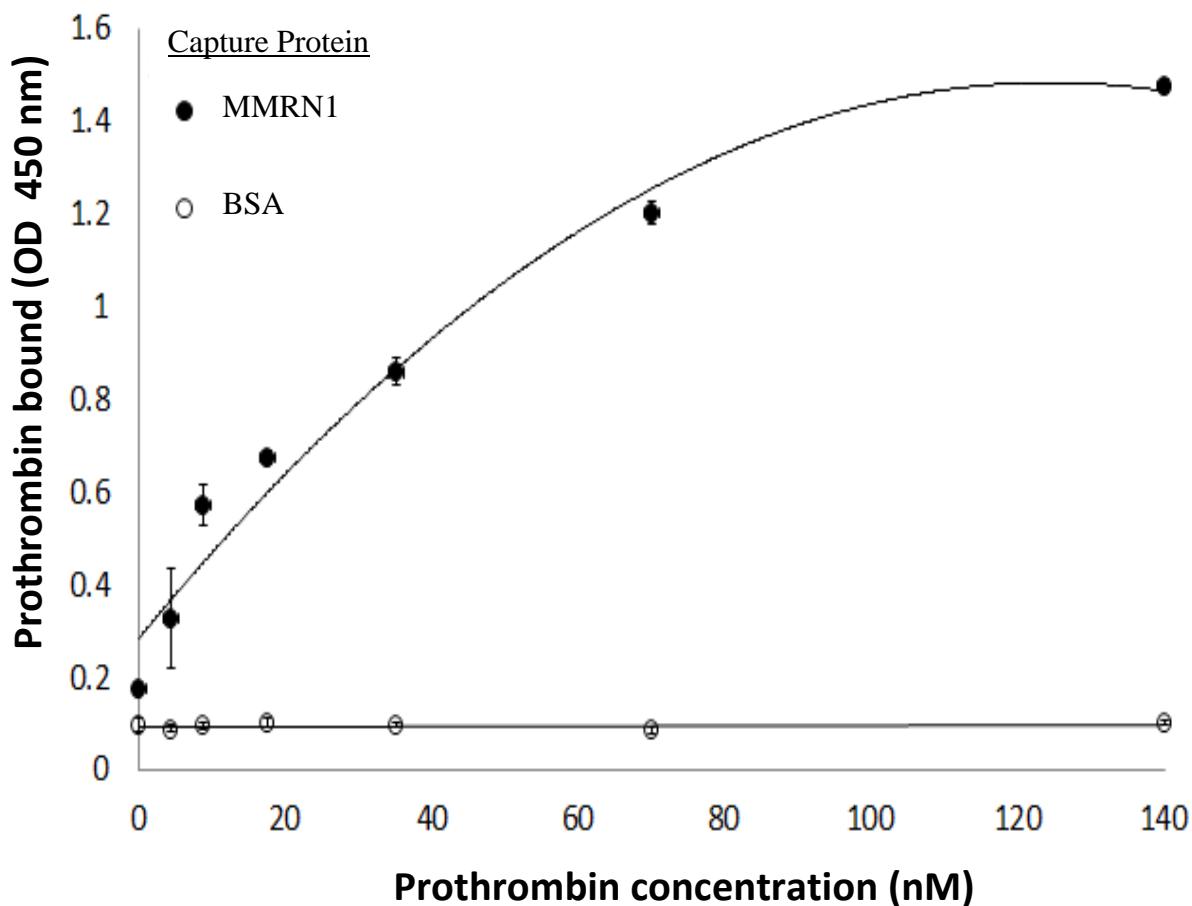
**Figure 11. The binding of MMRN1 to immobilized prothrombin (FII) or FII activation products, evaluated by ELISA.** Results show MMRN1 binding to immobilized FII, FII activation product (fragment 1, F1; fragment 2, F2; prethrombin 1, Pre1; and prethrombin 2, Pre2), or BSA, expressed as optical density (OD at 450 nm). Bars show means  $\pm$  S.E of three independent experiments, each done in triplicate. Prothrombin or prothrombin activation product that showed significantly more MMRN1 binding, above background binding to BSA-coated wells, are indicated (\*,  $p < 0.05$ ).

### **3.3 The binding of prothrombin and prothrombin activation products to immobilized MMRN1**

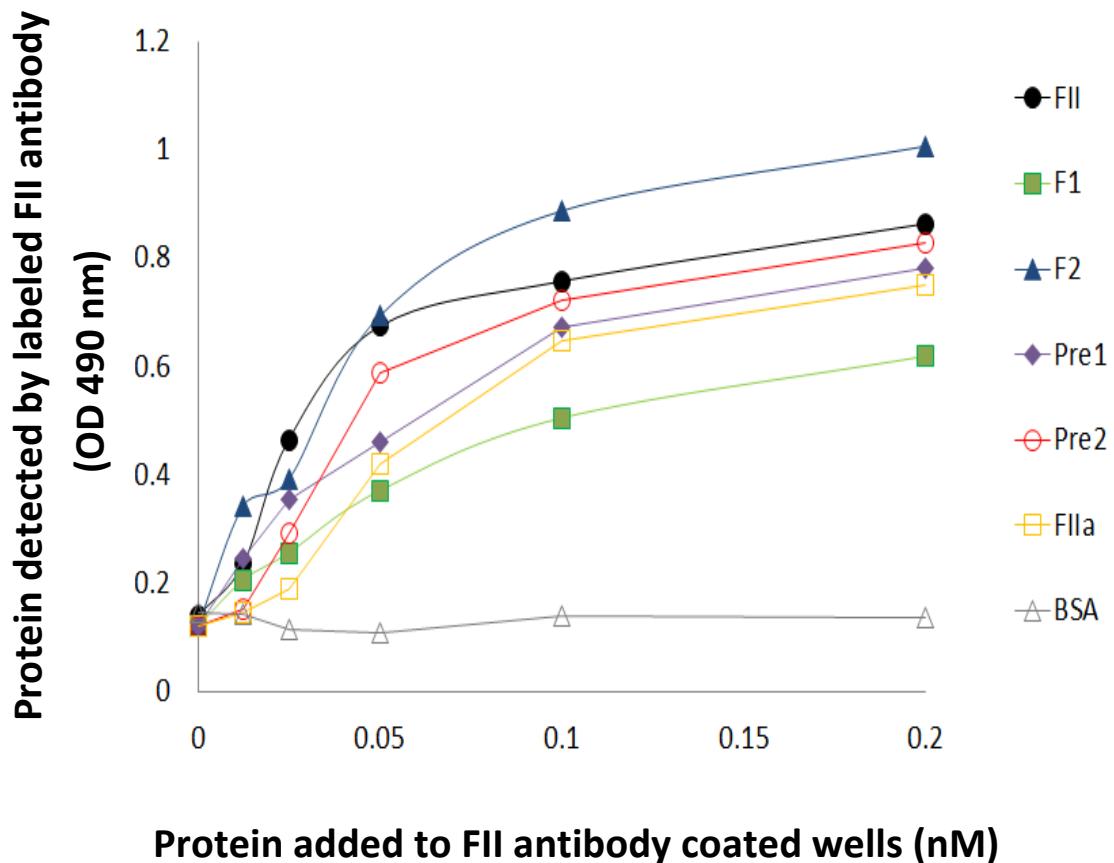
An ELISA-based protein binding assay indicated that prothrombin bound to immobilized MMRN1 was concentration-dependent and above the background binding to immobilized BSA ( $p<0.05$ ) (Figure 12). Based on these findings, a single concentration of prothrombin (35 nM) that was below saturation was selected for subsequent comparisons of the MMRN1 binding properties of prothrombin and prothrombin activation products.

The HRP-labelled polyclonal prothrombin antibody used for detection of prothrombin and prothrombin activation products (fragment 1, fragment 2, prethrombin 1, prethrombin 2, and thrombin), was verified to recognize these proteins, over a range of concentrations, bound to prothrombin antibody-coated wells (Figure 13). Based on this finding, prothrombin activation products were then tested for binding to immobilized MMRN1.

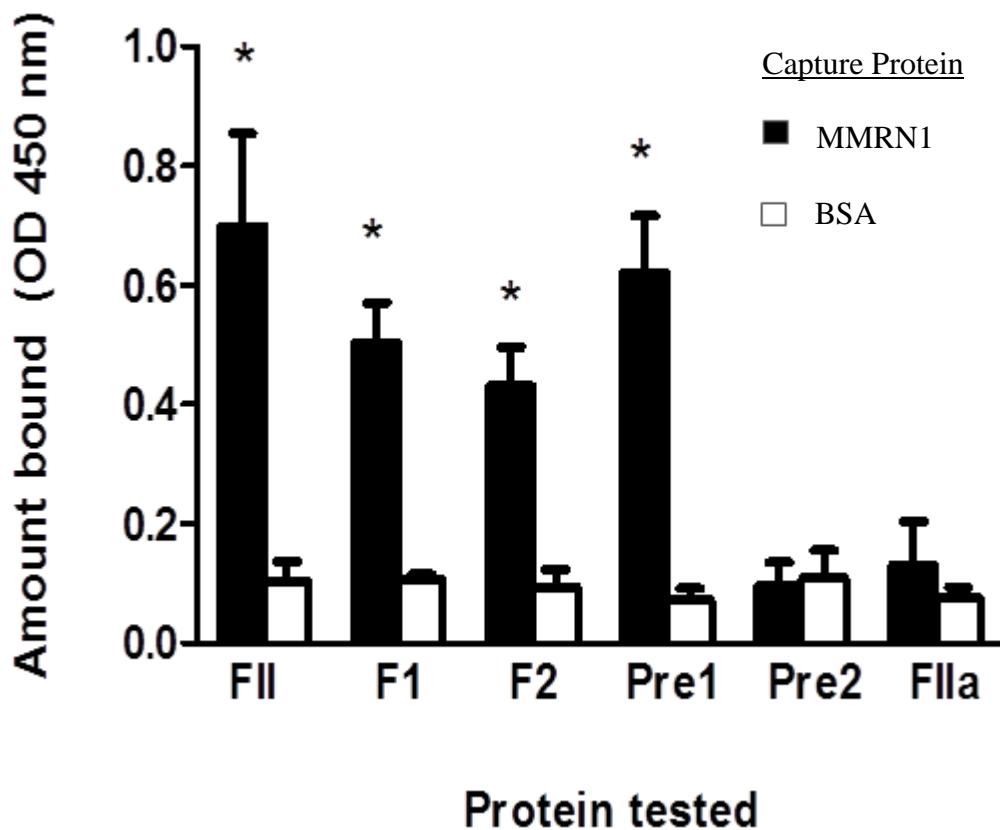
Prothrombin, fragment 1, fragment 2, and prethrombin 1 showed significant binding to immobilized MMRN1, above background binding to BSA ( $p<0.05$ ), as shown in Figure 14.



**Figure 12.** The binding of prothrombin to immobilized MMRN1, evaluated by ELISA. The binding of prothrombin to immobilized MMRN1 and BSA was compared over a range of prothrombin concentrations. The data shown represents the means  $\pm$  SE of a representative experiment, done in triplicate.



**Figure 13. Polyclonal prothrombin antibody detection of prothrombin and prothrombin activation products, evaluated by ELISA.** The capture of prothrombin (FII), prothrombin activation products (fragment 1, F1; fragment 2; F2; prethrombin 1, Pre1, prethrombin 2, Pre2, and thrombin, FIIa), or BSA to prothrombin antibody-coated wells, compared over a range of concentrations, was tested with polyclonal prothrombin antibody detection (peroxidase-conjugated). The data shown represents the means of a representative experiment, done in duplicate.



**Figure 14. The binding of prothrombin (FII) and FII activation products to immobilized MMRN1, evaluated by ELISA.** Results show FII or FII activation product (fragment 1, F1; fragment 2, F2; prethrombin 1, Pre1; prethrombin 2, Pre2; and thrombin, FIIa) binding to immobilized MMRN1 or BSA, expressed as optical density values (OD, at 450 nm). Bars show means  $\pm$  S.E. of three independent experiments, each performed in triplicate. Prothrombin or prothrombin activation product that showed significantly more binding to MMRN1 than to BSA are indicated (\*,  $p < 0.05$ )

### **3.4 Effect of MMRN1 on thrombin generation by factor Xa or prothrombinase in a purified system**

The thrombin standard curve shown in Figure 15 shows the relative fluorescence intensity (RFU) of DAPA measured over a range of thrombin concentrations. The data from the standard curve, which indicated a relationship of approximately 0.37 RFU/nM thrombin, was used to calculate the initial rates of thrombin generation (nM thrombin/second) by using the velocity (RFU/second) generated from the time course profiles of thrombin generation by factor Xa in prothrombinase, membrane-bound factor Xa, or factor Xa-factor Va.

The difference between the fluorescence intensity of DAPA with and without MMRN1 was approximately 3 percent, which suggests that MMRN1 has minimal effects on DAPA fluorescence (Figure 16). Further evaluations indicated that the initial rates of thrombin generation by prothrombinase with and without PBS were similar (0.68 and 0.67 nM thrombin/second, respectively), indications that the volume of PBS added with the MMRN1 has minimal affects (Figure 17).

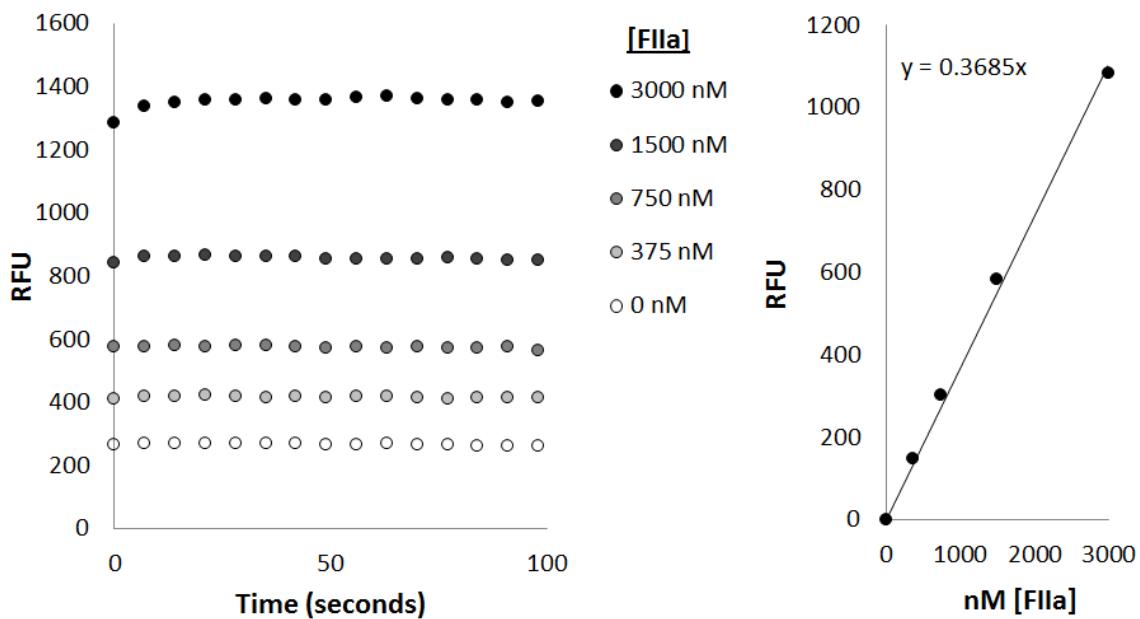
The effect of MMRN1-factor Va interaction on prothrombinase-catalyzed prothrombin activation was tested using thrombin generation assays with PCPS, the purified proteins prothrombin, factor Va and factor Xa and a concentration of MMRN1 predicted to bind 96% of the factor Va and 12 % of the prothrombin (Figure 18). For thrombin generation, the RFU signal above background DAPA fluorescence was observed. In these assays, MMRN1 significantly reduced the initial rate of thrombin

generation, compared to the control protein BSA ( $p=0.01$ ) (Table I). This data provides further evidence that MMRN1 inhibits factor Va-dependent thrombin generation. As all the components of prothrombinase are present in this assay, it is expected that the 1  $\mu\text{M}$  (1000 nM) prothrombin added to the assay, will be converted to thrombin relatively quickly. Based on the thrombin standard (Figure 15), 1000 nM thrombin generation would be represented by a change in fluorescence of approximately 300 RFU values. However, the observed change in RFU for thrombin generation was only approximately 80 RFU and thus lower than expected.

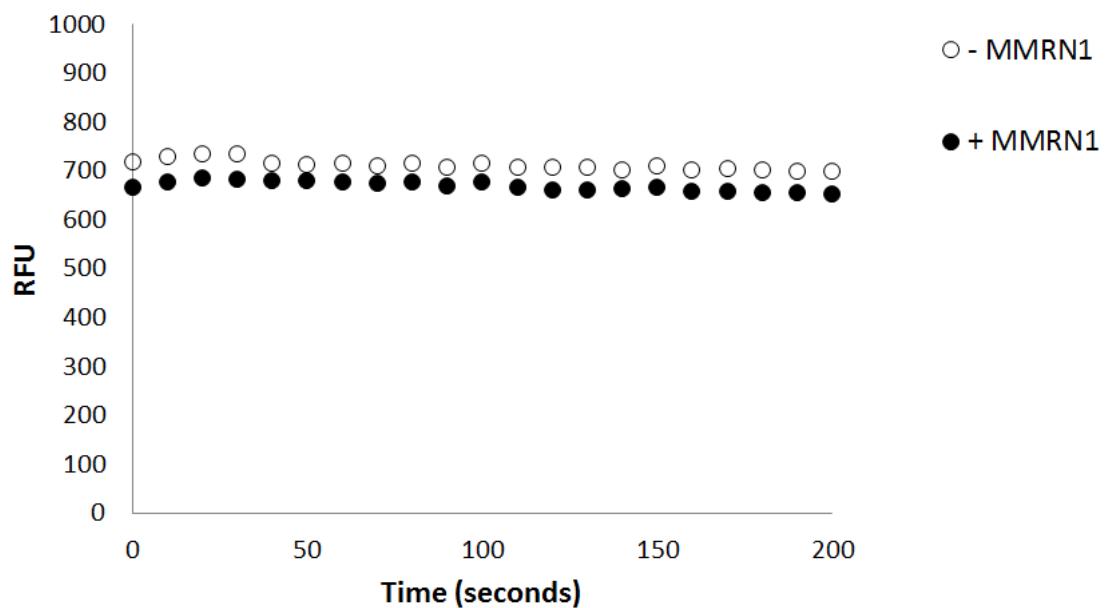
To examine if MMRN1 had inhibitory effects on thrombin generation independent of factor Va, prothrombin activation by factor Xa, in the absence of factor Va, was tested (Figure 19). Compared to BSA, MMRN1 significantly reduced the initial rate of thrombin generation when added at a concentration that was predicted to bind 74% of prothrombin ( $p=0.01$ ) (Table II). A minimal drift in RFU signal was observed at the beginning (but not towards the end) of the time course profiles of thrombin generation in the absence of MMRN1 or in the control “DAPA only” wells, and may be due to the relatively large amount of DAPA added.

Next, thrombin generation assays, with and without added MMRN1, were conducted in the absence of PCPS (Figure 20). In these experiments, MMRN1 had no significant effects on the initial rate of thrombin generation when added at a concentration predicted to bind 95% of the factor Va and 12% of the prothrombin ( $p=0.53$ ) (Table III). This suggest that the interaction of MMRN1 with factor Va, does not affect the factor Va

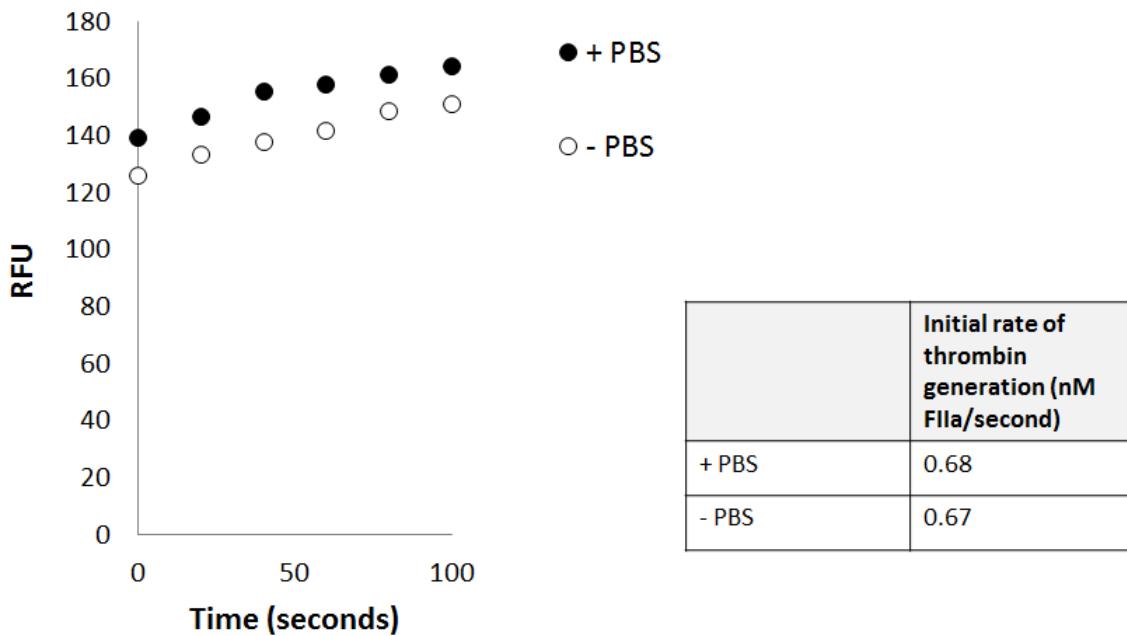
interactions with factor Xa that are required to activate prothrombin. Moreover, this suggests that the main inhibitory effect of MMRN1 on thrombin generation requires the presence of PCPS.



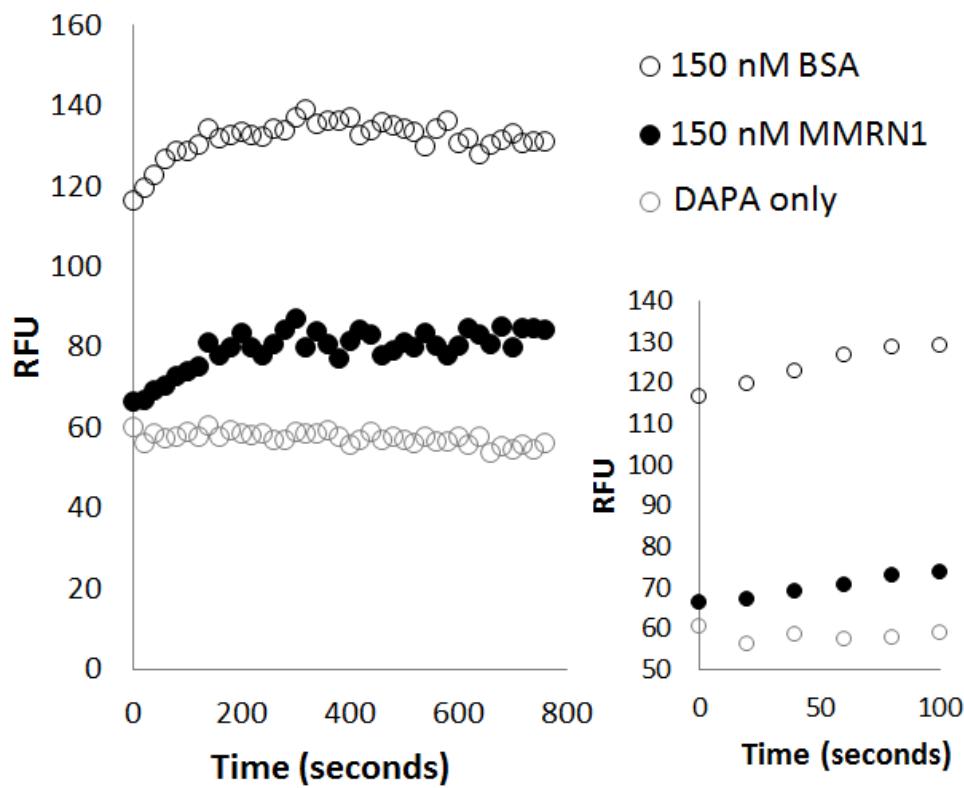
**Figure 15. Thrombin (FIIa) standard curve.** A FIIa standard curve was generated in order to measure the initial rates of FIIa generation, as outlined in Methods. The left figure shows the RFU values measured over a range of FIIa concentrations (final: 0-3  $\mu$ M) in the presence of DAPA (final: 4  $\mu$ M). The right figure shows the FIIa standard curve representing the RFU value measured for each FIIa concentration, following correction for background DAPA fluorescence signal. The relationship was determined to be approximately 0.37 RFU/nM FIIa.



**Figure 16. Effect of MMRN1 on the fluorescence intensity of DAPA.** The effect of MMRN1 on DAPA fluorescence intensity was determined by measuring the relative fluorescence units (RFU) of solutions containing thrombin and DAPA with or without MMRN1, as outlined in Methods.



**Figure 17. Effect of PBS on the initial rate of thrombin generation by prothrombinase.** As MMRN1 was purified into PBS, a thrombin generation assay was conducted to determine if PBS has an effect on the initial rates of thrombin generation, as outlined in Methods. The time course profile of prothrombin (final: 1  $\mu$ M) activation by prothrombinase (final concentrations: factor Xa, 10 nM; factor Va, 0.125 nM, PCPS, 50  $\mu$ M; DAPA, 2  $\mu$ M) in the presence or absence of PBS, over the first 100 seconds, is shown.

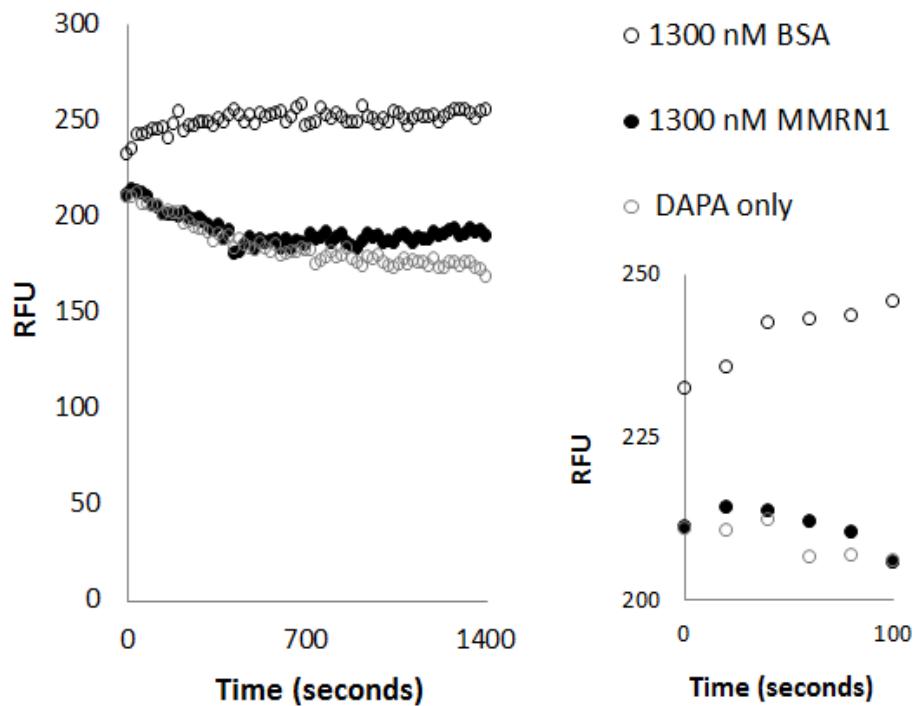


**Figure 18. Representative time course profile of thrombin generation by prothrombinase, in the presence of BSA or MMRN1.** Prothrombin (final: 1  $\mu$ M) was activated by prothrombinase (final concentrations: factor Xa, 10 nM; factor Va, 0.125 nM; PCPS, 50  $\mu$ M; DAPA, 2  $\mu$ M) in the presence of MMRN1 (closed black circles; final: 150 nM), or BSA (open black circles; final: 150 nM), that were preincubated with factor Va. The left profile shows data of the full time course profile of prothrombin activation, and the right profile shows data for prothrombin activation over the first 100 seconds, as outlined in Methods

**Table I. Effect of MMRN1 on the initial rate of thrombin generation by prothrombinase.** The initial rates of thrombin generation by prothrombinase were determined from continuous thrombin generation assays, with MMRN1 or the control BSA, that were preincubated with factor Va (described in Figure 18). The concentration of MMRN1 in the reactions was predicted (by binding affinities) to bind 12% of the prothrombin, and 96% of the factor Va. The data represent mean  $\pm$  S.E ( $n=3-4$ ).

	Protein preincubated with factor Va	
	BSA (final: 150 nM)	MMRN1 (final: 150 nM)
Mean initial rate of thrombin generation (nM thrombin/second) $\pm$ S.E.	0.56 $\pm$ 0.06	0.23 $\pm$ 0.03*
Relative activity	1.00	0.42

\* indicates significant difference between the mean initial rate of thrombin generation with MMRN1 compared to BSA ( $p=0.01$ ).



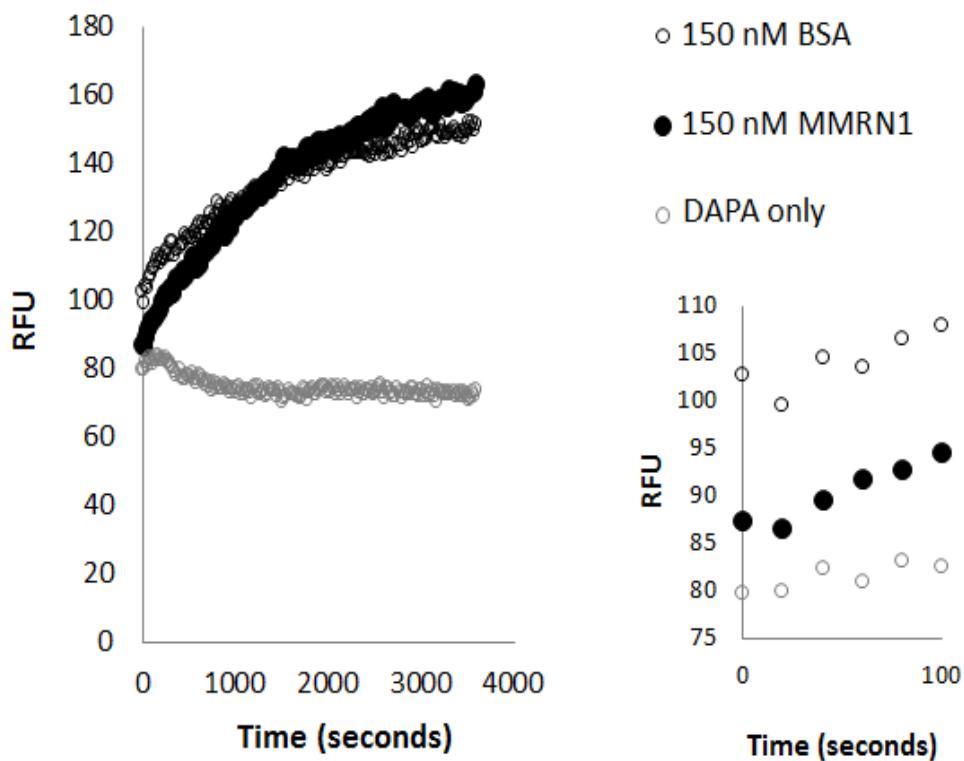
**Figure 19. Representative time course profile of factor Va-independent thrombin generation by factor Xa/PCPS vesicles, in the presence of BSA or MMRN1.**

Prothrombin (final: 1  $\mu$ M) was activated by factor Xa/PCPS vesicles (final concentrations: factor Xa, 200 nM; PCPS, 50  $\mu$ M; DAPA, 4  $\mu$ M) in the presence of MMRN1 (closed black circles; final: 1300 nM), or BSA (open black circles; final: 1300 nM), that were preincubated with prothrombin. The left profile shows data for the full time course profile of prothrombin activation, and the right profile shows data for prothrombin activation over the first 100 seconds.

**Table II. Effect of MMRN1 on the initial rate of factor Va-independent thrombin generation by factor Xa/PCPs.** The initial rates of thrombin generation assays with MMRN1 or the control BSA, that were preincubated with prothrombin (described in Figure 19). The concentration of MMRN1 in the reaction was predicted (by binding affinities) to bind 74% of the prothrombin. The data represent mean  $\pm$  S.E ( $n=3-6$ ).

	Protein preincubated with prothrombin	
	BSA (final: 1300 nM)	MMRN1 (final: 1300 nM)
Mean initial rate of thrombin generation (nM thrombin/second) $\pm$ S.E.	0.32 $\pm$ 0.06	0.00 $\pm$ 0.00*
Relative activity	1.00	0.00

\* indicates significant difference between the mean initial rate of thrombin generation with MMRN1 compared to BSA ( $p=0.01$ ).



**Figure 20. Representative time course profile of phospholipid-independent thrombin generation by factor Xa/factor Va, in the presence of BSA or MMRN1.** Prothrombin (final: 1  $\mu$ M) was activated by factor Xa/factor Va (final concentrations: factor Xa, 25 nM; factor Va; 30 nM; DAPA, 2  $\mu$ M) in the presence of MMRN1 (closed black circles; final: 150 nM), or BSA (open black circles; final: 150 nM), that were preincubated with factor Va, without PCPS vesicles in the assay. The left profile shows data for the full time course profile of prothrombin activation, and the right profile shows data for prothrombin activation over the first 100 seconds.

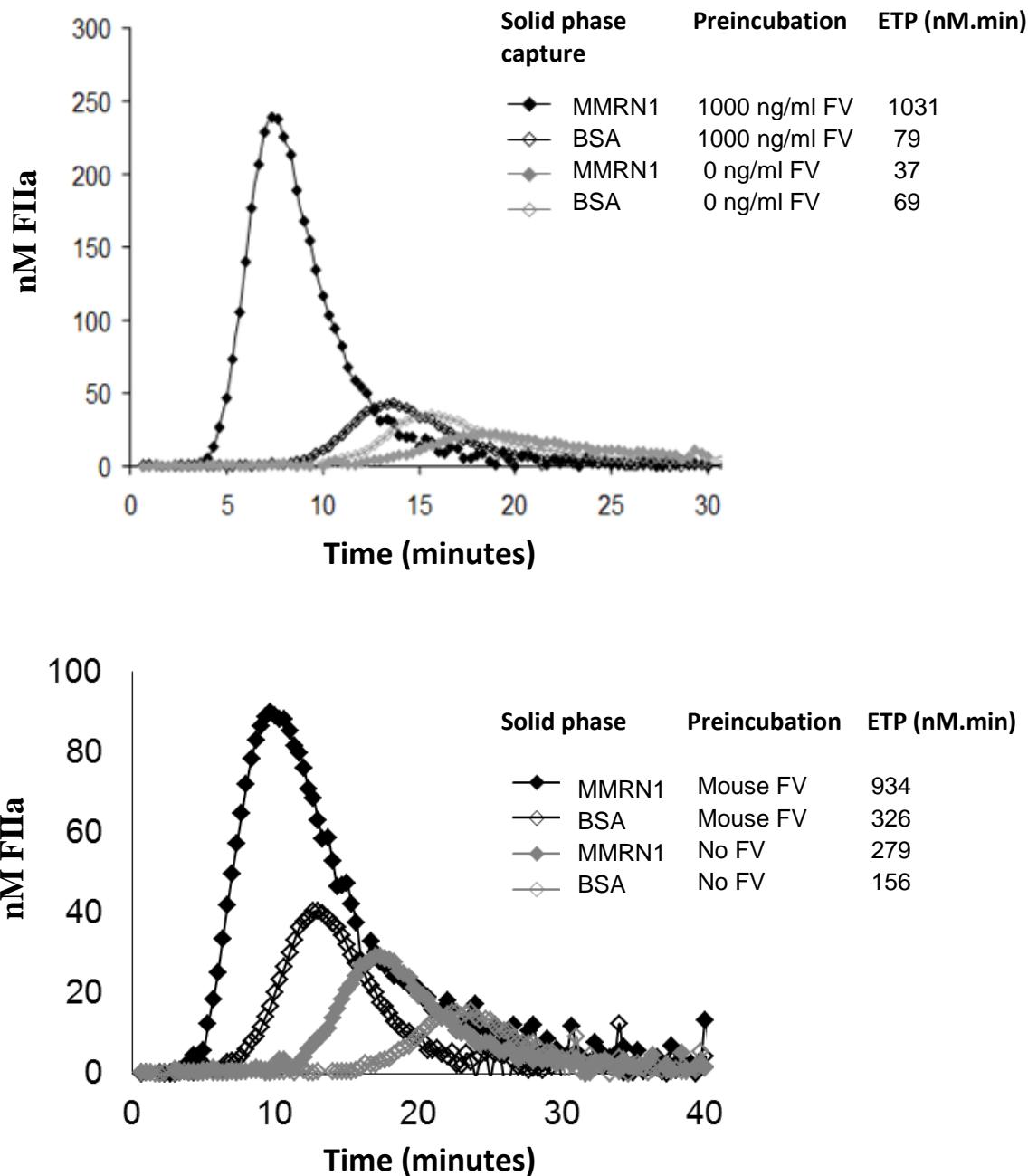
**Table III. Effect of MMRN1 on the initial rate of phospholipid-independent thrombin generation by factor Xa/factor Va.** The initial rates of thrombin generation by factor Xa/factor Va were determined from continuous thrombin generation assays with MMRN1 or the control BSA, that was preincubated with factor Va (described in Figure 20). The concentration of MMRN1 in the reactions was predicted (by binding affinities) to bind 12% of the prothrombin, and 95% of the factor Va.

	Protein preincubated with factor Va	
	BSA (final: 150 nM)	MMRN1 (final: 150 nM)
Mean initial rate of thrombin generation (nM thrombin/second) ± S.E.	0.30 ± 0.05	0.26 ± 0.04*
Relative activity	1.00	0.85

\* indicates that differences between the mean initial rate of thrombin generation with MMRN1 compared to BSA were not significant ( $p=0.53$ )

### **3.5 Study of matrix MMRN1 interaction with human or mouse plasma FV**

To determine if MMRN1, immobilized onto a surface, could bind human and/or mouse factor V in a manner that supports subsequent thrombin generation, modified thrombin generation assays were conducted. Immobilized MMRN1 was preincubated with human or mouse normal plasma, washed, and then thrombin generation was initiated in these wells using factor V-deficient plasma. Unlike BSA-coated wells, immobilized MMRN1 was able to capture sufficient factor V from human and mouse plasma to generate measurable thrombin when human factor V-deficient plasma was added to the wells (Figure 21). The small amount of thrombin generated in the absence of MMRN1 could reflect factor Va-independent thrombin generation or the binding of small amount of factor V/Va to BSA-coated wells. These data indicated that immobilized MMRN1 bind both mouse and human plasma factor V by mechanisms that allow factor V/Va to function in thrombin generation and suggest conservation of the regions of factor V/Va involved in MMRN1 binding.



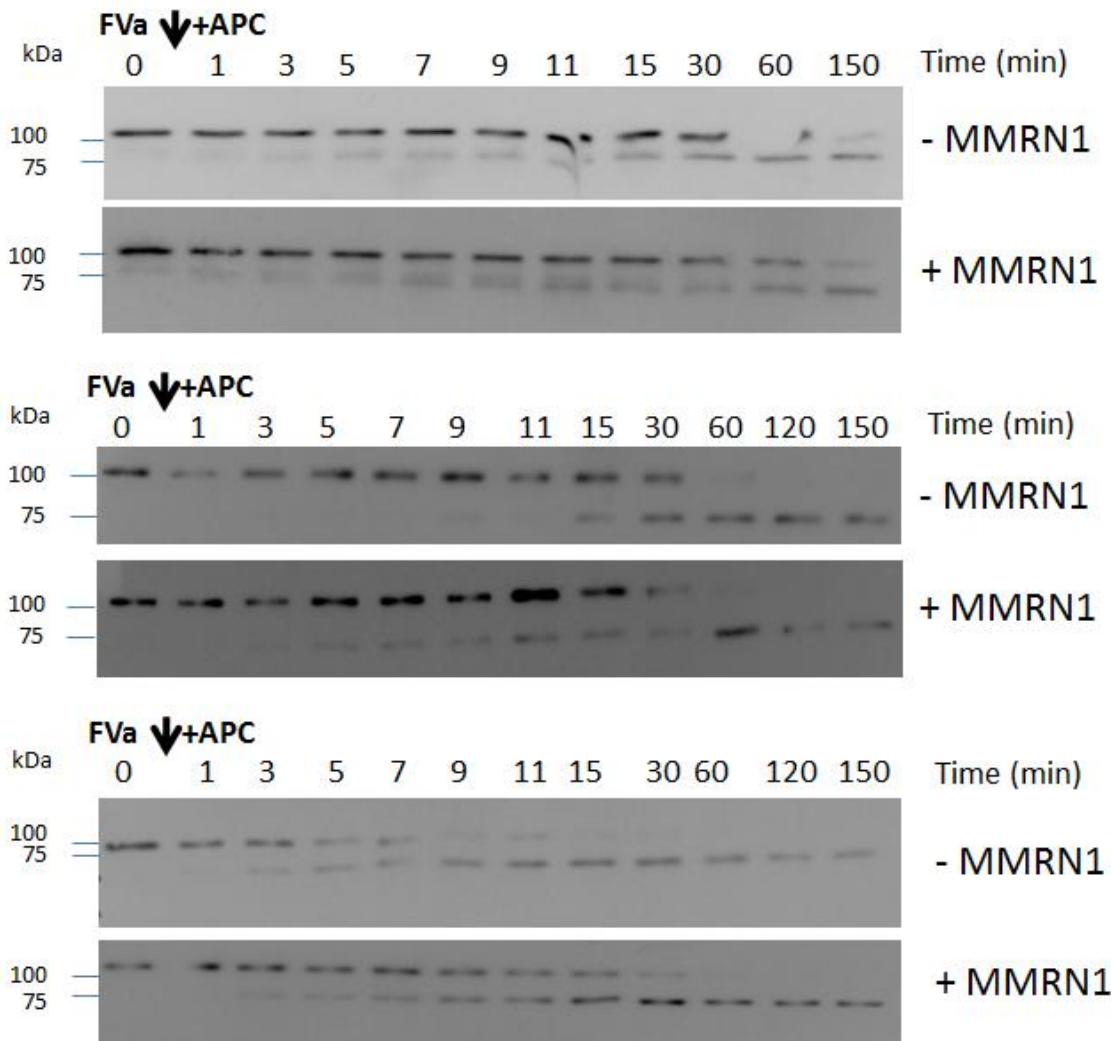
**Figure 21. Immobilized MMRN1 interaction with human or mouse plasma factor V.**

MMRN1 or BSA were added to the tests plate, preincubated with (A) human or (B) mouse normal plasma, washed, before testing thrombin generation with added human

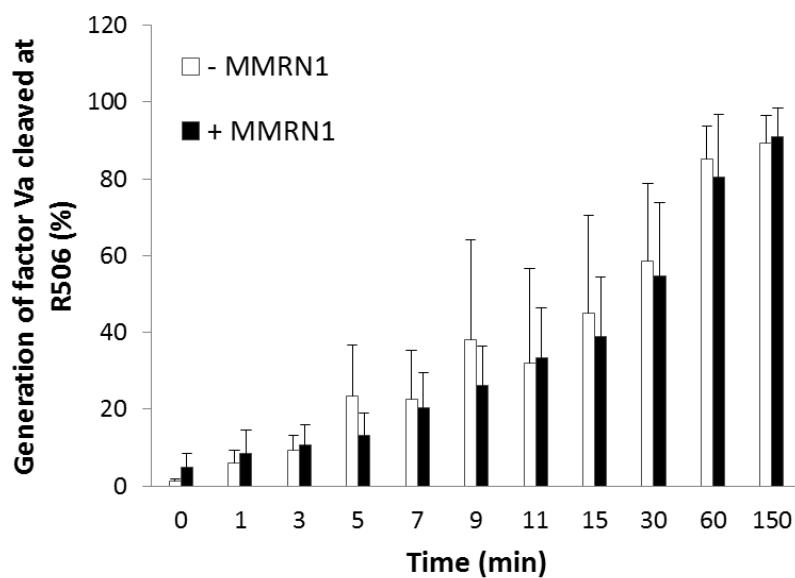
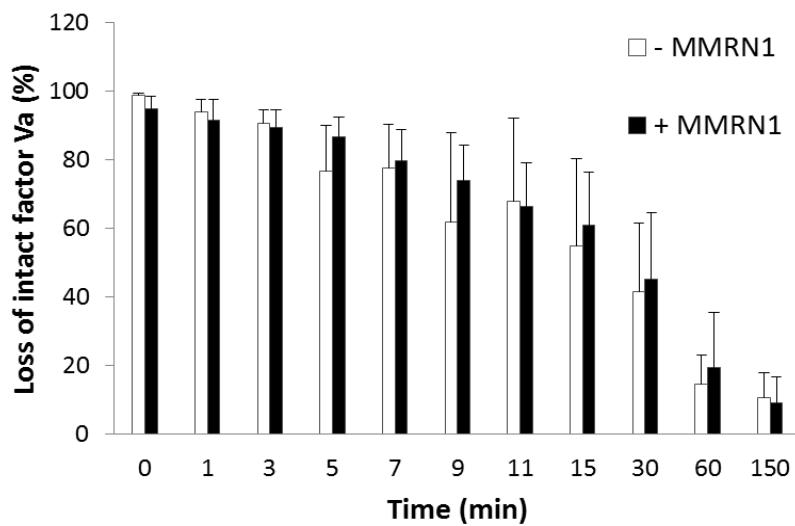
factor V-deficient plasma. Insets show data for the endogenous thrombin potential (ETP) measured from MMRN1- and BSA-coated wells (control).

### **3.6 Effect of MMRN1 on APC cleavage of factor Va**

The cleavage of factor Va by APC over time, in the presence and absence of MMRN1 (evaluated at concentrations predicted to bind 98% of factor Va in the experiment), was tested to determine if MMRN1 alters factor Va inactivation by APC. Western blot and densitometry analyses of factor Va inactivation indicated that the addition of MMRN1 did not significantly alter the loss of factor Va heavy chain, or the generation of factor Va fragment cleavage at Arg-506 by APC, at each time point (p values 0.36-0.96) (Figures 22 and 23).



**Figure 22. Effect of MMRN1 on factor Va cleavage by APC, evaluated by Western blotting.** Plasma-derived factor Va was preincubated with or without MMRN1, in the absence of PCPS vesicles, to assess Arg-506 cleavage, following the addition of APC. Aliquots taken at selected time intervals from the reaction mixture were analyzed by Western blotting, using 12% reduced SDS-PAGE. The factor Va heavy chain was detected using monoclonal antibody AHV5146 (epitope is between residues 307-506 on the heavy chain).



**Figure 23. Effect of MMRN1 on factor Va cleavage by APC, evaluated by densitometry analyses.** Plasma-derived factor Va was preincubated with or without MMRN1, in the absence of PCPS vesicles, to assess Arg-506 cleavage, following the addition of APC. Densitometry analyses of the Western blot bands (shown in Figure 23) was used to measure the loss of factor Va heavy chain and generation of Arg-506

cleavage product, from aliquots taken at the time intervals shown. The relative optical densities of the bands, obtained via densitometry analyses, are shown as a percentage. Data is represented as means  $\pm$  S.E. of three independent experiments.

**CHAPTER 4****DISCUSSION**

MMRN1 is a large, multimeric protein that is released from endothelial and platelet secretion granules into plasma for binding to their cell surfaces and to the extracellular matrix, at sites of vessel injury (26,164,165,173-175). MMRN1, in fluid phase, has an inhibitory effect on thrombin generation by prothrombinase in plasma (46). Presently, the molecular mechanism of this effect has not been determined, although MMRN1 is known to interact with several components of prothrombinase: the cofactor factor Va (46), substrate prothrombin (unpublished), and phosphatidyl serine of activated membrane surfaces (183). As platelet factor V/Va is stored in association with MMRN1, and platelet factor V has greater resistance to APC cleavage at Arg-506 compared to plasma factor V/Va, MMRN1 could have a role in modulating factor V/Va proteolysis by APC (47,89,164). The present studies were undertaken to characterize the regions of prothrombin that interact with MMRN1 and to investigate the role of MMRN1 binding to components of prothrombinase in modulating thrombin generation, at sites of vessel injury.

I used ELISA-based protein binding assays, and prothrombin activation products containing specific domains, to identify important regions of prothrombin involved in binding to MMRN1. These investigations indicated that prothrombin activation products, prethrombin 2 and thrombin, which contain only the serine protease domain, did not measurably bind to immobilized MMRN1. This observation is consistent with a previous

report using surface plasmon resonance (SPR), that thrombin does not bind to MMRN1 (46). However, like prothrombin, fragment 1, fragment 2, and prethrombin 1 (which respectively contain the Gla and kringle 1, kringle 2, or kringle 2 and serine protease domains), did bind to immobilized MMRN1. In ELISAs with immobilized prothrombin activation products, MMRN1 was confirmed to bind prothrombin and fragment 2 and not prethrombin 2, fragment 1 or prethrombin 1. The discrepancies between these assays in the data for fragment 1 and prethrombin 1 is not yet known and it could reflect differences in sensitivity. Potential explanation include limitations of the assay sensitivities, and/or a reduced access to MMRN1 binding sites when fragment 1 and/or prethrombin 1 are immobilized onto surfaces, as these proteins bound to immobilized MMRN1 in the other assay. As the kringle 2 domain is present in both fragment 2 and prethrombin 1, it is also unclear why MMRN1 bound to fragment 2 but not to prethrombin 1. Potential explanation include an altered conformation, with reduced access to the MMRN1 binding sites in the kringle 2 domain of prethrombin 1 compared to fragment 2. Collectively, this study has identified that the kringle domain(s), and possibly the Gla domain, as the sites in prothrombin that bind to MMRN1, and further exclude a role of the serine protease domain in MMRN1 binding. As the Gla and kringle domains of prothrombin interact with the membrane and factor Va (41,146), it is possible that MMRN1 inhibits these interactions. This could contribute to the inhibitory effects of MMRN1 on thrombin generation, observed in the previous (46) and current studies.

To investigate the molecular mechanism underlying the inhibition of MMRN1 on thrombin generation by prothrombinase, thrombin generation assays with purified

proteins, phospholipid vesicles, and DAPA were used. Analyses of the initial rates of thrombin generation (i.e. linear appearance of product over time) by prothrombinase, which proceeds via the meizothrombin pathway (55), showed that MMRN1 has an inhibitory effect on the initial rates of thrombin generation when added at a concentration that is predicted to bind 96% of the factor Va. This is consistent with observations from previous CAT studies that have shown that exogenous MMRN1 inhibits thrombin generation by prothrombinase in plasma (46).

MMRN1 was shown to have an inhibitory effect on the initial rates of thrombin generation in the absence of factor Va (by factor Xa/PCPS vesicles), which proceeds via the prethrombin 2 pathway (55), when added at a concentration predicted to bind a sufficient amount of prothrombin. It is unclear if MMRN1 inhibitory effects result from MMRN1 inhibiting prothrombin-membrane or prothrombin-factor Xa interactions. It is possible that MMRN1 causes steric inhibition to the prothrombin-factor Xa interactions required for prethrombin 2 formations, rather than inhibiting prothrombin-membrane interactions. As data from ELISAs suggested the kringle 2 domain as a potential region for MMRN1 binding, the inhibitory effect could reflect MMRN1 binding in proximity to the initial factor Xa cleavage site Arg-271 on prothrombin (in the absence of factor Va) (55). Further, there was an enhancement in prothrombin activation, in the presence of MMRN1, observed toward the end of the full time course profiles of thrombin generation. This suggests that MMRN1 may not affect the second Arg-320 cleavage site. In the presence of factor Va, the initial cleavage of prothrombin is thought to occur at Arg-320,

to form meizothrombin (55). Thus the physiological cleavage of prothrombin may be unaffected by MMRN1-prothrombin interactions.

In this study, MMRN1 was shown to have no effect on the initial rates of thrombin generation in the absence of PCPS vesicles (by factor Xa/factor Va), which proceeds via the prethrombin 2 pathway (201), when added at a concentration that was predicted to bind 95% of factor Va. This suggests that the predominant inhibitory effects of MMRN1 on thrombin generation are PCPS-dependent, and mediated by MMRN1 inhibiting factor Va-membrane interactions, rather than inhibiting factor Va-factor Xa interactions. This is consistent with observations that MMRN1 binding sites on factor Va overlap its binding sites for phospholipid residues (183,184). Collectively, the analyses of initial rates of thrombin generation suggest that MMRN1 also inhibits thrombin generation through its interactions with prothrombin.

Measurements of the initial rates of thrombin generation using DAPA have recognized limitations. As DAPA is a fluorogenic substrate, it can lose its fluorescence property overtime due to fluorescence quenching (202,203) which can affect the measurements of initial rates of thrombin generation. However, the observed drift in background DAPA fluorescence was minimal. In assays where prothrombin was activated by prothrombinase, thrombin generation appeared to reach saturation, but the amount of thrombin generated was lower than expected. Although the plates were pre-coated with BSA, and Prionex was added to the reaction mixture, it is possible that some prothrombin remained on the test plate and was not activated.

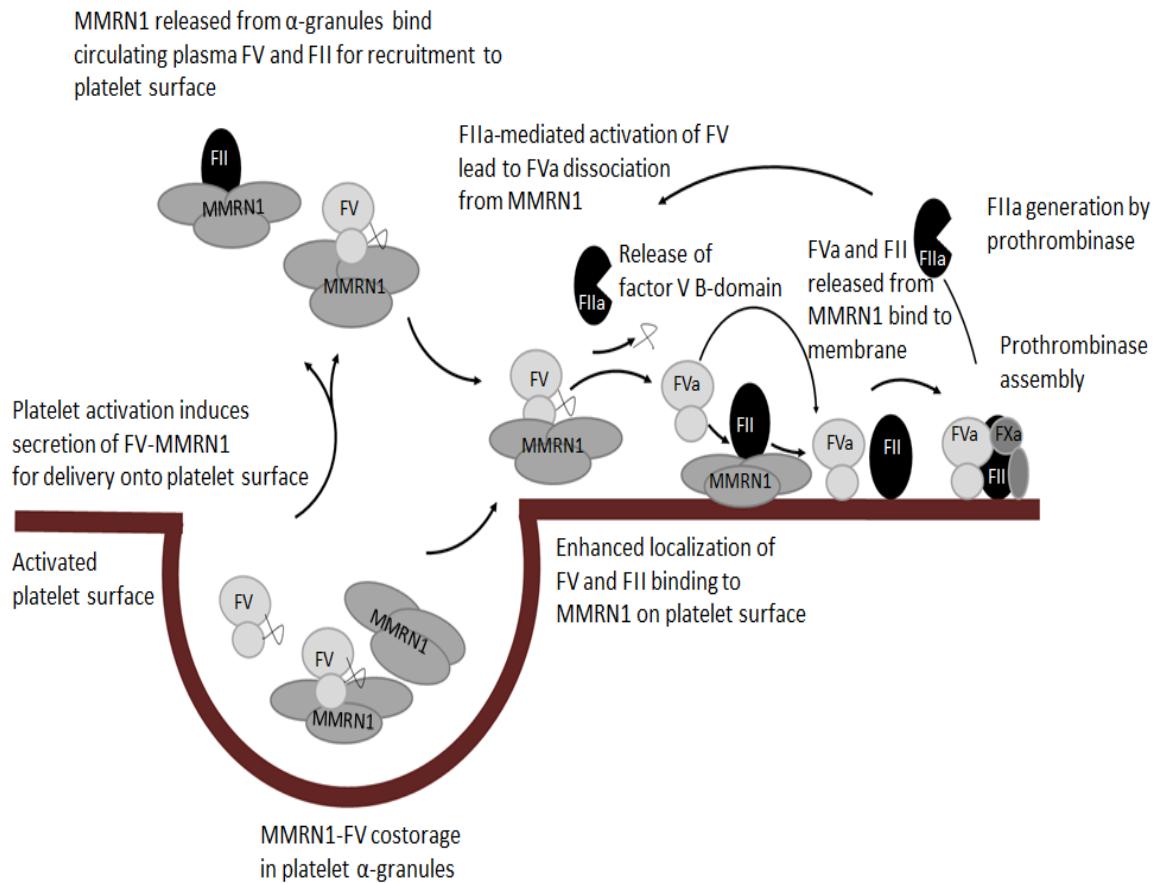
The thrombin generation assays in plasma, using the CAT method, was used to study the effect of immobilized MMRN1 on factor V/Va function in plasma. Analyses of ETP revealed that MMRN1 captured and released functional factor V/Va, of human and mouse origin, to support subsequent thrombin generation. This suggests that once MMRN1 becomes a constituent of the extracellular matrix, at sites of vessel injury, it can localize factor Va for its function in prothrombinase to generate thrombin. This observation is in contrast to the “anticoagulant” role or inhibitory effects of MMRN1 on thrombin generation. It is possible MMRN1 has a dual role in hemostasis, such that it functions as an anticoagulant in fluid phase, and functions as a procoagulant when immobilized onto the platelet surface and/or the extracellular matrix. The ability of MMRN1 to bind human and mouse plasma factor V is consistent with conservation of function, and this observation will allow future studies with selective *Mmrn1*-deficient mice to test *Mmrn1* role in modulating factor Va function *in vivo*. The proposed role of MMRN1 in modulating prothrombinase assembly and function during coagulation is illustrated in Figure 24.

Platelet factor V/Va exhibits procoagulant features and it has greater resistance to inactivation by APC than plasma factor Va due to slower cleavage at Arg-506 (89,128). As platelet factor V is stored and released complexed to MMRN1 (26), I tested if factor Va resistance to APC could be modified by its association with MMRN1, using factor Va inactivation assays, with Western blotting to follow APC cleavage of factor Va at Arg-506. The densitometry analyses of the bands revealed that MMRN1 did not alter factor Va cleavage by APC at Arg-506 suggesting that the association of platelet factor Va with

MMRN1 is not responsible for its increased APC resistance. This is supported by the observations that MMRN1 binds to the light chain of factor Va (183,184), whereas APC bind to the heavy chain to cleave Arg-506 (104,105,108). These data imply that other post-translational modifications of factor V that follow its uptake into platelets (47,89) accounts for its increased resistance to APC.

Future studies will be important to fully elucidate the role of MMRN1 as a potential modulator of prothrombinase. As the present studies explored regions of prothrombin with MMRN1 binding sites, future studies to consider include further mapping of the MMRN1 binding sites on prothrombin. As MMRN1 does not bind to factor Xa, which shares a homologous Gla domain with prothrombin, this indirectly suggests that MMRN1 binding sites are not located in the Gla domain of prothrombin. It would be interesting to test the binding of MMRN1 to Gla-domainless prothrombin, which can be generated *in vitro* (204), and to prothrombin constructs missing specific regions in the kringle domains, to further localize MMRN1 binding sites on prothrombin. In addition, as MMRN1 does not bind to thrombin or prethrombin 2, this suggests MMRN1 binding sites are not located in the serine protease domain of prothrombin. It would be worthwhile to further evaluate this possibility by testing prethrombin 2 activation by factor Xa, with and without MMRN1. Further binding studies, with SPR analysis, could also be useful to further test the regions of prothrombin involved in MMRN1 binding. It is possible that the inhibitory effect of MMRN1 on thrombin generation is due to MMRN1 binding of factor Va that reduces factor Va availability to bind to membranes and incorporate into prothrombinase, which could change the pathway

for prothrombin activation pathway to that of the factor Va-independent pathway. This can be confirmed by repeating the experiment using SDS-PAGE, to analyse the sequence of prothrombin cleavage, with and without MMRN1. In addition, SDS-PAGE analysis can be used to further examine the effect of MMRN1 on prothrombin activation at specific cleavage sites by factor Xa, in the absence of factor Va or PCPS. As immobilized MMRN1 is able to capture plasma factor V to support subsequent thrombin generation, it would be interesting to determine if MMRN1 can similarly capture and localize prothrombin for subsequent thrombin generation. As MMRN1 binding to factor Va did not influence APC proteolysis, it is possible that MMRN1 does not alter platelet factor Va phosphorylation by casein kinase II, which contributes to platelet factor V APC resistance (89). A future study could test the phosphorylation of platelet factor V by casein kinase II, with and without MMRN1. As mouse factor V has been identified to bind to MMRN1, and retain its function, future studies with a selective *Mmrn1* knockout mouse would help clarify the role of MMRN1-factor V/Va and MMRN1-prothrombin interactions in modulating coagulation. Studies may include measuring thrombin generation using blood from mice that are, or are not, deficient in *Mmrn1*; and testing if any defects that exist in *Mmrn1* knockout mice can be corrected by the addition of MMRN1.



**Figure 24. Proposed model of MMRN1 effect on blood coagulation.** Under normal conditions, MMRN1, factor V (FV) and MMRN1-FV complexes are stored in platelet α-granules, with MMRN1 in molar excess (46). In response to vessel injury, platelet activation induces the secretion of its α-granule proteins including MMRN1-FV complexes, for enhanced delivery of FV onto external platelet surface. MMRN1 released into the plasma binds to circulating plasma FV and prothrombin (FII), for enhanced localization onto the platelet surface. FV and FII dissociate from MMRN1, and membrane-bound factor Xa (FXa) converts a small amount of FII to thrombin (FIIa). Thrombin catalyzes the activation of FV (delayed by MMRN1) leading to FV

dissociation from MMRN1 in its active form (FVa). FVa binds to membrane bound FXa to form the prothrombinase complex to enhance thrombin generation or FVa binds to MMRN1 to delay prothrombinase assembly and limit thrombin generation. Figure is adapted from Jeimy et al. (46).

## CHAPTER 5

### CONCLUSION

In the present study, the regions of prothrombin important for MMRN1 binding were characterized. The findings from ELISA binding assays strongly suggest that the prothrombin kringle 2 domain, is involved in MMRN1 binding. However, there were discrepancies between the data from the binding assays in which fragment 1 (Gla and kringle 1 domain) and prethrombin 1 (which like fragment 2 also contains the kringle 2 domain, in addition to the serine protease domain) was detected for binding to immobilized MMRN1, but MMRN1 was not detected for binding to immobilized fragment 1 or prethrombin 1. Although it is possible, fragment 1 and prethrombin 1 had reduced access to MMRN1 binding. The results also strongly suggest that, prethrombin 2 and thrombin, which consists of the serine protease domain, is not involved in MMRN1 binding. Therefore, the data suggests that the kringle 2 domain present in prothrombin fragment 2 and prethrombin 1 has a potential MMRN1 binding site, whereas the Gla and kringle 1 domain present in prothrombin fragment 1 may be involved in MMRN1 binding. As the prothrombin Gla and kringle domains are involved in membrane and factor Va binding, respectively (41, 146), the interference of MMRN1 to these interactions may contribute to the inhibitory effect of MMRN1 on thrombin generation.

In thrombin generation assays with purified proteins and phospholipid vesicles, MMRN1 inhibited thrombin generation by (a) factor Xa in prothrombinase, through interactions with factor Va, and (b) by factor Xa-PCPS, in the absence of factor Va,

through interactions with prothrombin. However, MMRN1 did not inhibit thrombin generation by factor Xa-factor Va, in the absence of PCPS, which strongly suggests the MMRN1 inhibitory effects are PCPS-dependent. Therefore, the inhibitory effects of MMRN1 on thrombin generation are mediated by the interaction of MMRN1 with both factor Va and prothrombin, and require the presence of phospholipids (PCPS vesicles).

In thrombin generation assays with plasma, immobilized MMRN1 was determined to bind human and mouse plasma factor V for subsequent thrombin generation. This suggests MMRN1, as an extracellular matrix component, could have roles in localizing and recruiting factor V/Va to sites of vessel injury for prothrombinase assembly and function. It is possible that MMRN1 could similarly bind and localize prothrombin from plasma as well. Therefore, the findings from the thrombin generation assays using purified proteins and phospholipid vesicles, or with plasma, suggest that MMRN1 has a dual role in coagulation: MMRN1 functions as an inhibitor of thrombin generation in fluid phase, and MMRN1 functions as a carrier protein for factor V/Va to promote thrombin generation, in an immobilized form.

As the binding of MMRN1 to factor Va does not alter APC proteolysis at Arg-506, it is likely that platelet factor Va APC resistance reflects other post translational modifications such as the presence of an *O*-linked sugar residue at Thr-402 (89).

Overall, the findings of this study have added novel information to the function of MMRN1 as a factor V/Va and prothrombin binding protein, and the mechanism by which MMRN1 modulates thrombin generation. As the findings suggest MMRN1 inhibits

thrombin generation, through its interactions with both factor Va and prothrombin, it would be interesting to determine if MMRN1 could be used as an anticoagulant in order to prevent and treat hypercoagulable disorders. Also, as my findings strongly suggest the mechanism by which MMRN1 inhibits thrombin generation involves phospholipids, novel anticoagulant therapies can be designed to mimic MMRN1 function of specifically interfering with factor Va-phospholipid interactions. In addition, by further characterizing the MMRN1 binding sites in prothrombin fragment 2 (kringle 2 domain), potential anticoagulant therapies can be designed to specifically target these binding sites in prothrombin. Now that MMRN1 has been identified to bind mouse factor V/Va, and *Mmrn1* knockout mice are being developed, it will be interesting to verify the inhibitory role of fluid phase MMRN1 on coagulation *in vivo*.

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