Structure and function of human coagulation factor V : a multidisciplinary approach combining bioinformatics and molecular biology

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Structure and function of human coagulation factor V

a multidisciplinary approach combining
bioinformatics and molecular biology

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Structure and function of human coagulation factor V

A multidisciplinary approach combining
bioinformatics and molecular biology

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
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The most exciting phrase to hear in science,
the one that heralds the most discoveries,
is not 'Eureka!' (I found it!) but "that's funny..."

Isaac Asimov

Contents

Chapter I:	Introduction: Structural and functional properties of human coagulation factor V	1
Chapter II:	Coagulation FV and thrombophilia: Background and mechanisms	27
Chapter III:	Structural models of the snake venom FV activators from <i>Daboia russelli</i> and <i>Daboia lebetina</i>	53
Chapter IV:	The role of thrombin exosites I and II in the activation of human coagulation factor V	89
Chapter V:	Design of protein-membrane interaction inhibitors by virtual ligand screening, proof of concept with the C2 domain of factor V	117
Chapter VI:	Identification of surface epitopes of human coagulation factor Va that are important for interaction with activated protein C and heparin	139
Chapter VII:	General discussion and conclusions	163
	Summary	187
	Samenvatting	191
	Dankwoord	195
	Curriculum vitae	199
	List of publications	200

Chapter I

Introduction:

Structural and functional properties of human blood coagulation factor V

1.1 Introduction

Blood performs many important functions within our body. It not only serves as a medium for the transport of oxygen, nutrients, metabolites and waste products throughout the body via the vascular system, but it is also involved in many biological processes including body heat regulation and immune function. It is therefore essential for survival that blood flow is tightly regulated. In the course of evolution, a highly specialized and complex biological system has developed to regulate blood fluidity: the haemostatic system. The system allows blood to remain in fluid form under normal conditions, but ensures prompt arrest of bleeding in case of vascular injury.

In small vessels, blood loss is mainly limited by vasoconstriction, whereas in larger vessels bleeding is stopped via the complex interplay of (sub)endothelial cells, activated platelets and plasma components. After injury, damaged endothelial cells expose subendothelial proteins (most notably collagen), which bind and induce the activation of circulating platelets. Activated platelets release a number of different coagulation factors and platelet-activating factors, which in turn activate other platelets and eventually promote their aggregation. Platelet aggregation results in the formation of an unstable platelet plug at the site of vascular injury and the arrest of bleeding. This fragile primary plug is reinforced by deposition of insoluble cross-linked fibrin, which is the end-product of the coagulation cascade. The coagulation cascade is a series of reactions in which inactive coagulation factors (pro-enzymes) are activated to become active proteins that then catalyze the next reaction in the cascade, ultimately resulting in cross-linked fibrin. Initiation of this complex series of reactions is triggered by tissue factor, a subendothelial protein which is exposed to flowing blood following vascular injury.

Because it is undesirable that a small, local injury leads to systemic coagulation or to continued coagulation after the arrest of bleeding, coagulation has to be controlled spatially and temporarily. Spatial restriction of coagulation is achieved by the requirement for a negatively charged membrane surface (provided by the activated platelets) and the presence of activated coagulation factors for the assembly of the membrane-bound macromolecular complexes that catalyze the activation reactions in the coagulation cascade.

Both requirements are only fulfilled after the exposure of a trigger (collagen and tissue factor) as a result of vascular damage, which restricts coagulation to the site of injury. Besides, control of coagulation in time is achieved by the presence of circulating coagulation factor inhibitors and a proteolytic anticoagulant pathway that acts via inactivation of cofactor proteins that are involved in the coagulation cascade.

A pivotal protein for the maintenance of the haemostatic balance is blood coagulation factor V (FV). In contrast to most coagulation factors, FV is implicated both in the procoagulant and anticoagulant pathways. Hence, it is often described as a Janus-faced protein [1], in reference to the dual-faced Roman god Janus. The research presented in this thesis focuses on the structure-function relationship of FV with respect to its activation by thrombin, its membrane-binding properties and the inactivation of the activated cofactor FVa by activated protein C (APC). This chapter will provide a brief background into the structure of FV and how its structure is related to the pro- and anticoagulant properties of the protein.

1.2 Human blood coagulation factor V

1.2.1 Factor V: from gene to protein

The gene encoding human FV spans a chromosomal region of about 80 kb on the long arm of chromosome 1 (1q23) and consists of 25 exons [2]. Transcription of the FV gene in the cell nucleus results in a 6.8 kb mature mRNA, which is translated in the cytosol into a preproprotein of 2224 amino acid residues [3]. After translocation into the endoplasmatic reticulum, the 28-amino acid signal peptide is removed by a signal peptidase. The mature FV protein then undergoes several post-translational modifications, including N- and O-linked glycosilation, phosphorylation and sulfation [4]. Although the functional impact of the individual modifications still has to be clarified, it is known that post-translational modification of FV is required for efficient folding and secretion [5] and that these modifications influence the activation of FV (see also section 1.2.3).

Several cell types have been reported to synthesize FV when grown in culture [6-8], but it is widely accepted that hepatocytes are the primary source of FV [9,10]. After secretion, most FV circulates in blood as a 330 kDa-glycoprotein at a concentration of approximately 21 nM [11]. However, about 20% of the FV present in blood resides in platelet α -granules [12] where it is stored in complex with multimerin 1 via interactions with the FV B and C2 domain [13-15]. The platelet fraction of FV is partly synthesized in the megakaryocytes and partly derived from internalization of plasma FV via endocytosis [16,17]. Following platelet activation, the FV-multimerin complexes are released, which causes a local increase in the FV concentration at sites of vascular injury. Thrombin-catalyzed FV activation induces the dissociation of FVa from the multimerin complex [15], after which FVa promotes the assembly of the prothrombinase complex on the activated platelet membrane surface [18].

1.2.2 Factor V primary structure

The primary structure of FV was determined from the cloned FV cDNA and shows that the protein is composed of multiple domains, organized in a A1-A2-B-A3-C1-C2 domain structure (Figure 1.1) [19]. Sequence analysis revealed that the three A domains of FV are highly conserved between species and that they show about 40% sequence identity to the corresponding A domains of coagulation factor VIII and the copper-binding protein ceruloplasmin [3,19]. The two C domains of FV belong to the family of discoidin-like domains, a structural motif that is found in both eukaryotic and prokaryotic proteins and is characterized by a considerable functional diversity [20,21]. Both FV C domains are 37% identical to each other and also show a high degree of sequence identity (46%) with their FVIII counterparts [19]. In contrast to the A and C domains, the FV B domain displays no significant homology to any known protein. The sequence of the FV B domain is poorly conserved and shows only 35% overall amino acid identity between human, bovine and murine sequences [22,23]. The similarity with the FVIII B domain is even lower (only 14%) and confined to the amino- and carboxy-terminal region of the domain [3].

Figure 1.1 (A) Schematic representation of the exon-intron structure of the F5 gene. Exons are represented by grey boxes, introns are drawn as lines. The scale is indicated at the bottom of the figure. **(B) Diagram of the domain organization of the human FV molecule.** The amino-terminal signal peptide (28 amino acids) is represented in black, the homologous A domains in light grey, the B domain in white and the two homologous C domains in dark grey. Grey rhombs represent the position of N-glycosylation sites. Free cysteines and cysteines involved in disulfide bridges are also indicated.

1.2.3 FV is activated to a procoagulant cofactor by thrombin or FXa

Single-chain FV is almost completely devoid of procoagulant activity and has to be activated by limited proteolysis to express its cofactor activity in FXa-catalyzed prothrombin activation [24]. Thrombin and FXa are the major physiological activators of FV, but several other proteases of different origin have been shown to activate FV *in vitro* [25-29]. Although not relevant for thrombin formation *in vivo*, attention has been paid to FV activators that are present in the venom of a wide variety of snakes. It so happens that snake venom FV activators are not only excellent tools in the study of the structure-function relationship of FV(a), but they are also used in diagnostic tests for quantification of plasma FV levels and for screening of defects in the anticoagulant protein C pathway [30,31].

FV activation by factor Xa

FXa-catalyzed FV activation is dependent on negatively charged phospholipids and proceeds via initial cleavage at Arg¹⁰¹⁸, which generates two intermediates of 220 and 150 kDa [32] (Figure 1.2). A second cleavage in the 150 kDa-fragment results in the release of the FVa heavy chain (105 kDa) and an activation peptide of 71 kDa. Monkovic and Tracy reported cleavage at Arg¹⁰¹⁸ to be sufficient for full expression of FVa cofactor activity, but this finding has been challenged by FV mutagenesis experiments [33]. The latter experiments demonstrated that a third cleavage at Arg¹⁵⁴⁵, which correlates with the formation of the FVa light chain, is crucial for maximal expression of FVa procoagulant activity. Moreover, FXa was found to cleave FVa at Arg¹⁷⁶¹ and Arg¹⁷⁶⁵, two sites that are located in the A3 domain of the heavy chain [33,34]. In the absence of functional cleavage sites at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵, the single cleavage at Arg¹⁷⁶⁵ yielded a partially active FVa intermediate with 14% cofactor activity [33].

It has been proposed that the activation of FV by FXa is particularly important in the initiation phase of coagulation, when virtually no thrombin is available [35]. The low levels of (meizo)thrombin that are generated by this mechanism would then allow for full prothrombinase activity by virtue of the thrombin-catalyzed feedback activation of FV. However, kinetic evaluation and Western Blot analysis of the extrinsic blood coagulation reactions in a reconstituted system indicated no detectable FV activation in the absence of prothrombin or active thrombin during the initiation phase of coagulation [36]. In the same experimental system however, the formation of low levels of thrombin (0.6-0.7 pM) resulted in rapid cleavage of intact FV. This suggests that the concentrations of FXa that exist during the early phase of coagulation may be insufficient to fully explain the observed amounts of FVa that are generated and that not phospholipid-bound FXa, but α -thrombin and/or meizothrombin may be the initial activator of FV.

Figure 1.2. Activation of human FV by FXa. FXa-mediated FV activation proceeds via initial cleavage at Arg¹⁰¹⁸, followed by limited proteolysis at Arg⁷⁰⁹, Arg¹⁵⁴⁵ and Arg^{1761/Arg¹⁷⁶⁵}. The molecular weight of the activation fragments is listed below the intermediate. Scissors symbols designate the FXa cleavage sites at Arg⁷⁰⁹, Arg¹⁰¹⁸, Arg¹⁵⁴⁵ and Arg^{1761/Arg¹⁷⁶⁵}. Numbers indicate amino acid position.

FV activation by thrombin

Thrombin activates FV by limited proteolysis at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵, a process that does not require the presence of phospholipids [32]. Thrombin-catalyzed activation of FV follows a kinetically preferred order of bond cleavage, in which cleavage at Arg⁷⁰⁹ occurs first, followed closely by cleavage at Arg¹⁰¹⁸ and finally a slow cleavage at Arg¹⁵⁴⁵ [33,37] (Figure 1.3). These three cleavages result in the removal of the large B domain and the association of the heavy (105 kDa) and light chain (71/74 kDa). FV mutagenesis studies with FV variants that are resistant to thrombin cleavage at one or more of the three activation cleavage sites have demonstrated that proteolysis at Arg⁷⁰⁹ and Arg¹⁰¹⁸ results in a FVa intermediate with ~30% procoagulant cofactor activity [33,38]. Moreover, cleavage at these sites appeared to facilitate the cleavage at Arg¹⁵⁴⁵ [39]. It has been shown that formation of the light chain, resulting from a single cleavage at Arg¹⁵⁴⁵, is the only

requirement for expression of full FVa cofactor activity [33,34,38].

Structural and functional studies have identified two highly acidic regions upstream from the Arg⁷⁰⁹ and Arg¹⁵⁴⁵ cleavage sites [3]. Both regions contain sulfated sequences homologous to hirudin, an anticoagulant peptide isolated from the bloodsucking leech *Hirudo medicinalis*. Because the hirudin peptide is a high-affinity ligand of thrombin exosite I, this exosite was proposed to be involved in the recognition of the Arg⁷⁰⁹ and Arg¹⁵⁴⁵ cleavage sites during thrombin-catalyzed FV activation [40]. Several studies have demonstrated that thrombin exosite I is indeed of major importance for FV activation, whereas the role of thrombin exosite II in this process remained less clear [41-45].

In addition to the acidic regions preceding the Arg⁷⁰⁹ and Arg¹⁵⁴⁵ cleavage sites, a thrombin-binding site has been identified in the FV C2 domain that is important for FV activation at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ [46].

Figure 1.3. Activation of human FV by thrombin. Thrombin-catalyzed FV activation proceeds via sequential cleavage at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. The molecular weight of the activation fragments is listed below the intermediate. Scissors symbols designate the thrombin cleavage sites at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. Numbers indicate amino acid position.

FV activation by snake venom proteases

Although they are not relevant for thrombin formation *in vivo* (unless after envenomation), attention has been paid to FV activators that are present in the venom of a variety of snakes. Snake venom proteases have not only proven to be

excellent tools for the study of the structure-function relationship of FV(a), but they are also used in diagnostic tests for quantification of plasma FV levels, for screening of defects in the anticoagulant protein C pathway and they even have therapeutic potential [30,31].

In particular, the FV activator from the venom of Russell's viper (RVV-V) has been extensively studied [47,48]. RVV-V is a 27 kDa-serine protease capable of activating FV via a single cleavage at Arg¹⁵⁴⁵ [3,19]. Like in thrombin-catalyzed FV activation, the presence of phospholipids is not required for FV activation by RVV-V. Remarkably, RVV-V-activated FV (FVa_{RVV-V}) and thrombin-activated FV (FVa_{IIa}) display similar procoagulant cofactor activities despite their different structure [37,49] (Figure 1.4). This observation illustrates once more that cleavage at Arg¹⁵⁴⁵ appears to be the only requirement for expression of full FVa cofactor activity in FXa-catalyzed prothrombin activation.

Figure 1.4. Activation of human FV by Russell's Viper Venom FV activator (RVV-V). In contrast to FXa and thrombin, RVV-V activates FV by a single cleavage at Arg¹⁵⁴⁵. The molecular weight of the activation fragments is listed below the intermediate. Scissors symbols designate the RVV-V cleavage site at Arg¹⁵⁴⁵. Numbers indicate amino acid position.

1.2.4 Three-dimensional structure of activated FV

In-depth studies of the three-dimensional structure of FV(a) have long been hampered by the lack of soluble, properly folded and homogeneous proteins in quantities sufficient for crystallographic or NMR analysis. In addition, the high molecular weight of the protein, together with its complex multidomain structure and high degree of glycosylation appeared to be major bottlenecks in obtaining high-quality crystals suitable for X-ray analysis. In this context it is not surprising that for a long time, theoretical models of the A and C domains of FVa were the only structural information available at atomic level. Molecular models of the three A domains of FVa are based on the homologous copper-binding protein ceruloplasmin [50,51]. Because the C-terminal region of the FVa A2 domain (residues 664-709) is missing from the template structure ceruloplasmin, no

structural information of this segment is available in these homology models [52,53]. The structures of the two discoidin-like domains of FV were originally modeled based on the X-ray structure of the binding domain of galactose oxidase from *Dactylium dendroides* [54].

The first experimental structural data at atomic level became available with the elucidation of the X-ray structures of the isolated human FV C2 domain and APC-inactivated bovine FVa (FVai) [55,56]. A molecular model of complete human FVa was proposed based on the atomic coordinates of the bovine FVai structure, but this required a structural rearrangement of the A1 and A3 domains to fit the A2 domain and an *ab initio* approach to predict the structure of the carboxy-terminal region of the FVa heavy chain [57]. Although there has been considerable progress in low-resolution *ab initio* protein structure prediction, both the accuracy and the reliability of the structural models produced by these methods is fairly low [58]. Accordingly, caution should be taken in the interpretation of structural data that were obtained by *ab initio* protein structure prediction approaches.

Theoretical models of the FVa-FXa prothrombinase complexes have been developed based on the X-ray structure of FXa and a theoretical FVa model that was constructed using the crystallographic coordinates of ceruloplasmin (for modeling of the A domains) and bovine FVai (for modeling of the C domains) [59,60]. Recently, we have determined the first experimental structure of complete, membrane-bound human FVa at low resolution by transmission electron microscopy (EM) studies [61]. Together, these three-dimensional structures have considerably improved and expanded our understanding of FVa function at the molecular level.

In this section, the overall three-dimensional structure of FVa will be outlined and some structural features important for FVa function will be discussed in more detail.

Overall structure

The initial homology models of FVa resemble a windmill-like structure in which the three A domains are arranged in a triangular fashion, with the two C domains extending from the A3 domain. Such domain arrangement, with the C1 domain stacked on top of the C2 domain, was predicted on the basis of electron microscopy data [62]. The recently solved crystal structure of APC-inactivated bovine FVa however revealed a rather different domain arrangement compared with the earlier models [57]. The three A domains are still arranged around a pseudo-threefold axis, but contrary to the earlier FVa models the two C domains are aligned edge-to-edge and form a platform upon which the A domains rest. As a result, the heavy chain of membrane-bound FVa is located from 40 to 115 Å

above the plane of the membrane, a height that is appropriate for interaction with its physiologic partners FXa, APC and prothrombin (Figure 1.5).

Figure 1.5. Three-dimensional model of FVa. FVa is shown with the backbone displayed in ribbon and the solvent-accessible surface in dot envelope representation. Each domain of FVa is represented by a different color: A1, green; A2, cyan; A3, brown; C1, pink; C2, purple. The membrane is shown in grey wire representation (phosphorus atoms are indicated in red). The major APC cleavage sites (Arg³⁰⁶ and Arg⁵⁰⁶) are shown in CPK representation (red). Putative locations of calcium and copper ions are represented as orange and blue spheres, respectively. The distance information is given as approximate values and was calculated using ICM Pro, the program the figure was created in. Coordinate files of the FVa structure were adapted from Autin *et al.* [59].

The ceruloplasmin-like A domains

Each A domain is formed by two linked cupredoxin-like β -barrels which are also found in copper-binding proteins like ceruloplasmin and plastocyanin. The A1 and A2 domains are covalently associated by the loop that contains the Arg³⁰⁶ APC cleavage site, whereas the two β -barrels of the A2 domain are connected by the Arg⁵⁰⁶ cleavage site loop (see also figure 1.5). The A3 domain is attached to the amino-terminus of the C1 domain via a short loop segment and is involved in hydrophobic and electrostatic interactions with this domain. In contrast, the A1 domain does not make substantial interactions with the C2 domain, suggesting that the association of the light and heavy chain is entirely mediated by means of interactions with the A3 domain. Chain association is required for FVa cofactor activity and has been shown to be calcium-dependent [63]. Historically, it was believed that the two chains of FVa are bridged by a single calcium ion, but site-directed mutagenesis experiments on the basis of the molecular model of the A domains have demonstrated that the FVa calcium-binding site is located within the

A1 domain [64]. These data were later confirmed by X-ray crystallography, indicating that this single calcium ion is entirely coordinated by ligands (Lys⁹³, Glu¹⁰⁸, Asp¹¹¹ and Asp¹¹²) in the A1 domain. The loop comprising these ligands is involved in several essential interactions between the A1 and A3 domains and therefore it has been hypothesized that disruption of the conformation of the calcium-binding loop may be sufficient to force the dissociation of the FVa light and heavy chain [56]. Besides this high-affinity calcium binding site in the A1 domain, several low-affinity calcium-binding sites have been demonstrated [65] and putative locations of these sites were proposed to be in the A3 domain [53]. In addition, FV and FVa contain one copper-binding site within the buried interface of the A1 and A3 domains [56,66]. The functional role of this copper ion is not known, but for FVIII it has been shown that copper-binding is crucial for chain association and activity [67]. Analogously, the copper ion in FVa has been proposed to fulfill a structural role in the stabilization of the A1-A3 domain interface [56].

The discoidin-like C domains

The FV C1 and C2 domains each fold as a distorted β -barrel structure in which a five-stranded antiparallel β -sheet faces another three-stranded antiparallel β -sheet [55,56] (Figure 1.6).

Figure 1.6. Proposed membrane-binding mode of the FVa C1 and C2 domains. The FVa C1 and C2 domains are shown in grey ribbon representation. Secondary structure elements, including the eight-stranded β -sandwich core, are represented as arrows. The three loops that delineate the PS binding pocket in each domain are colored red. Side chains of hydrophobic (orange) and positively charged (blue) residues that are important for FV membrane binding are shown and labeled. Disulfide bridges are shown in green.

The global shape is a cylinder of about 45 Å in length and 30 Å in diameter. The upper part of the barrel surface contains several salt bridges and one disulfide bridge, which links the amino- and carboxy-terminal end of the domain. A striking feature of the C domains is the presence of three solvent-exposed loops that protrude like spikes from the bottom of the β -barrel. These three loops delineate a deep groove that represents the putative phosphatidylserine (PS) binding pocket. In the C2 domain, spike 1 and spike 3 (corresponding with amino acid sequences Ser²⁰⁵⁸-Trp²⁰⁶⁸ and Gly²¹¹²-Tyr²¹²¹, respectively) consist of separate β -hairpin loops with hydrophobic residues (Trp²⁰⁶³, Trp²⁰⁶⁴ and Leu²¹¹⁶) that are located at the apex of the loop (Figure 1.6). The second spike (Asn²⁰⁷⁶-Asn²⁰⁸²) is a wider irregular loop and is capped by the polar residue Arg²⁰⁸⁰. Molecular, immunologic and structural studies have identified several hydrophobic and positively charged residues that are important for binding of the C2 domain to PS-containing membranes [68-72]. In addition, aromatic and hydrophobic side chains located at the apex of spike 3 in the C1 domain (Tyr¹⁹⁵⁶ and Leu¹⁹⁵⁷) and positively charged residues located at the bottom of the C1 β -barrel (see also Figure 1.6) were found to contribute to the binding of FVa to PS and to the assembly of the prothrombinase complex [73].

The exact mechanism of FVa membrane binding is not yet fully understood, but the elucidation of the structure of the isolated FV C2 domain has shed more light on the mechanism by which this domain interacts with phospholipid membranes [55]. The structure of the isolated FV C2 domain was solved in two different crystal forms in which two distinct conformations were observed (Figure 1.7). In the 'closed' conformation, the three spikes are tilted inwards to the PS binding pocket and the hydrophobic residues located at their apices are shielded from unfavorable interactions with the solvent.

As a result of a large (~7 Å) conformational movement of the first spike, the PS specificity pocket and the hydrophobic residues that are important for phospholipid binding are more solvent-accessible in the second ('open') conformation. It was hypothesized that the FV C2 domain circulates in the closed form and converts to the open conformation upon interaction with the negatively charged phospholipids exposed by activated platelets [55]. Membrane binding of the open C2 conformation would then be mediated by insertion of hydrophobic amino acid side chains into the phospholipid bilayer and by specific electrostatic interactions with the PS head group. Molecular dynamics simulations of the open and closed form of the FV C2 domain in aqueous or lipid environment have confirmed this hypothesis, but in addition show that the closed conformation is also energetically compatible with membrane binding [74].

Figure 1.7. Open and closed conformation of the human FV C2 domain. (A,C) Ribbon representation of the open and closed conformation, viewed from the membrane side. Side chains of hydrophobic (Trp²⁰⁶³, Trp²⁰⁶⁴ and Leu²²¹⁶) and positively charged residues (Lys²⁰⁶⁰, Lys²⁰⁶¹, Arg²⁰⁸⁰, Lys²¹¹⁴ and Lys²¹⁵⁷) important for FV membrane binding are shown in CPK representation and colored orange and blue, respectively. The N-linked glycosylation site at Asn²¹⁸¹, which is glycosylated in the FV₁ isoform, is shown in green. (B,D) Electrostatic surface potential representation of Fig. 1.7 A and C, respectively. Positively charged regions are colored blue, negatively charged regions are in red.

Partial glycosylation of an N-linked glycosylation site at Asn²¹⁸¹ in the first spike of the FV C2 domain generates two FV isoforms (FV₁ and FV₂) that differ significantly in their membrane-binding properties [75-78]. The presence of a large carbohydrate chain at Asn²¹⁸¹ in the FVa₁ isoform interferes with phospholipid binding and results in a 45-fold lower affinity for negatively charged phospholipid membranes when compared to the less-glycosylated isoform FVa₂ [75].

1.2.5 FVa is a cofactor of FXa in the prothrombinase complex

It is only after its activation by thrombin or FXa (discussed in section 1.2.3) that FV behaves as a procoagulant molecule and substantially contributes to thrombin generation by serving as a cofactor to FXa in prothrombin activation [79,80]. The large B domain of FV has been proposed to keep the FV molecule in the inactive procofactor state by sterically and electrostatically preventing the interaction with FXa [81,82]. FV activation and the concomitant release of the B domain then would result in the exposure of regions in the FV molecule that are important for its interaction with FXa [34]. However, the removal of these inhibitory constraints is not the only prerequisite for the formation of a productive prothrombin-activating complex, because the FVa-FXa interaction is weak in solution ($K_d \sim 0.8 \mu\text{M}$ [83]). Therefore, prothrombin activation by FXa (and its non-enzymatic cofactor FVa) becomes only biologically relevant in the presence of a negatively charged phospholipid membrane surface, which lowers the K_d of the FXa-FVa interaction approximately 1000-fold [83]. Such an appropriate membrane surface is typically provided by activated platelets, and it is believed that FVa and FXa first independently bind to the platelet surface after which they associate to form an active prothrombinase complex [18,84]. Membrane-bound FVa increases the affinity of FXa and prothrombin for phospholipids by about two orders of magnitude and additionally causes a 1000-fold increase of the catalytic activity of FXa [18,83,85]. Hence, the rate of prothrombin activation by the prothrombinase complex is about 300,000 times higher compared to the rate of prothrombin activation in solution catalyzed by FXa alone [79,85]. It should be noted that prothrombinase assembly not only results in a dramatic increase in the catalytic efficiency of prothrombin activation by FXa, but also restricts the thrombogenic response to the site of vascular injury.

1.2.6 Downregulation of FVa procoagulant activity

In addition to the restriction of the blood coagulation reactions to the site of vascular injury, regulation of coagulation is achieved at each level of the pathway by circulating protease inhibitors or by negative feedback mechanisms [86]. Whereas protease inhibitors are constitutively present and act by the formation of irreversible enzyme-inhibitor complexes which are rapidly cleared from the circulation, negative feedback inhibition of thrombin formation is initiated by thrombin itself and is mainly achieved by the irreversible proteolytic inactivation of factors Va and VIIIa via the protein C pathway. This pathway is initiated by the binding of thrombin to thrombomodulin, a transmembrane protein that is constitutively expressed on the surface of intact vascular endothelial cells.

Thrombomodulin binding alters the thrombin specificity from procoagulant substrates toward activation of the anticoagulant protease protein C [87,88].

Activated protein C (APC) inactivates FVa through limited proteolysis of the heavy chain at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹ [89,90]. These cleavage reactions are calcium-dependent and also require the presence of negatively charged phospholipids [91]. A dual pathway model for the inactivation of FVa by APC has been proposed [90]. A first option for FVa inactivation is via initial cleavage at Arg⁵⁰⁶, the preferred cleavage in FVa, that is subsequently followed by cleavage at Arg³⁰⁶. Alternatively, direct inactivation of the FVa molecule can occur by initial cleavage of Arg³⁰⁶. Cleavage at Arg⁵⁰⁶ results in a 40-fold reduction in the affinity of FVa for FXa, whereas the subsequent cleavage at Arg³⁰⁶ results in complete FVa inactivation.

Complete loss of FVa cofactor activity is associated with the dissociation of the A2 domain, which is also likely to occur after cleavage of FVa at Arg³⁰⁶ [89,90,92]. A three-dimensional model of the FVa-APC complex suggests that a positively charged region in the serine protease domain of APC (comprising the 37, 60, 70 and 148 loops) interacts with a negatively charged region adjacent to the Arg⁵⁰⁶ cleavage site in FVa [53,93]. The cleavage at Arg³⁰⁶ is likely mediated by different interactions and it appears that far less intermolecular contacts are involved compared to the interaction of APC with the Arg⁵⁰⁶ cleavage site [93]. In line with this, it has been observed that the elimination of the positive cluster in the serine protease domain of APC had no effect on the rate of the Arg³⁰⁶ cleavage, but reduced the rate of cleavage at Arg⁵⁰⁶ [94,95].

Several factors have been reported that modulate the APC-catalyzed inactivation of FVa. One of these factors is the vitamin K-dependent coagulation factor protein S, which acts as a non-enzymatic cofactor of APC by stimulating the cleavage at Arg³⁰⁶ about 20-fold [96]. The mechanism by which protein S selectively stimulates FVa inactivation at Arg³⁰⁶ by APC is still unclear, but it appears that several partial enhancing steps and interactions are involved. Protein S enhances the affinity of APC for negatively charged phospholipid membranes [97], which may in part explain the stimulating effect of protein S on APC-catalyzed FVa (and FVIIIa) inactivation. Another possible mechanism is based on the observation that protein S positions the active site of APC closer to the membrane [98]. It has been proposed that such relocation of the active site of APC could specifically facilitate the interaction of APC with the Arg³⁰⁶ cleavage site [53].

Another factor that influences APC-catalyzed FVa inactivation is FXa. When FVa is incorporated in the prothrombinase complex, FXa protects FVa from APC-catalyzed inactivation by specifically inhibiting the Arg⁵⁰⁶ cleavage [96,99]. Other factors that have been reported to influence the APC-mediated inactivation of FVa include heparin [93], phospholipid composition [100], prothrombin [101], FVa origin [16], the extent of FVa glycosylation [76] and ionic strength (unpublished observation).

1.2.7. FV, a protein with anticoagulant properties

Besides its procoagulant function in prothrombin activation, FV also acts as an anticoagulant by stimulating the APC-mediated inactivation of FVIIIa [102-104]. While thrombin converts FV to the procoagulant cofactor FVa, limited proteolysis of intact FV by APC is required for the anticoagulant activity. The APC cleavage sites in FV are identical to those in FVa, but it has been demonstrated that only the APC-mediated cleavage at Arg⁵⁰⁶ converts FV into an anticoagulant protein (FVac) [105]. The molecular mechanism by which FVac acts as a cofactor of APC in the degradation of membrane-bound FVIIIa remains largely unknown, but it has been shown that the carboxy-terminal region of the B domain is important for the interaction with APC [106]. The observation that FVac and protein S act synergistically in the APC-mediated inactivation of FVIIIa further suggests that the regulation of the intrinsic tenase complex requires the assembly of a multimolecular complex composed of membrane-bound FVIIIa, FVac, APC and protein S [107].

Just as APC can inactivate the procoagulant cofactor FVa, thrombin and FXa can inactivate the anticoagulant cofactor FVac by limited proteolysis. FV mutagenesis studies show that cleavage at Arg¹⁵⁴⁵ in FVac is sufficient to abolish the anticoagulant activity of FVac [106]. It is noteworthy that FV activation intermediates with partial procoagulant activity as a result of thrombin cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸ (but not at Arg¹⁵⁴⁵) still can be converted into active APC cofactors by the APC-mediated cleavage at Arg⁵⁰⁶ [106]. These findings show that the biological fate of FV is determined by the local concentrations of thrombin and APC [108]. Under normal physiologic conditions, the balance between procoagulant and anticoagulant forces is shifted in favour of anticoagulation, and circulating APC may direct FV in an anticoagulant direction. At sites of vascular injury however, procoagulant forces prevail and intact FV or FVac are converted to the procoagulant cofactor FVa.

Given the dual role of FV in the coagulation cascade, genetic or acquired alterations in FV antigen or activity levels may result in either hemorrhagic or thrombotic tendencies [1,109,110]. FV-related risk factors that contribute to an increased risk for venous thrombosis, like the well-known FV_{Leiden} mutation, will be discussed in detail in the next chapter.

1.3 Outline of this thesis

The generation of sufficient amounts of thrombin by the prothrombinase complex is a prerequisite for the formation of a stable fibrin clot at the site of vascular injury and the arrest of bleeding. Thrombin is not only responsible for the proteolytic conversion of soluble fibrinogen into insoluble fibrin, but is also directly involved in the activation of platelets and feedback activation of other components of the coagulation cascade.

Factor Va, the non-enzymatic cofactor of FXa in the prothrombinase complex, is of crucial importance in the generation of thrombin as it accelerates prothrombin activation by FXa by several orders of magnitude. Considering the importance of FVa in the procoagulant pathway, it is not surprising that regulation of thrombin formation proceeds in large part through the regulation of FVa cofactor activity by the protein C system. Because the procofactor FV serves as a cofactor of APC in the inactivation of FVIIIa, it is evident that FV is not only vital for thrombin formation but also contributes considerably to the regulation of thrombin formation. It is therefore beyond doubt that malfunctioning of FV may influence both the pro- and anticoagulant pathways which can lead to either severe bleeding tendencies or thrombotic disease. Inherited defects as well as acquired factors that modulate FV antigen or activity levels and therefore are considered as risk factors for thrombosis are discussed in chapter 2.

Snake venom proteins affect many steps of the blood coagulation cascade. Because each of the venom components usually acts selectively on one coagulation factor, they are potentially useful to study the mechanism of action, the regulation and the structure-function relationship of human coagulation factors. In chapter 3, we have predicted the three-dimensional structures of two FV activators that are present in the venom of Russell's and Levant's viper (RVV-V and LVV-V respectively) using comparative modeling techniques. Electrostatic potential calculations reveal the presence of two positively charged regions on opposite sides of the active site of RVV-V and LVV-V. In analogy with thrombin, these exosites may account for the substrate specificity of both proteases for the Arg¹⁵⁴⁵ cleavage site in FV.

The role of thrombin exosites in FV activation was further investigated in chapter 4, where we have used FV cleavage site mutants in combination with exosite I- or exosite II-specific ligands to assess the contribution of each exosite to the individual activation cleavages in FV.

The activation of FV by thrombin yields the procoagulant molecule FVa, which promotes the formation of the prothrombinase complex and enhances the FXa-catalyzed conversion of prothrombin to thrombin by several orders of magnitude. Because thrombin generation by the prothrombinase complex is a crucial step in

haemostasis, it represents an attractive target for anticoagulant therapy. In chapter 5, we have applied structure-based virtual ligand screening and high-throughput screening to discover small-molecules that block the interaction of FV(a) with the phospholipid membrane and thus prevent the assembly of the prothrombinase complex.

Thrombin formation by the prothrombinase complex is under physiological conditions regulated by the protein C pathway via the proteolytic inactivation of FVa by APC. Three-dimensional molecular models of APC docked at the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites have identified several FVa amino acids that may be important for FVa inactivation by APC. In chapter 6, we have employed a site-directed mutagenesis approach to probe the importance of these residues in APC-catalyzed FVa inactivation.

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Chapter II:

FV and thrombophilia: background and mechanisms

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2.1 Summary

Human coagulation factor V (FV) is an essential coagulation protein with functions in both the pro- and anticoagulant pathways. Failure to express and control FV functions can either lead to bleeding, or to thromboembolic disease. Both events may develop into a life-threatening condition. Since the first description of APC resistance, and in particular the description of the so-called factor V_{Leiden} mutation, in which a prominent activated protein C cleavage site in FV has been abolished through a mutation in the FV gene, FV has been in the center of attention of thrombosis research. In this review we describe how the functions of FV are expressed and regulated and provide an extensive description of the role that FV plays in the etiology of thromboembolic disease.

2.2 Introduction

In the Western world, thrombotic disease is a major cause of mortality. The clinical definition of thrombosis is that of the pathological presence of a clot (thrombus) in a blood vessel or in the heart that causes the obstruction of blood flow through the circulatory system. Depending on the location where thrombus formation takes place (i.e. in the venous or arterial part of the vessel tree), thrombotic disease can be classified into venous and arterial thrombosis. Both types of thrombosis are considered as distinct disease states that are characterized by different pathogenic mechanisms and underlying risk factors [1,2]. However, central to the pathogenesis of both venous and arterial thrombosis is the perturbation of the normal haemostatic balance. In healthy individuals, haemostasis is carefully controlled by several anticoagulant mechanisms that balance the procoagulant forces and thus prevent inappropriate vascular blood clotting. Several observations are even in favor of a slight dominance of the anticoagulant forces during homeostasis [3]. This dynamic equilibrium between pro- and anticoagulant factors can be rapidly shifted in favor of coagulation in case of a physiological need for cessation of blood loss. However, this proneness towards clot formation implies a risk to develop thrombosis. As early as in the mid 1800's, the German pioneer in the field of haemostasis, Rudolf Virchow, postulated that three major causes contribute to thrombin formation (Virchow's triad): changes in blood composition, the vessel wall or the blood flow [4]. Whereas arterial thrombosis is dominated by vessel wall changes (atherosclerosis), the known risk factors for venous thrombosis can be divided into those that are attributable to changes in the blood flow (stasis) or to changed blood composition. Although Virchow's broad classification is still valid overall, the view of thrombogenicity in present day medicine is more refined as a result of progress that has been made in this field of research. A more common

view is to divide those factors that contribute to thrombosis into two groups: the *inherited* and *acquired (environmental)* thrombosis risk factors. Acquired risk factors for thrombosis include changes induced by various influences such as pregnancy, surgery, immobilization, hypobaric travel, autoimmune disease, diet, smoking and the use of oral contraceptives, or by intercurrent disorders such as diabetes mellitus, dyslipidemia, hypertension and hyperhomocystinemia [1,2,5]. Perturbation of the haemostatic balance towards coagulation may also be influenced by genetic changes, most of them affecting the protein C anticoagulant system, such as activated protein C (APC) resistance caused by the factor V_{Leiden} mutation, or other polymorphic forms of FV, deficiencies of protein S, protein C or antithrombin or by increased levels of procoagulant factors such as FVIII or prothrombin [3,6-8]. Other distinct factors are age and sex, each affecting an individual's cumulative thrombotic risk.

For a long time, thrombosis was commonly regarded as a single gene disorder. This view was conditioned largely by a seemingly obvious thrombosis etiology that was concluded from studies with small numbers of patients or families with inherited deficiencies in the anticoagulant proteins antithrombin [9], protein C [10] and protein S [11] and the associated high prevalence or severity of thrombosis. Over the last decade however, additional risk factors for thrombosis have been described and our understanding of the underlying mechanisms that contribute to the actual development of thrombosis has increased. It has now become widely accepted that thrombosis is a multifactorial disease that may occur as the result of the interplay between two or more genetic, environmental or behavioral risk factors [1,5]. These factors together are capable of synergistically passing a certain anticoagulant threshold, thereby tipping the natural haemostatic balance between pro- and anticoagulant forces. When the threshold is passed, the natural anticoagulant systems are insufficient to balance the procoagulant factors, resulting in the development of a thrombotic event [1].

Recently, it has become evident that hypercoagulability not only results in an increased tendency to thrombosis, but also triggers intracellular signaling for inflammation, given that the pathways of coagulation and inflammation are intertwined at several points. For many years it has been known that C4 binding protein (C4BP), a regulator from the complement pathway, is also a major regulator of the *in vivo* activity of protein S [12]. Furthermore, the procoagulant clotting factors FVIIa, FXa and thrombin can activate members of the protease-activated receptor (PAR) superfamily, which in turn initiate intracellular signaling pathways [13]. The elicited inflammatory response includes cytokines, adhesion molecules and growth factors, which upregulate the expression of the coagulation initiator tissue factor (TF), through their corresponding receptors. This feedback mechanism sustains the coagulation-inflammation cycle and thus contributes to an increased

thrombotic risk. Therefore, increasing attention has been recently devoted to the development of PAR antagonists that may be useful in anti-thrombotic therapy [13-15]. Lastly, in the process of inflammation the protein C pathway likely is of less importance, as is evidenced by the reduction in mortality after i.v. infusion of APC in patients that have developed life threatening sepsis. The exact mechanisms have not been clarified, but the contribution of APC and EPCR (the endothelial cell protein C receptor molecule) appear evident [16].

Since thrombin is the dominant enzyme in so many humoral and cellular processes and since for the formation of thrombin in plasma there is an absolute requirement for the presence of FV, regulation of FV activities is a major regulatory mechanism for the control of thrombin formation. FV not only plays a role in procoagulant processes, but as will be detailed below, FV also plays an important role in the protein C pathway, and thus plays a dual role with its involvement in both pro- and anticoagulant processes.

This chapter will focus on inherited defects in blood coagulation factor V (FV) that are considered as risk factors for thrombosis. Particular attention will be paid to the molecular mechanism by which FV polymorphisms contribute to APC resistance. Interactions with acquired risk factors that are known to synergistically increase the thrombotic risk that is related to FV genetic defects will also be discussed.

2.3 Haemostasis

Haemostasis is a physiological mechanism that controls blood fluidity and has the potential to rapidly induce haemostatic plug formation at sites of injury in order to stop or limit bleeding. The three distinct phases of the haemostatic process (primary haemostasis, coagulation and fibrinolysis) are closely linked to each other and are strictly regulated in order to efficiently close vessel wounds and promote vascular healing.

Primary haemostasis is initiated by the adhesion of platelets to collagen fibers underlying the vascular endothelium, which have become exposed following vascular injury. This adhesion is mediated by a specific platelet collagen receptor (glycoprotein Ia/IIa) and von Willebrand factor (vWF), which forms links between the platelet and collagen fibers [17]. As a result of the interaction with collagen, the platelets are activated and release a number of different coagulation factors and platelet activating factors, which in turn activate other platelets and white blood cells. The platelets adhere to each other via adhesion receptors (integrins) that bind to the same receptor on adjacent platelets via a central fibrinogen molecular bridge, which results in the formation of a haemostatic platelet plug. Platelet activation further results in the activation of several phospholipid transporter proteins, among which the protein scramblase, which causes the transport of

negatively charged phospholipids from the inner to the outer leaflet of the platelet membrane. These negatively charged phospholipids provide a catalytic surface that is crucially important for efficient propagation of the coagulation through binding of several blood coagulation factors.

Initiation of blood coagulation (also known as secondary haemostasis) *in vivo* is triggered by the exposure of the transmembrane glycoprotein tissue factor (TF) to blood [18]. Exposed TF from subendothelial layers binds with high affinity and specificity to both the zymogen and activated forms of coagulation factor VII (FVII and FVIIa respectively). A small fraction of FVII circulates in blood as an active protease (FVIIa) and activates factors IX and X when it is bound to TF. Activated FIX (FIXa) subsequently interacts with its non-enzymatic cofactor FVIIIa on the activated platelet surface to form the tenase complex, which efficiently activates FX. After its formation by the tenase complex, FXa assembles on the platelet membrane with its cofactor FVa to form the prothrombinase complex. The prothrombinase complex efficiently activates prothrombin to thrombin, which feedback amplifies its own formation by activating FV, FVIII and FXI [19-25]. Activation of FXI results in the generation of additional FIXa, which in turn activates FX, thus enforcing thrombin formation [26]. Most importantly, thrombin converts soluble fibrinogen into insoluble fibrin fibers which aggregate to a soft fibrin clot.

The third phase of haemostasis involves fibrinolysis. The role of fibrinolysis is to remove fibrin deposits, after the damaged vessel wall has been restored. To this end, circulating plasminogen is activated by interactions between fibrin, plasminogen and the activator tPA. Plasmin, the product of plasminogen activation, is able to proteolytically degrade fibrin, such that the fibrin network is dissolved.

2.4 Anticoagulant mechanisms that regulate haemostasis

The formation of a stable plug by platelet aggregation and fibrin polymerization following vascular injury is a very rapid and important process that prevents extensive blood loss. However, intrinsic to the capacity of the blood clotting system to respond quickly to vascular injury is the danger of excessive clotting that may contribute to thrombosis. Therefore, nature has provided us with several anticoagulant mechanisms that at different levels control the coagulation pathway and that prevail over procoagulant mechanisms under normal conditions. Two major systems can be discriminated that provide efficient control of the activity of pro- and anticoagulant pathways. A first system comprises circulating inhibitors (e.g. TFPI [18,27], antithrombin [28], α_2 -macroglobulin [29,30] and antitrypsin [31]) which can directly neutralize activated coagulation factors. A second important negative regulatory pathway of the coagulation cascade is the protein C (PC)

pathway [32-34]. A key event in the initiation of the PC pathway is the formation of the thrombin-thrombomodulin complex [35]. Thrombomodulin (TM) is a transmembrane protein present on intact endothelium, primarily that of the smaller vessels, that acts as a thrombin receptor by binding to thrombin exosite I and provides a binding platform for the substrate protein C. This thrombin exosite I plays an important role in the thrombin-mediated recognition and activation of FV, FVIII and fibrinogen [36]. As a consequence, the procoagulant properties of thrombin molecules that escape a site of ongoing coagulation and migrate into the microvasculature are lost upon binding to TM. In addition, TM binding causes conformational changes near the active site of thrombin which alter substrate specificity [35,37] such that thrombin becomes an anticoagulant protein that can efficiently activate PC in a process that is enhanced by the endothelial cell protein C receptor (EPCR) [35].

In contrast to inhibitors that directly target protease activity, the PC pathway regulates the formation of the activated procoagulant enzymes FXa and thrombin by proteolytic inactivation of FVIIIa and FVa respectively, both of which are the essential cofactors of the tenase and prothrombinase complex, respectively [38]. Proteolytic inactivation of FVIIIa and FVa (as opposed to the spontaneous inactivation through cofactor dissociation which is particularly important in the case of FVIIIa) is accomplished by activated protein C (APC) and is stimulated by the non-enzymatic cofactor protein S [39-41]. During the degradation of FVIIIa, FV acts as an additional cofactor to APC and the stimulating effect of FV and protein S on FVIIIa inactivation was found to be synergistic rather than additive [42,43].

Under physiological conditions, the presence of the circulating inhibitors and the protein C pathway together form the earlier mentioned thrombosis threshold that prevents the initiation and propagation of thrombin formation. When the trigger of coagulation is such that sufficient amounts of proteolytically active enzymes are formed that cannot be controlled by the inhibitors or the protein C pathway, the formation of large amounts of thrombin cannot be prevented anymore. At this stage the threshold is surpassed. The physiological importance of the PC pathway is illustrated by the observation that deficiencies of protein C or protein S result in an increased risk for venous thrombosis [6,44].

2.5 FV and thrombophilia

Given the key role of FV in haemostasis, regulation of its pro- and anticoagulant cofactor activities is of prime importance for maintaining the haemostatic balance. As a consequence, perturbed FV functions can result in both thrombotic and haemophilic clotting disorders [45,46]. Genetic and acquired defects that affect the

activity and/or expression level of the FV molecule therefore are considered as risk factors for thrombosis and will be discussed in the following paragraphs.

2.5.1 APC resistance

APC resistance is an *in vitro* phenomenon that is characterized by a poor anticoagulant response to APC in plasma. Reduced susceptibility to APC of a plasma sample results in inefficient regulation of thrombin formation *in vitro* making a plasma APC resistant. *In vitro* APC resistance is as such associated with an increased risk for thrombosis [47]. The phenomenon of APC resistance was first reported by Dahlbäck and co-workers, who found that the plasma of a group of patients with a family history of venous thrombosis showed a reduced anticoagulant response to the addition of APC [48]. Later, it was found by several groups simultaneously that a single point mutation in the FV gene, which results in the substitution of arginine at position 506 by glutamine (FV_{Leiden} mutation), was responsible for the observed APC-resistant phenotype of these patients [49-52]. The R506Q mutation is the hallmark of the FV_{Leiden} phenotype, and is a missense mutation at a dominant APC cleavage site in the heavy chain of FVa (Table 2.1). APC resistance represents the most frequent independent risk factor for (venous) thrombosis known to date [53]. In most cases, it is associated with the FV_{Leiden} mutation, but several other genetic and acquired risk factors such as the prothrombin G20210A mutation, pregnancy or the use of oral contraceptives are known to affect the anticoagulant response to APC. It should be noted that APC resistance as such is an independent risk factor for the development of thrombosis, even in the absence of the FV_{Leiden} mutation [47].

2.5.2 The FV_{Leiden} mutation as a major cause of APC resistance

Since its discovery in 1994, the FV_{Leiden} mutation has been demonstrated to represent the most frequent cause of APC resistance [54]. In healthy individuals of Caucasian origin, the prevalence of FV_{Leiden} is between 2 and 10%, making the factor V_{Leiden} mutation at least 10 times more common than other genetic factors that cause thrombosis [55]. The highest prevalence of the mutation is found in Europe, most notably in Cyprus, southern Sweden and Germany, but the mutation is also very common in Saudi Arabia and in the Arab and Jewish populations of Israel [56]. Although widely present in Caucasians, the FV_{Leiden} mutation is not found in other ethnic groups such as in individuals of African, Chinese or Japanese origin. Haplotyping of FV_{Leiden} homozygotes suggested a founder effect and that a single mutational event occurred approximately 21,000 years ago [57] .

Among patients with venous thromboembolism, FV_{Leiden} occurs in 20% of all cases and in up to 50% of selected patients with thrombophilia. The FV_{Leiden} mutation seems to be a risk factor of much the same strength as the deficiencies of coagulation inhibitors, increasing the risk about 5-fold in heterozygous carriers [58]. Homozygous carriers of the mutation are considered to have a 50- to 100-fold increased risk of thrombosis.

Amino acid substitution	Alternative name	Domain	Exon	db SNP rs number	MAF African	MAF Caucasian	MAF Oriental
Arg ⁵⁰⁶ Gln	FV Leiden	A2	10	rs6025	0	1	0
Arg ³⁰⁶ Thr	FV Cambridge	A2	7	/	/	/	/
Arg ³⁰⁶ Gly	FV Hong Kong	A2	7	/	/	/	/
Ile ³⁵⁹ Thr	FV Liverpool	A2	8	/	/	/	/
Arg ⁴⁸⁵ Lys	/	A2	10	rs6020	32	0	70
Met ³⁸⁵ Thr	R2 haplotype	A2	8	rs6033	0	6	3
His ¹²⁹⁹ Arg		B	13	rs1800595	-	-	-
Met ¹⁷³⁶ Val		A3	16	rs6030	19	32	22
Asp ²¹⁹⁴ Gly		C2	25	rs6027	0	7	5
Ser ⁷³⁹ Ser	A/G allele	B	13	rs6021	7*	0*	?
Lys ⁸³⁰ Arg		B	13	rs4524	17	26	17
His ⁸³⁷ Arg		B	13	rs4525	20	21*	27*
Lys ⁸⁹⁷ Asp		B	13	rs6032	17	26	17

Table 2.1. List of missense variations in FV that were discussed in the text. The common allele is given before the coordinate, the rare allele after the coordinate. Numbering is according to the cDNA sequence of *F5* (Jenny *et al.* [77]; GenBank accession number M16967). Population frequency data are obtained from an online database of mutations and polymorphisms in the FV gene, accessible at http://www.lumc.nl/4010/research/factor_V_gene.html [78]. Data from the HapMap project (<http://www.hapmap.org/index.html.en>), in which Africans, Caucasians, Han Chinese and Japanese were typed, have been used. The Oriental MAF (Minor Allele Frequency) represents the average from the Chinese and Japanese data. If the HapMap data are not available, data were obtained from Seattle SNPs (<http://pga.mbt.washington.edu/>), in which Afro-Americans and Caucasians were typed. Question marks have been entered when data were not available from the HapMap or Seattle project. Because of its presence in a repeated region, the H1299R-variant has not been typed in any of the projects.

The most common clinical manifestations of APC resistance caused by the FV_{Leiden} mutation are venous thrombosis and pulmonary embolism. There is a much less clear link between the mutation and arterial thrombosis. Recent studies however have identified factor V_{Leiden} as an important risk factor for arterial thrombosis [59,60]. Yet other studies have linked the FV_{Leiden} mutation to atherosclerosis [61] and myocardial infarction [62]. At the same time however, several other studies have been unable to directly associate the FV_{Leiden} mutation to arterial thrombotic disease [63,64].

2.5.3 Molecular basis of FV_{Leiden} related APC resistance

The FV_{Leiden} mutation, i.e. the substitution of a glutamine for an arginine at position 506, has important consequences for the function of FV in the pro- and anticoagulant pathways of the blood coagulation process. First, the loss of one of the three APC cleavage sites in FVa_{Leiden} impairs efficient downregulation of procoagulant FVa activity (Figure 2.1) [65].

It was long believed that the reduced APC-mediated inactivation of FVa_{Leiden} provides a rational molecular mechanism to explain the increased thrombogenicity in carriers of this mutation. In a purified system however, it was demonstrated that protein S and FXa can almost completely eliminate the differences between the APC-mediated inactivation of normal FVa and FVa_{Leiden}. This was explained by the observed protective effect of FXa against FVa cleavage at Arg⁵⁰⁶ by APC [66], together with a stimulating effect of protein S on mainly the APC-mediated cleavage at Arg³⁰⁶ [67,68].

The discovery that FV serves as a cofactor during the APC-catalyzed inactivation of FVIIIa provided an important clue to possible mechanisms by which the FV_{Leiden} mutation contributes to the APC resistance phenotype *in vivo*. It was found that FV isolated from patients carrying the FV_{Leiden} mutation is a much weaker APC cofactor in the inactivation of FVIIIa than normal FV [69,70]. Later, it was demonstrated using recombinant FV that cleavage at Arg⁵⁰⁶ is required for FV in order for FV to express its anticoagulant cofactor activity [71]. Thus, because FV_{Leiden} cannot be cleaved at Arg⁵⁰⁶, it cannot be transformed into an anticoagulant FV molecule (see also figure 2.1) and therefore it is a poor cofactor of APC in the proteolytic inactivation of FVIIIa.

The pathophysiologic relevance of the FV_{Leiden} mutation was further illustrated by thrombin generation experiments from our laboratory, in which FV-deficient plasma was reconstituted with increasing concentrations of normal FV or FV_{Leiden}. In the presence of APC, thrombin generation was progressively decreased when increasing the concentration of normal FV, whereas the addition of FV_{Leiden} had no effect on thrombin generation [72]. Moreover, addition of purified normal FV, was

Figure 2.1. FV and FV_{Leiden} in the pro- and anticoagulant pathways of coagulation. Native FV has little procoagulant activity until it is activated through limited proteolysis by thrombin or FXa at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. Following activation, the activated FV molecule (FVa) is a heterodimer that consists of a 105 kDa heavy chain (A1-A2 domains) and a 71/74 kDa light chain (A3-C1-C2 domains) that are non-covalently associated in a calcium-dependent manner. FVa promotes FXa-catalyzed prothrombin activation within the prothrombinase complex and is subsequently inactivated by the APC/protein S complex via proteolytic cleavage at Arg³⁰⁶ and Arg⁵⁰⁶. Alternatively, native FV can be directly cleaved by APC at Arg⁵⁰⁶ to yield an anticoagulant molecule (FV_{ac}) which serves as a cofactor in the APC-mediated cleavage of FVIIIa. APC-mediated cleavages of FV(a) that are suppressed by the FV_{Leiden} mutation are marked by a cross.

able to reduce thrombin generation in pseudohomozygous carriers of the FV_{Leiden} mutation (who express 50% FV_{Leiden} only since they also carry a null-allele). These individuals have an associated thrombosis risk that is in the same range as that of homozygous carriers of the FV_{Leiden} mutation (50- to 80-fold increased). Addition of purified FV to pseudohomozygous plasma brings the response to APC in the same range as that of heterozygous carriers of the FV_{Leiden} mutation, who have an associated risk of thrombosis that is ~5-fold increased. It therefore appears as if

the increased risk of thrombosis in homozygous and pseudohomozygous carriers of the FV_{Leiden} mutation is not so much a defect in the FV_{Leiden} that is present, more likely it illustrates the lack of anticoagulant potency of normal FV in these individuals.

2.5.4 Other FV mutations associated with APC resistance

Studies validated by FV gene analysis revealed that up to 20% of randomly selected individuals exhibiting APC resistance do not carry the FV_{Leiden} mutation [73]. Since the discovery of the FV_{Leiden} mutation, several other genetic defects in the FV gene have been identified as possible risk factors for thrombosis.

Some of these mutations affect the APC cleavage site at position Arg³⁰⁶. A first variant, *FV Cambridge*, contains a missense mutation in the FV gene resulting in the replacement of Arg³⁰⁶ by threonine. The FV Cambridge mutation was first discovered in a British patient and his mother who were found to be APC resistant [7]. A second mutation at Arg³⁰⁶ is caused by a missense mutation in the FV gene resulting in an Arg to Gly substitution at position 306. This variant was first described in two Chinese thrombotic patients from Hong Kong and has been named FV Hong Kong. Although phenotypically very similar to FV Cambridge, the *FV Hong Kong* mutation has not been associated with APC resistance [8]. However, *in vitro* experiments employing recombinant FV Cambridge and FV Hong Kong molecules have shown that both variants cause mild APC resistance due to a moderately impaired APC cofactor activity [74]. It is not known what has caused the different observations in the proband plasma, but other plasma factors that contribute to APC resistance are likely candidates to explain the apparent difference between the plasma derived proteins and their recombinant counterparts. Why FV Cambridge and FV Hong Kong are less resistant to APC than FV_{Leiden} remains unclear in particular since from studies with purified proteins it is evident that both cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ are important for the loss of FVa activity [65]. It has been proposed though that in the absence of the normal Arg³⁰⁶ cleavage site, alternative APC cleavage sites close to this region become relevant [75]. Likewise it is possible that reduced Arg³⁰⁶ cleavage in plasma can remain masked as a result of low levels of FXa generated during the coagulation assay: at low FXa concentration the activity of FVa cleaved at only Arg⁵⁰⁶ is virtually indiscriminable from completely inactivated FVa [65]. Due to the rare occurrence of both FV variants [76], extensive epidemiological studies are not available and as a result it remains unanswered whether or not both mutations are risk factors for venous thrombosis.

Apart from the mutations affecting the APC cleavage sites described so far, other mutations in the heavy chain of FV have been reported that are associated with mild APC resistance.

The *FV Liverpool* mutation, a missense mutation that results in the substitution of Ile³⁵⁹ by threonine, introduces a novel glycosylation consensus sequence at Asn³⁵⁷ (Figure 2.2) [79]. The presence of a carbohydrate side chain at this position hampers APC cleavage at Arg³⁰⁶ and Arg⁵⁰⁶ and also impairs the APC cofactor function of FV in the degradation of FVIIIa presumably by steric hindrance [80].

Figure 2.2. FVa missense mutations associated with APC resistance. FVa is shown as a ribbon (domains A1, green; A2, cyan; A3, brown; C1, pink and C2 purple). The membrane is shown in grey wire representation (phosphorus atoms are indicated in red). Polymorphisms in the FVa molecule that were discussed in the text are displayed in red CPK representation. The H1299R mutation is present in the B domain of FV for which no structure is available at present. The figure was created with ICM Pro (Molsoft LLC).

The *R485K mutation* (Figure 2.2) has been found in several Oriental populations and has been associated with venous thrombosis [81], coronary artery disease [82] and pre-eclampsia [83]. Unfortunately, no data are available that might explain the molecular mechanism that explains the observed APC resistance in carriers of the R485K mutation.

A further common genetic variation in the FV gene is the *R2 haplotype*, which is hallmarked by a His to Arg substitution at position 1299 in the FV B domain [84]. A number of other mutations in the FV gene are tightly linked to the H1299R single nucleotide polymorphism (SNP). These mutations are collectively referred to as the R2 haplotype and predict silent and missense amino acid substitutions in both the heavy and light chain as well as the B domain [85,86]. Based on its worldwide distribution and high prevalence in both the Caucasian and non-Caucasian population, it was proposed that the origin of the R2 haplotype dates back further than the FV_{Leiden} mutation [86]. The Asp²¹⁹⁴Gly mutation, which is tightly linked to the R2 haplotype, has been associated with reduced FV levels and a shifted FV₁:FV₂ ratio in favour of the more thrombogenic FV₁ isoform [85]. It has earlier [87] been shown that differential glycosylation of Asn²¹⁸¹ in the C2 domain of FV is the molecular cause of the coexistence of two forms of FV: FV₁ and FV₂. Yamazaki et al. [88] later proved that the Asp²¹⁹⁴Gly mutation and not Met³⁸⁵Thr, His¹²⁹⁹Arg or Met¹⁷³⁶Val, plays a key role in the partial FV deficiency of the R2 haplotype, due to impaired secretion of recombinant molecules that carry this missense mutation. *In vitro* experiments using recombinant FV molecules further indicate that the presence of a glycine at position 2194 stimulates glycosylation at Asn²¹⁸¹ (unpublished data, Yamazaki and Nicolaes).

Molecular dynamics simulations have demonstrated that the Asp²¹⁹⁴Gly mutation results in a less stable FV C2 domain as a result of the loss of a salt bridge network between Asp²¹⁹⁴ and surrounding Lys residues [89]. Possibly, the non-glycosylated Asp²¹⁹⁴Gly variant is cleared differently from the circulation as compared to its glycosylated counterpart, which could explain the observed lower FV levels and increased FV₁:FV₂ ratio in FV R2 carriers, likewise the glycosylation at Asn²¹⁸¹ in the C2 domain may be an epi-phenomenon that is not directly linked to the levels of circulating FV, but merely is an illustration of the overall intracellular processing of FV.

The molecular mechanism that causes the observed (mild) decrease in APC resistance in R2 carriers has still not been completely elucidated. The reduced level of expression of R2-FV is the most likely explanation for the enhanced thrombophilic phenotype in R2 carriers, in particular in FV_{Leiden} carriers. It is now indeed widely accepted that compound heterozygosity of the R2 and the FV_{Leiden} mutations is associated with a higher risk of venous thrombosis than heterozygous FV_{Leiden} alone [90-92]. Until now however, there are no conclusive data available

that associate the R2 haplotype *per se* with a relevant increased risk of thrombosis [93]. Recently, it was observed that male carriers of the FV R2 polymorphism have increased circulating levels of FVIII [94]. No biochemical explanation has been given for this observation, but it is tempting to speculate that altered FV anticoagulant activity of R2 FV results in diminished proteolytic control of FVIII levels by the protein C pathway. Since increased levels of FVIII are an independent risk factor for the occurrence of venous thrombosis [95], this may explain a potential association between R2 FV and thrombosis.

Given the poor conservation of the FV B domain among different mammalian species and thus that mutations are obviously tolerated within this part of the FV gene, it is to be expected that more missense mutations are present in this domain. Besides the H1299R mutation, a number of other missense mutations in the B domain have been reported [78]. Most of these mutations are however not associated with functional defects and together with the overall poor conservation of the B domain, this may indicate that the amino acid sequence of the B domain is not an important determinant for FV function. Nevertheless, a number of polymorphisms in the FV B domain have been described that are always co-inherited in Caucasians and that contribute to the APC resistance phenotype [96]. Because these mutations result from the A>G transitions at nucleotide positions 2391, 2663, 2684 and 2863, the terms A-allele and G-allele were introduced for the wild type and variant allele respectively. Of these 4 mutations, only the last three changes result in amino acid substitutions (K830R, H837R, K897E). The molecular mechanism involved in the APC resistance phenotype that is associated with these polymorphisms is still unknown. It may be anticipated that the G-allele mutations affect the anticoagulant function of FV rather than its procoagulant function, because they are present in a region that is removed upon activation. In the absence of direct proof of the effect that these mutations have on FV function, caution is to be taken with these FV variants. The changed APC resistance phenotype that is observed in the plasma of individuals carrying this haplotype may merely be a reflection of yet another (unknown) genetic variation outside the FV gene that causes the APC resistance to vary.

2.5.5 Acquired thrombotic risk factors that affect FV function

Apart from genetic defects, other factors that influence FV function and impair the response to APC have been reported. Over the last decade, several individuals have been described who have developed auto-antibodies against FV [97-101]. In most instances, these antibodies inhibit the expression of the normal functions of FV in haemostasis. The most common cause of FV inhibitors has been exposure to bovine thrombin. Bovine thrombin is commonly mixed with fibrinogen derived

from cryoprecipitate to make fibrin sealant, a haemostatic preparation that is commonly used in cardiovascular surgery [102]. The presence of small amounts of contaminating proteins (among which FV) in the thrombin preparation that has been purified from bovine plasma may elicit a strong immune response in recipients. Although antibodies against FV generally have no clinical consequences or induce a bleeding tendency, a number of cases have been reported in which the development of FV auto-antibodies was associated with thrombosis. Sufficient data to establish a direct causal link between FV antibodies and thrombosis have only been obtained in a couple of instances [98-101]. What the underlying mechanisms are that result in the increased thrombotic risk in these patients is unknown. It is possible that the auto-antibodies block the anticoagulant properties of FV, similar to the confirmed effect that a monoclonal FV antibody, directed against an epitope in the B domain of FV, has on the *in vitro* APC cofactor activity of FV [42]. Since patients with FV antibodies had additional risk factors for thrombosis, it cannot be excluded that the thrombotic phenotype is to be attributed to the presence of these risk factors.

2.5.6 Risk factor interactions determine thrombotic risk

Although the pathogenesis of venous thrombosis is still far from being fully elucidated, substantial progress has been made over the last decade towards a better understanding of the pathophysiological mechanisms involved in this disease. The most significant finding was the confirmation of the concept that inherited hypercoagulable conditions are present in a large proportion of patients with venous thrombosis or pulmonary embolism. It has been estimated that inherited genetic risk factors may account for more than 60% of the total risk to develop thrombosis [103]. The high prevalence of the FV_{Leiden} mutation and other frequently occurring prothrombotic mutations (e.g. the G20210A prothrombin mutation) have made it possible to study the cumulative effects of individual risk factors on the overall thrombotic risk. In individuals where multiple risk factors for thrombosis occur, the risk factors appear to have a synergistic effect. Among thrombotic families with antithrombin, protein C, protein S deficiency, the HR2 haplotype or the prothrombin G20210A mutation, it was found that the prevalence of the FV_{Leiden} mutation was much higher than in the general population. Moreover, the combined risks in these families are much higher than the risk conferred by the sum of the single thrombophilic defects [91,104-107]. It is now generally accepted that interactions between more than one genetic or acquired risk factor are a prerequisite for the development of thrombosis [1,94]. Given the ubiquitous presence of FV prothrombotic genetic variants, FV is one of the important factors that contribute to development of thrombosis worldwide.

The earlier mentioned FV_{Leiden} pseudohomozygosity (a combination of the FV_{Leiden} mutation and a null mutation on different alleles (*in-trans*)) is in fact a special type of gene-gene interaction, where the presence of a nonsense and a missense mutation on different alleles coincide within the same gene. Alternatively, the lack of expression of the FV null allele can mask a detrimental effect of the FV_{Leiden} mutation when both mutations are present on the same allele (*in-cis*). This can cause a discrepancy in the FV genotype/phenotype, which in this case appears to be in favor of the carrier: the FV_{Leiden} mutation is silenced by another null-mutation of the same allele [108] and therefore only normal FV is expressed from the remaining normal FV allele. Notably, both these cases illustrate that APC resistance should preferably be evaluated with a functional assay since PCR based mutation analysis of the FV_{Leiden} mutation may result in test outcomes that do not represent the biologically relevant phenotype in case of *in-trans* or *in-cis* combinations of the FV_{Leiden} allele and a null mutation.

Besides gene-gene interactions, interactive effects between genetic and acquired risk factors also contribute significantly to a higher thrombotic risk. Such interaction is well illustrated when analyzing the risk for venous thrombosis in women carrying the FV_{Leiden} mutation who use oral contraceptives [109,110]. It has been estimated that the relative risk for the development of thrombosis in FV_{Leiden} carriers taking oral contraceptives is approximately 5 times higher than in FV_{Leiden} carriers who do not use oral contraceptives [111]. The factors that increase thrombosis risk in women taking oral contraceptives are clearly not independent of the mechanism that increases APC resistance in FV_{Leiden} carriers, as is concluded from the overall multiplicative risk of thrombosis (as compared to the risk increases that are conveyed by each of the factors separately).

Other examples of synergistic interactions between environmental factors and FV genetic risk factors are those observed between FV_{Leiden} and the antiphospholipid syndrome [112] and long-distance travel [113]. Also in these cases there is an apparent interaction between the processes that are the cause of the thrombosis risk increase for each of the factors separately and the combined risk increase outweighs the sum of the increases due to individual risk factors.

2.6 Conclusions

This chapter has concentrated on FV, its functions and inherited defects in the FV gene that may contribute to an increased thrombotic risk. It should be kept in mind however that thrombosis is a multifactorial disease in which genetic and acquired risk factors interact dynamically. Therefore the role that FV plays in the etiology of thrombosis is largely dependent on other factors that influence the impact of any altered FV phenotype. Although an increasing number of genetic risk

factors have been identified in recent years, less is known about the combined effect of multiple genetic and acquired risk factors on the overall thrombotic risk. Future studies will need to focus on gene-gene and gene-environment interactions to appreciate, and possibly predict, the overall impact of a given polymorphism in venous and arterial thrombosis. The central role that FV plays both in pro- and anticoagulant pathways suggests that any alterations in the FV gene or its expression may have more pronounced systemic functional effects than alterations in the genes of other coagulation factors.

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Chapter III:

Structural models of the snake venom FV activators from *Daboia russelli* and *Daboia lebetina*

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3.1 Summary

Blood coagulation factor V (FV) is a multifunctional protein that circulates in human plasma as a precursor molecule which can be activated by thrombin or activated factor X (FXa) in order to express its cofactor activity in prothrombin activation. FV activation is achieved by limited proteolysis after Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ in the FV molecule. The venoms of *Daboia russelli* and *Daboia lebetina* contain a serine protease (RVV-V and LVV-V respectively) that specifically activates FV by a single cleavage at Arg¹⁵⁴⁵. We have predicted the three-dimensional structure of these enzymes using comparative protein modeling techniques. The plasminogen activator from *Agkistrodon acutus*, which shows a high degree of homology with the venom FV activators and for which a high-quality crystallographic structure is available, was used as the molecular template. The RVV-V and LVV-V models provide for the first time a detailed and accurate structure of a snake venom FV activator and explain the observed sensitivity or resistance toward a number of serine protease inhibitors. Finally, electrostatic potential calculations show that two positively charged surface patches are present on opposite sides of the active site. We propose that both FV activators achieve their exquisite substrate specificity for the Arg¹⁵⁴⁵ site via interactions between these exosites and FV.

3.2 Introduction

Blood coagulation factor V (FV) is a multifunctional protein which plays a central role in the pro- and anticoagulant pathways of blood coagulation [1]. FV is a 330 kDa single-chain glycoprotein that has a mosaic domain structure A1-A2-B-A3-C1-C2 formed by three homologous A-type domains, two smaller homologous C-type domains, and a heavily glycosylated B domain [2]. FV circulates in human plasma as a precursor molecule and can be activated by thrombin or activated factor X (FXa) in order to express its activity in the prothrombin-activating complex that further consists of FXa, prothrombin and calcium, assembled on a phospholipid surface. FV activation is achieved by three specific proteolytic cleavages which result in the removal of the B domain and the exposure of epitopes in the FV molecule that are necessary for expression of its procoagulant activity [3,4]. Thus, thrombin-activated FV (FVa) is a heterodimer consisting of a heavy chain of 105 kDa (A1-A2 domains) and a 71/74 kDa light-chain doublet (A3-C1-C2 domains) [5]. FVa acts as the non-enzymatic cofactor in the prothrombinase complex which accelerates the activation of prothrombin more than 1000-fold [6,7].

FV is not only activated by thrombin and FXa, but also by proteases present in the venom from a wide variety of snakes [8]. One group of venom proteases are the thrombin-like enzymes found in the viper family (*Viperidae*). Snake venom

proteases from the family of the *Viperidae* belong to the most extensively studied, as they have proven to be useful tools in thrombosis and haemostasis studies [9] and are of interest to both biochemists and clinical chemists.

The FV activator from the venom of Russell's Viper (*Daboia russelli* formerly *Vipera russelli*) has been purified to homogeneity by Kisiel and co-workers [10,11]. This protease, generally known as Russell's Viper Venom FV activator (RVV-V), is a 26 kDa-serine protease that activates FV via a single cleavage of the Arg¹⁵⁴⁵-Ser¹⁵⁴⁶ peptide bond [2,12] (Figure 3.1). Noteworthy, activation of FV by its physiological activator thrombin coincides with the proteolysis of three peptide bonds after Arg⁷⁰⁹, Arg¹⁰¹⁸ and the same Arg¹⁵⁴⁵, with only the cleavage after Arg¹⁵⁴⁵ being required for full expression of FV procoagulant activity [13]. As a result, FVa obtained by incubation of FV with RVV-V has the same procoagulant activity as thrombin-activated FV [14,15]. The relative rates of FV activation at Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ appear to be Arg⁷⁰⁹ > Arg¹⁰¹⁸ > Arg¹⁵⁴⁵ for thrombin (see also Figure 3.1) and Arg¹⁰¹⁸ > Arg⁷⁰⁹ ~ Arg¹⁵⁴⁵ for FXa, thus indicating that Arg¹⁵⁴⁵ is not the preferred cleavage site for these proteases [16].

The FV activator from the venom of *Daboia lebetina* (LVV-V) was isolated and characterized [17,18]. Like RVV-V, this single-chain glycoprotein of 28.4 kDa is a serine protease which cleaves the FV molecule after Arg¹⁵⁴⁵ [19].

Figure 3.1. Activation of human FV by thrombin and the snake venom FV activators RVV-V and LVV-V. Thrombin-catalyzed FV activation proceeds via three sequential cleavages at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. The resulting activated FV molecule (FVa) is a heterodimeric protein consisting of a light chain doublet (71/74 kDa) and a heavy chain (105 kDa). The snake venom FV activators from *Daboia russelli* and *Daboia lebetina* cleave FV at Arg¹⁵⁴⁵, giving rise to a FVa molecule with a light chain of 71/74 kDa and a heavy chain of around 290 kDa.

Despite their widespread use in the field of thrombosis and haemostasis, little or no functional and structural data on snake venom FV activators are available. Therefore, in the absence of a crystal structure or other available structural data for a snake venom FV activator, we have modeled the three-dimensional structures of RVV-V and LVV-V in order to obtain structural information on the cleavage requirements of FV, so as to correlate the activators' structure to their preferred FV cleavage site. We have investigated the structural and functional features of our RVV-V and LVV-V models and found that both models provide insight into the resistance of RVV-V and LVV-V toward a number of serine protease inhibitors. Further, the presence of two highly positively charged surface patches located on opposite sides of the active site cleft suggests that exosite-mediated interactions are responsible for the enzymes' specificity for the Arg¹⁵⁴⁵ site. Finally, we have confirmed that both enzymes activate FV by a fast cleavage after Arg¹⁵⁴⁵, generating a FVa heavy chain of 290 kDa.

3.3 Materials and methods

Materials

Bovine brain phosphatidylserine, egg-yolk phosphatidylcholine, ovalbumin, bovine serum albumin, benzamidine-HCl, and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma (St. Louis, MO). EDTA and 2-mercaptoethanol were from Merck (Darmstadt, Germany). Poly ethylene glycol 8000 (MW =7000-9000) was from Fluka (Buchs, Switzerland). Pro-Phe-Arg-CH₃Cl (FPRCK) was from Calbiochem (La Jolla, CA). Pefabloc TH was a product of Pentapharm Ltd (Basel, Switzerland). The chromogenic substrate S2238 was supplied by Chromogenix (Milan, Italy). Micro BCA protein assay kits were from Pierce Biotechnology (Rockford, IL). Column materials for protein purification were purchased from Amersham Biosciences (Uppsala, Sweden). Tris-Glycine polyacrylamide gradient gels (4%-12%) were from Bio-Rad (Hercules, CA). The Mark12 wide-range unstained protein standard for SDS-PAGE and the colloidal blue staining kit were from Invitrogen (Carlsbad, CA).

Proteins

The crude venom of *Daboia lebetina turanica* was a kind gift of the snake farm of the Institute of Biochemistry, Uzbek Academy of Sciences, Tashkent, Uzbekistan. The venom of *Daboia russelli* was supplied by Haematologic Technologies (Essex Junction, VT). Human thrombin was purchased from ERL (Swansea, United Kingdom).

Human FV was purified essentially according to the method of Dahlbäck [5], modified as described [20]. Bovine factor X was purified according to Fujikawa et al. [21]. FXa was prepared from FX after activation by RVV-X and further purified as described by Fujikawa [22]. Human prothrombin was purified according to the method of Discipio et al. [23]. Bovine antithrombin was purified according to Thaler and Schmer [24]. All proteins were stored at -80°C in 25 mM Hepes (pH 7.5) and 175 mM NaCl. FV was stored at -80°C in the same buffer with 5 mM CaCl₂.

Purification of LVV-V from crude venom

The FV activator from *Daboia lebetina turanica* was purified to homogeneity as described elsewhere [8]. In brief, crude venom was dissolved in 25 mM Hepes, 50 mM NaCl, pH 7.5 to a final concentration of 50 mg/ml and centrifuged at 3500 g for 10 min. The clear supernatant was filtered through a 0.22 µm filter. In two identical runs, 0.2 ml of this solution was injected onto a Superose 12 column (HR 10/30), which was first equilibrated with the aforementioned buffer. Fractions containing FV activator were pooled and applied to a mono S column (HR 5/5), which was first equilibrated in 25 mM Hepes (pH 7.5). Aspecifically bound proteins were eluted with the washing buffer (25 mM Hepes, 25 mM NaCl, pH 7.5). Bound proteins were eluted by applying a 40 ml linear gradient from 25 to 500 mM NaCl. Fractions were screened for the presence of FV activator using the FVa assay described below. The purified protein appeared as a single band of 29 kDa as judged by SDS-PAGE analysis [8]. After determination of the protein concentration, the purified protein was diluted in 50 mM Hepes, 175 mM NaCl, 0.5 mg/ml ovalbumin (pH 7.7) and aliquots were stored at -80°C.

Protein concentration determination

The concentration of the snake venom FV activators was measured using the Micro BCA protein assay kit (Pierce). Thrombin concentrations were measured using the chromogenic substrate S2238 [7].

FVa assay

Activation of FV by the *Daboia* venom activators and thrombin was monitored via its increase in cofactor activity in the activation of prothrombin by factor Xa. Unless otherwise indicated, FVa was quantified as follows: to 230 µl of a buffer solution (prewarmed at 37°C) containing prothrombin, phospholipids and Ca²⁺ ions, 10 µl FXa was added and after 15 sec prothrombin activation was started with the addition of a 10 µl aliquot containing FVa. Final concentrations reached in the

reaction mixture were: 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl_2 , 0.5 μl prothrombin, 0.5 nM FXa, 20 μl phospholipid vesicles (10% brain phosphatidylserine/90% egg-yolk phosphatidylcholine; M/M), 0.5 mg/ml ovalbumin, 1 μl Pefabloc TH, and the factor V/Va from the aliquot. Phospholipid vesicles were prepared as described by Rosing et al. [7]. The reversible thrombin inhibitor Pefabloc TH was present to prevent feedback activation of FV by thrombin formed during the course of the assay. Rates of prothrombin activation were determined by measuring the thrombin formed after 1 min using the chromogenic substrate S2238 [7]. The amount of thrombin generated in the assay mixture was linear in time and FVa concentration (up to 60 pM). The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 mol of prothrombin activated per minute per mole of factor Xa-Va complex [25]. FVa was diluted in 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl_2 and 5 mg/ml bovine serum albumin in such amounts that the final concentration of FVa in the assay mixture did not exceed 20 pM. The Pefabloc TH present in the aliquots taken from the FVa assay mixtures did not interfere with the thrombin determination since it is a reversible inhibitor whose inhibitory action in the thrombin assay was eliminated by dilution and by the presence of a large excess of the thrombin-specific substrate S2238.

FV activation by RVV-V and LVV-V

FV (75 nM) was activated at 37°C in a reaction mixture containing 25 mM Hepes (pH 7.5), 110 mM NaCl, 5 mM CaCl_2 , 1 mg/ml PEG and 5 nM snake venom FV activator or 1 nM thrombin. At different time intervals, aliquots were taken and diluted 1/500 in ice-cold HNBSACa buffer and the FVa activity was measured in the FVa assay as described above. Full FV activation was achieved by incubation of 75 nM FV with 30 nM snake venom FV activator or 5 nM thrombin for 120 min. Activities were expressed as percentages of the full FVa cofactor activity.

Visualization of FV activation fragments by colloidal blue staining

To correlate peptide bond cleavages in FV with the generation of FVa activity during the RVV-V and LVV-V catalyzed activation of FV, 75 nM plasma-purified human FV (in 50 mM Hepes, 110 mM NaCl, 5 mM CaCl_2 and 1 mg/mL PEG, pH 7.4) was incubated with 5 nM of the purified snake venom proteases or 1 nM thrombin. Maximal cofactor activity in the activation mixture was determined by incubation of an equal amount of FV with 30 nM snake venom FV activator or 5 nM thrombin. At various time points, aliquots were removed and subjected to SDS-polyacrylamide gel electrophoresis (4%-12%) under reducing conditions. FV fragments were

visualized by colloidal blue staining. Simultaneously, FVa activities were determined as described above.

Inhibition studies

Purified RVV-V or LVV-V was incubated for 1 h at 37°C in 950 µl buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl and 0.5 mg/ml ovalbumin at a concentration that permitted reproducible estimation of chromogenic substrate conversion (8.3 and 0.9 µg/ml, respectively). One of the following inhibitors was included in the reaction mixture: EDTA (10 mM), antithrombin (6 µM), PMSF (0.1 mM), benzamidine (10 mM), BPTI (10 µM) or FPRCK (50 µM). After incubation, 50 µL 3.9 mM S2238 was added to quantify the residual amidolytic activity of the venom activator which was expressed as a percentage of the amidolytic activity of the venom activators measured in the absence of inhibitors.

Sequence alignment

All sequences were obtained from the MEROPS peptidase database (<http://merops.sanger.ac.uk>) [26]. The BLAST program [27] was used to identify the best template for modeling. Coordinates of the following crystal structures were obtained from the Protein Data Bank [28]: AAV-SP-II (1OP2.PDB), factor Xa (1G2L.PDB), thrombin-FPRCK (1PPB.PDB) and trypsin-BPTI (1TPA.PDB). Sequence alignments were created with the ICM package (Molsoft LLC), using the following parameters: gonnet scoring matrix; protein gap open and protein gap extension penalties of 2.4 and 0.15, respectively.

Homology modeling

Computer modeling and other graphical approaches were performed on a Dell workstation using the ICM Pro modeling package (Molsoft LLC) and the YASARA/WHAT IF Twinset [29]. As a template for modeling we used the 2.1 Å crystal structure of AAV-SP-II, a glycosylated snake venom plasminogen activator from *Agkistrodon acutus* (Protein Data Bank entry code 1OP2). Both snake venom FV activators are highly homologous to AAV-SP-II and share approximately 60% sequence identity with this serine protease. Compared to the template structure, RVV-V and LVV-V both show an insertion of two amino acids at position 38 (chymotrypsinogen numbering), located on a solvent-exposed loop. The conformation of this insertion loop (residues 35-41) was predicted using Loopy loop simulation [30]. This approach involves generating a large number of random backbone conformations (at least 2000) while the loops were closed using the

random tweak method [31]. For each loop, side chains were generated from a rotamer library and were grafted onto the loop using the program SCAP [32]. After energy minimization, the best candidates were selected and re-ranked after computations of the so-called colony energy. The 10 lowest energy structures were kept and analyzed interactively. Of the loops that fitted well the C α anchor groups of the residues adjoining the insertion (Tyr³⁴ and Cys⁴²), the energetically most favorable one was selected for insertion in the model.

No severe atomic clashes were detected during the construction of the models. Minor atomic bumps were removed using the WHATIF server (<http://swift.cmbi.kun.nl/WIWWWI/>). In brief, side chain torsion angles for the complete model are first rotated around in steps of 120° and the rotamer that leads to the smallest overlap with the rest of the molecule is kept. In a second round steps of 2°, 4°, or 6° are used. Further energy refinement of the models was achieved by a standard regularization protocol using the ICM program (Molsoft LLC). Stereochemistry, geometry and overall structural validity were checked at pertinent stages of the modeling procedure using the program WHATCHECK [33] and the protein health analysis tool as incorporated in the ICM Package [34]. Coordinates of the RVV-V and LVV-V models have been deposited to the Protein Data Bank (accession codes: 2FJO for RVV-V, 2FJQ for LVV-V).

Model evaluation

Several approaches were used to evaluate model quality. A Ramachandran plot of the minimized models was generated using the STRIDE web server (<http://webclu.bio.wzw.tum.de/stride/>) [35]. Secondly, model accuracy was assessed by the inverse protein folding verification approach, using the program Verify3D [36]. For experimentally determined high-resolution structures, the Verify3D scores are positive and consistently high (>0.2), indicating that they provide a reliable means to assess the quality of a protein structure [36,37]. Thirdly, the root-mean-square (RMS) deviation between the initial and the minimized model structures was calculated. The appropriateness of the charged, conserved, variable, and hydrophobic residues was also considered. Finally, we verified whether our models were compatible with available biological data such as the location of putative glycosylation sites.

Calculation of the active site volume

The active site volumes of RVV-V, LVV-V, human α -thrombin and human FXa were calculated as follows. First, using the YASARA/WHATIF Twinset package, the corresponding refined model or PDB structure was placed inside a simulation cell.

The active site inhibitor present in the thrombin or FXa structure file (1PPB.pdb and 1G2L.pdb, respectively) was removed manually. Next, the unit cell was completely filled with water and water molecules more than 10 Å away from at least one of the three catalytic site residues (His⁵⁷, Asp¹⁰² and Ser¹⁹⁵) were removed. Finally, the volume occupied by the molecular surface of the residual water molecules was calculated.

Inhibitor docking studies

We constructed four docking complexes (RVV-V and LVV-V in complex with either FPRCK or BPTI) using the X-ray crystal structures of thrombin-FPRCK (PDB entry code 1PPB) and trypsin-BPTI (PDB entry code 1TPA) as templates. Initially, the RVV-V and LVV-V models were superposed on the crystal structure complexes, guided by the C α atoms. Then, the original enzyme from the crystal (thrombin or trypsin) was deleted using the YASARA/WHATIF Twinset program and the snake venom FV activator-ligand complex was analyzed for the presence of steric overlaps. In the structural overlays of both proteases with the BPTI structure from 1TPA, severe steric clashes were observed between inhibitor residues and parts of the modeled protease, which disqualified these complexes for further evaluation. The overlay complexes harboring FPRCK were subjected to subsequent energy minimization using a standard minimization protocol in the YASARA/WHATIF program.

Electrostatic calculations

Electrostatic surface potentials were generated in order to visualize the surface charge distributions on both protein models and to compare them with thrombin (PDB entry 1PPB). Molecular surfaces and electrostatic potentials were calculated using the REBEL (Rapid Exact Boundary Element) module of the ICM package. Electrostatic potentials were visualized on the surface: red regions indicate excessive negative charge density ($e < -5$ kcal/electron units), blue regions excessive positive charge density ($e > 5$ kcal/electron units) and white regions are charged in between ($-5 \leq e \leq 5$ kcal/electron units).

3.4 Results and discussion

Visualization of FV activation fragments by SDS-PAGE analysis

Time courses of FV activation by thrombin (1 nM) and the FV-activating enzymes from *Daboia russelli* and *Daboia lebetina* (5 nM) were followed as described in the Materials and Methods section (Figure 3.2A). At varying time intervals, samples were taken and subjected to SDS-PAGE analysis to investigate the FV fragments formed during FV activation and to correlate fragment generation to the observed increase in FVa cofactor activity (Figure 3.2B).

Figure 3.2. Activation of factor V by RVV-V, LVV-V and thrombin.

(A) Time course of factor V activation by RVV-V (Δ), LVV-V ($\diamond$) and thrombin ($\circ$). Factor V (75 nM) was activated with 5 nM snake venom factor V activator or 1 nM thrombin. After the indicated time intervals, aliquots were taken and diluted 1/500 in ice-cold HNBSA/Ca buffer and the FVa activity was measured in the prothrombinase assay as described in the Materials and Methods section. Values are represented as percentage of the maximal cofactor activity that accompanies complete cleavage after Arg¹⁵⁴⁵.

(B) From the same reaction mixtures as described in (A), aliquots were withdrawn at 0, 1, 3, 5, 7, 10, 20, 45, 60, and 90 min and subjected to SDS-PAGE under reducing conditions. An aliquot of fully activated FV (after 120 min incubation with 30 nM snake venom FV activator or 5 nM thrombin, indicated as "120+") was also taken for comparison. Proteins were visualized by colloidal blue staining.

After 120 min incubation of an equal amount of FV with 30 nM snake venom FV activator or 5 nM thrombin, identical cofactor activities were obtained (data not shown). From this, it can be concluded that, like FVa_{RVV-V} [38], FVa_{LVV-V} expresses similar cofactor activity in prothrombin activation as FVa_{IIa}. In line with earlier observations, RVV-V-mediated FV activation resulted in the formation of a 71/74 kDa light chain and a heavy chain of around 290 kDa, resulting from the cleavage at Arg¹⁵⁴⁵ in the FV molecule [2,12]. A similar pattern was observed for the LVV-V-catalyzed reaction (Figure 3.2B). This is in agreement with the observation that LVV-V is able to cleave peptide fragments containing amino acid sequences identical to those around the Arg¹⁵⁴⁵ cleavage site in the FV molecule [19].

Inhibition studies

Both snake venom FV activators were tested for their sensitivity towards a number of inhibitors, which are representative for different protease classes. In Table 3.1, the effect of these inhibitors on the amidase activity of both enzymes is summarized. As shown, RVV-V and LVV-V were only significantly inhibited by the serine protease inhibitors PMSF, benzamidine, and FPRCK. This is in agreement with the decrease of the esterase and coagulant activity observed for both FV activators in the presence of DFP [10,17]. However, the *Daboia*-activators were not inhibited by antithrombin and bovine pancreatic trypsin inhibitor.

Inhibitor	RVV-V	LVV-V
None	100	100
EDTA (10 mM)	92	94
PMSF (100 µM)	24	7
Benzamidine (10 mM)	50	33
PPACK (50 µM)	3	2
BPTI (10 µM)	98	99
Antithrombin (6 µM)	99	98

Table 3.1. Effect of various inhibitors on the amidolytic activity of RVV-V and LVV-V. The FV activators from the venom of *Daboia russelli* (250 nM) or *Daboia lebetina* (30 nM) were incubated at 37°C in 950 µl buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin, pH 7.9 and inhibitor as indicated. After 1 hour incubation, 50 µl of the chromogenic substrate S2238 (3.9 mM) was added and the residual amidolytic activity was determined by measuring the increase in absorbance (405-490 nm) over 15 minutes. Residual activities are presented as a percentage of the activity measured in the absence of inhibitors, which was taken as 100%.

Model building

Comparison of the RVV-V- and LVV-V-catalyzed FV activation patterns with those of thrombin shows that both snake venom proteases exhibit remarkable high substrate selectivity. This is also reflected by the fact that unlike thrombin, FXa or thrombocytin (a FV activator from *Bothrops atrox* venom [11]), RVV-V has no apparent proteolytic effect on factor VIII [39], factor XIII [11], fibrinogen [40] or prothrombin [40]. Also for LVV-V, no protein substrate other than FV has yet been identified. To gain a better insight into the underlying mechanism that renders these enzymes their exquisite substrate specificity and their resistance towards natural inhibitors like BPTI, a three-dimensional structure is absolutely necessary. However, little or no structural data on snake venom FV activators are available. Therefore, we have determined the three-dimensional structure of RVV-V and LVV-V by homology modeling.

Sequence alignment

A BLAST search of the RVV-V and LVV-V sequences against the Protein Data Bank (PDB) revealed that both proteins show the highest sequence identity (59 and 58%, respectively) with AAV-SP-II, a snake venom protease from *Agkistrodon acutus*.

RVV-V	16	VVGGDECNINEHPFLVALY	TSSSTTH	CGGALINREWVLTAAHCDRRNIRIKLGMHSKNI
LVV-V	16	VVGGDECNINEHPFLVALY	TSSSSTVHC	CAGTLINQEWVLTAVHCDRKNIRIKLGMHSKNI
AAV-SP-II	16	VIGGNECDINEHRFLVAFFNTG	--	FFCGGTLINEWVTAAHCDSTNFQMLGVHSKKV
AAV-SP-II				
RVV-V	76	RNEDEQIRVPRGKYFCLNTKFPNGLDKDIMLIRLRFPV	TYSTHIAPVSLPSRSRGVGSRC	
LVV-V	76	RNEDEQIRVPRRKFFCLNTKFPNGDKDIMLIRLRFPV	KNSAHIAPISLPSSPSSPSSRC	
AAV-SP-II	76	LNEDEQTRNPKEKFCPNKNNNEVL	DKDIMLIKLDKPISNSKHIAPLSLPSSPSSVGSVC	
AAV-SP-II				
RVV-V	137	RIMGWGKISTTEDTYPDVPHCTNIFIVKHKWCEPLYPWVP	ADSRTLCA	GILKGGRTDCHG
LVV-V	137	RIMGWGKISTTEETYPDVPHCAKIFIVKHAWCEALYPWVP	ADSRTLCA	GILQGGKDTCEG
AAV-SP-II	137	RIMGWGSITPVKETFPDVPYCANINLLDHAVCQAGYPELL	AEYRTLCA	GIVQGGKDTCEG
AAV-SP-II				
RVV-V	194	DSGGPLICNGQIQGIVAGGSEPCGQHLKPAVYTKVFDYNN	WIQNI	IAGNRTVTCPP
LVV-V	194	DSGGPLICNGQIQGIVSGGSDPCGQRLKPAVYTKVFDYTD	WISII	IAGNTTATCP-
AAV-SP-II	194	DSGGPLICNGQFQGI	VSYG	AHPCGQGPKEGIYTNVFDYTDWIQNIAGNTDATCPP
AAV-SP-II				

Figure 3.3. Multiple sequence alignment of the snake venom FV activators from *Daboia russelli* (RVV-V) and *Daboia lebetina* (LVV-V) and the plasminogen activator from *Agkistrodon acutus*, which was used as the structural template for modeling. Identical residues are shaded in grey. The secondary structure is indicated below the sequence: α -helices as black cylinders, 3^{10} -helices as grey cylinders and β -strands as arrows. The variable surface loop that contains two insertions compared to the template structure is boxed. Numbering is according to chymotrypsinogen [64]. The alignment was created using the ICM program (Molsoft LLC).

Therefore, the recently published 2.1 Å crystal structure [41] of this fibrinogen-converting serine protease was used as the structural template for the generation of homology models for RVV-V and LVV-V. Sequence alignment of the RVV-V and LVV-V model sequences with the template (Figure 3.3) shows an insertion of two amino acids in RVV-V and LVV-V at position 38, which is located on a solvent-exposed loop in the template structure.

Loop building

The computer programs ICM and the WHAT IF/Yasara Twinset were used for the construction of three-dimensional models of RVV-V and LV-V, as described under Materials and Methods. The most difficult part of the model building was the construction of the above-mentioned insertion loop, because no structural information of this region is available in the template structure. A geometric search through the PDB high-resolution structures provided no loop that fitted properly into the model protein framework, probably because of the length of this loop (eight residues). Therefore, we have generated the loop using the colony energy approach [30]. Of all conformations that were generated in this way, the 10 most frequently generated loops were further inspected manually. Loops with improper phi-psi torsion angles, as judged by Ramachandran plot analysis, were not selected for further analysis. From the remaining subset, the energetically most favorable loop was inserted into the model. The homology models were subsequently subjected to a short energy minimization (as described in the Materials and Methods section) to obtain the final three-dimensional structure of both snake venom proteases.

Validation of the models

No major steric clashes were observed in the models before minimization whereas minor unfavorable side chain contacts were removed by rotating side chain torsion angles, as described in the Materials and Methods section. The RMS-deviations for the backbone and for all heavy atoms between the minimized and non-minimized models were 0.67 and 0.89 for RVV-V, and 0.54 and 0.78 for the LVV-V model. Furthermore, the final models exhibit excellent stereochemical quality, as judged by their Ramachandran plots (Figure 3.4), with 83.2% and 86.5% of the residues falling in the most favored region for the RVV-V and LVV-V model, respectively.

Figure 3.4. Ramachandran plot of the RVV-V and LVV-V homology models. Ramachandran plot of the main-chain phi-psi dihedral angles of the RVV-V (A) and LVV-V model (B). The areas of the plots are defined as most favored (dark grey), additionally allowed (grey), and generously allowed (light grey). Upper case G's represent glycine residues. Plots were generated using the PROCHECK software [88].

Since these two criteria alone provide no full proof that a structural model is valid [42], the quality of the models was further assessed by using the structure verification program Verify3D [36,37]. The Verify3D profile of the template structure and both models are graphically represented by the numerical scores as a function of the residue number (Figure 3.5). For the RVV-V and LVV-V model, 92 and 94 percent of the residues had an averaged 3D-1D score greater than 0.2. This indicates that both models have an overall acceptable conformation [36,37].

Figure 3.5. Verify3D profiles of the RVV-V and LVV-V models and comparison with the template structure. Comparison of the calculated 3D profiles of the RVV-V (dashed line) and LVV-V (solid line) models with the template structure AAV-SP-II (dotted line). The vertical axis depicts the average 3D-1D score and the residue number (chymotrypsinogen numbering) is indicated on the horizontal axis. This plot shows that both models have an overall acceptable conformation (positive scores). A 21-residue sliding window was used to calculate the 3D-structures. Scores for the first nine and the final nine sequence positions have no meaning.

Next, the appropriate location of charged, conserved and hydrophobic residues was checked. Most of the charged residues (Glu, Asp, Arg and Lys) are located at the surface of the protein and are solvent-exposed. Of the 11 and 13 aspartic acid residues present in RVV-V and LVV-V, only two are not fully accessible to the solvent. The catalytic Asp¹⁰² interacts with His⁵⁷, whereas Asp¹⁹⁴ stabilizes the N-terminus by making a salt bridge with the N-terminal residue Val¹⁶. Among the glutamic acid residues (eight in RVV-V, nine in LVV-V), only one is partially buried. This residue, Glu⁸⁰, forms a salt bridge with Lys⁶⁷ in both models, indicating conservation of this interaction between species and thus its possible structural importance. One lysine residue (out of a total of 11 and 14 in RVV-V and LVV-V, respectively) is partially buried. This residue, Lys⁸⁸, interacts with Asp⁵⁹ and is further stabilized by making a hydrogen bond with the backbone carbonyl group of Arg⁶⁰. The latter residue is the only arginine residue (out of a total number of 17 in RVV and 14 in LVV) that is partially buried in both model structures.

The six disulfide bridges characteristic for snake venom serine proteases [43] are also conserved in RVV-V and LVV-V. Disulfide bridges are formed between Cys²²-Cys¹⁵⁷, Cys⁴²-Cys⁵⁸, Cys⁹¹-Cys^{245E}, Cys¹³⁶-Cys²⁰¹, Cys¹⁶⁸-Cys¹⁸² and Cys¹⁹¹-Cys²²⁰.

Finally, both RVV-V and LVV-V have been reported to be glycoproteins [11,17]. Potential N-linked glycosylation sites are located between amino acid residues 245 and 245B in RVV-V and LVV-V and are different from the putative glycosylation site in the template structure (Asn³⁵). In both models, this putative glycosylation site is fully solvent-exposed. This observation, together with the above results, provides strong evidence that both models are valid and suitable for further structural analysis.

Overall structure

Both FV-activating snake venom proteases display the β/β hydrolase fold typical of serine proteases from the chymotrypsin family [44,45]. The three active site residues His⁵⁷, Asp¹⁰² and Ser¹⁹⁵ are found at the interface of the two six-stranded β -barrels. The surface is made up of various turns and loops, and two short α -helices (Figure 3.6A).

The six disulfide bonds, which are strongly conserved among snake venom serine proteases, are arranged in a way topologically equivalent to that of trypsin. The Cys¹²⁸-Cys²³² bond of trypsin is replaced by a hydrophobic stacking between Pro¹²⁸ and Phe²³² in LVV-V, which helps to stabilize the long Pro¹²⁸-Ser¹³⁴ unstructured segment. In RVV-V however, no interaction is noted between Ser¹²⁸ and Phe²³².

The disulfide bond formed between Cys⁹¹ and Cys^{245E} substitutes the Cys¹²⁸-Cys²³² bond of trypsin and connects the C-terminal region with the main body of the protein. Compared to trypsin, the C-terminal part of RVV-V and LVV-V is extended

with seven and six residues, respectively. This extended region is further stabilized by a salt bridge between the amino group of Lys¹⁰¹ and the C-terminal proline residue. This extension is present in all snake venom serine proteases and its amino acid sequence is highly conserved. The biological function of this extended region is still unknown, but has been correlated to serine protease function since it makes up most of the surface of the S1-S3 specificity sites that interact with the substrate [44]. Interestingly, the putative N-linked glycosylation site of RVV-V and LVV-V (Asn²⁴⁵) is located in this extended region. The presence of a bulky glycan structure at the C-terminal extended region could interfere with substrate binding, thus contributing to the narrow substrate specificity of both enzymes.

Figure 3.6. Overall structures of RVV-V and LVV-V and comparison with thrombin. (A) Structural superposition of the backbone of the FV activators from *Daboia russelli* (red), *Daboia lebetina* (dark blue) and thrombin (cyan). Representation is according to thrombin's standard orientation, i.e., with the active site cleft facing the viewer and the 60-loop to the north. The side chains of the catalytic site residues His⁵⁷, Asp¹⁰², and Ser¹⁹⁵ are shown in yellow. The surface loops discussed in the text are indicated. The N- and C-terminal ends are also labeled. (B) Access to the active site of both snake venom FV activators is less restricted due to a large reduction in the 60-loop (colored according to the color scheme that was used in Figure 3.6A). Figure B was obtained by rotating figure 3.6A 90° around the X-axis. All figures were prepared using the YASARA/WHATIF program.

Active site

An internal salt bridge between the α -amino group of the valine at the N-terminus and the γ -carboxyl group of Asp¹⁹⁴ is involved in the formation of the active site of both venom FV activators. Cleavage of the Arg¹⁵-Ile¹⁶ bond of serine protease zymogens typically leads to the formation of this particular salt bridge and to

spontaneous ordering of the active site [46]. Together with Gly¹⁹³ and Ser¹⁹⁵, Asp¹⁹⁴ creates the oxyanion hole which stabilizes the negative charge on the carbonyl oxygen of the P1 residue of the substrate in the transition state. Further, the spatial organization of the catalytic triad residues His⁵⁷, Asp¹⁰², and Ser¹⁹⁵ at the active site cleft between the two subdomains of the β -barrel structure is preserved. A 3_{10} helix is present between residues 56 and 59 (see also Figure 3.3). This short helical segment contains the catalytic His⁵⁷ and provides a helical dipole moment that helps to stabilize the negative charge of Asp¹⁰² [47]. Remarkably, the S1 specificity pocket of both snake venom proteases is larger than that of trypsin, thrombin, and FXa. This is due to the insertion of a proline residue at position 219, which is conserved among all snake venom proteases and members of the kallikrein family [43]. In both models, Pro²¹⁹ adopts a *cis*-conformation, similar to that seen in the template structure AAV-SP-II and TSV-PA. The unusual *cis*-conformation makes the carbonyl group of Pro²¹⁹ pointing away from the specificity pocket, in contrast with other trypsin-like serine proteases in which this carbonyl group provides an anchoring point for guanidium groups entering the specificity pocket.

Non-specific substrate binding site

A remarkable feature of the RVV-V and LVV-V models is the non-conserved region between residues 214 and 216, which is often referred to as the non-specific substrate binding site [48]. The backbone of this segment forms the opening to the primary specificity pocket and is also involved in secondary substrate binding. The Ser²¹⁴-Trp²¹⁵-Gly²¹⁶ segment is strictly conserved among serine proteases [49] and plays a major role in substrate and inhibitor recognition. Upon binding, the backbone of this region forms an antiparallel β -sheet with the backbone of the substrate and hydrogen bonds are formed with the backbone of P2 and P3 residues [50]. However, this conserved tripeptide sequence is replaced by Ala²¹⁴-Gly²¹⁵-Gly²¹⁶ in RVV-V and Ser²¹⁴-Gly²¹⁵-Gly²¹⁶ in LVV-V.

In trypsin and chymotrypsin, Trp²¹⁵ interacts intramolecularly with a highly conserved aromatic residue (Trp or Tyr) at position 172, which has been shown to be a determinant of substrate specificity [48,51]. In both FV activators, Trp²¹⁵ is replaced by a glycine residue. One of the other determinants of the non-specific substrate binding site, Ser²¹⁴, is conserved in LVV-V, but replaced by alanine in RVV-V (see also Figure 3.3). In all serine proteases, the hydroxyl group of Ser²¹⁴ serves as a hydrogen donor to one of the oxygen atoms of the γ -carboxylate group of Asp¹⁰². It has been suggested that the hydroxyl group of Ser²¹⁴ may stabilize the negative charge on the γ -carboxylate group of Asp¹⁰², thereby facilitating proton transfer to His⁵⁷ [52]. The lack of interaction between Ala²¹⁴ and Asp¹⁰² in RVV-V may allow the side chain of this catalytic site residue to move away

from the active site, as observed in the crystal structure of human factor D [53]. Furthermore, Ser²¹⁴ has been ascribed a crucial role in substrate recognition, ensuring optimal diffusion into the active site and proper induced fit rearrangement of the enzyme-substrate complex [44]. The observation that, compared to LVV-V, RVV-V has an almost 10-fold lower amidase activity toward the chromogenic substrate S2238 can therefore likely be explained by the presence of the non-conserved alanine residue at position 214.

Among typical trypsin-like serine proteases, only a few exceptions lacking the conserved 214-216 tripeptide sequence have been reported: mouse killer T cell proteinase I (Ser-Tyr-Gly), *Streptomyces griseus* proteinases A and B (Ser-Gly-Gly), complement factor D (Thr-Ser-Gly) and rat submaxillary tonin (Ser-Gly-Gly). The crystal structures of the latter two proteins reveal a shift of the segment between residues 215-219, compared to all other serine proteases of known structure [53,54]. This shift results in the closure of the normal binding pocket and creates another opening between this strand and the strand containing residues 225-228. It has been suggested that the atypical conformation of the backbone of loop 214-219 probably is a major determinant of the low reactivity of tonin and factor D with synthetic esters [55,56]. We hypothesize that the region between residues 214 and 219 may potentially adopt a similar conformation in the snake venom FV activators, which can explain the observed low amidase activity of these enzymes toward chromogenic substrates and their very narrow substrate specificity. However, because it is still unclear what exactly causes this shift in tonin and factor D [57,58] and because there are no structural or biochemical data that support a similar movement of this region in RVV-V or LVV, the standard conformation of the Gly²¹⁵-Pro²¹⁹ segment is proposed in our models.

Substrate specificity

The primary substrate binding site, which is built of three β -strands (residues 189-192, 214-216 and 226-228) and the oxyanion binding site (Gly¹⁹³ and Ser¹⁹⁵) both belong to the C-terminal β -barrel. In analogy with trypsin and thrombin, an aspartic acid residue present at the bottom of the specificity pocket (Asp¹⁸⁹) is positioned to make a salt bridge with basic P1 residues of substrates and inhibitors [59], which explains the preference of both FV activators for an arginine residue at the P1 position. The replacement of the highly conserved Trp²¹⁵ by a glycine residue in both FV activators results in a larger and less hydrophobic P2 specificity pocket. Moreover, Gly²²⁶, which is located at the back of the S2 pocket and which is also highly conserved among serine proteases, is replaced by an alanine in both models. Together with Gly²¹⁶, this small residue at position 226 permits access of large amino acids to the base of the pocket. Secondary specificity is further

determined by the residue at position 99, being a leucine in RVV-V and a lysine in LVV-V. As a consequence, it is very likely that RVV-V will prefer large hydrophobic residues at the P2 position, whereas LVV-V probably will have a preference for negatively charged residues at this position.

Of the three residues that normally form the highly conserved non-specific substrate binding site in serine proteases, Gly²¹⁶ is the only one that is conserved in both snake venom proteases. This residue is also part of the S3 subsite, and its backbone is involved in two hydrogen bonds with the P3 residue of the substrate. Selectivity for P3 residues is poor in nearly all chymotrypsin-like serine proteases as the enzyme-substrate interaction is limited due to solvent exposure of the P3 side chain. Besides Gly²¹⁶, the S3 specificity pocket consists of His¹⁹² in RVV-V and Glu¹⁹² in LVV-V. In blood coagulation proteases, residue 192 is known to exhibit a high degree of flexibility that permits interactions with both the P3 and P3' residues. The high side chain mobility of this amino acid weakens its ability to act as a specificity determinant [60].

The S4 binding pocket is created by the side chain of residue 215 and the 172-loop. In both models, this loop adopts a conformation close to the core structure due to the burial of Tyr¹⁷² and Trp¹⁷³. Compared to other chymotrypsin-like serine proteases, the S4 specificity pocket of both snake venom proteases is less hydrophobic due to the replacement of Trp²¹⁵ by a glycine. However, few chymotrypsin-like peptidases possess selectivity for P4 residues.

Substrate interactions for residues C-terminal to the scissile bond (prime side) is limited in S1 peptidases. Selectivity of the S1' subsite is dependent on the composition of the 60-loop. Typically, a small residue at P1' is preferred based on steric hindrance, rather than a larger residue that introduces a specific interaction between enzyme and substrate. In both models, the side chain of Arg⁶⁰ points towards the active site cleft. Arg⁶⁰ is probably the major determinant in RVV-V's and LVV-V's preference for small hydrophilic residues at the P1' position. Indeed, both FV activators cleave their substrate in a sequence containing a serine residue at the P1' position.

Loops surrounding the active site

Snake venom proteases combine a high degree of sequence identity with a remarkably broad substrate diversity (cf. AAV-SP-II and RVV-V/LVV-V) and high sensitivity towards inhibitors. The difference in substrate specificity is only partly due to the nature of the active site environment. Unique surface loops surrounding the active site cleft play a more important role in substrate recognition and specificity [61]. In the following discussion, the loops surrounding the active site will be described and discussed clockwise, where quarters are denoted according to

thrombin's standard orientation [62] as shown in Figure 3.6A. The northern rim of the active site is formed by the *99-loop*. This β -hairpin loop is held in place by a disulfide bridge between Cys⁹¹ and Cys^{245E} from the C-terminal extension. In LVV-V, the loop is further stabilized by a hydrogen bond between the amide group of Lys⁹⁹ and the carbonyl backbone atom of Trp¹⁷³, which is part of the *174-loop*.

To the northeast of the active site, the *60-loop* presents a 10-residue deletion compared to thrombin. In thrombin, this loop is found to partially occlude the active site cleft and restricts access to substrates and inhibitors. In addition to this role in excluding interactions with certain ligands, residues of the *60-loop* have been described to be involved in the formation of the unique S2 subsite and aryl binding site of thrombin [63,64]. Inhibitor studies with RVV-V and LVV-V (which are further discussed below) show that both enzymes behave differently from thrombin. Some of these results can be explained by the dramatic decrease in length of the *60-loop* in RVV-V and LVV-V.

Access to the active site is further restricted by the *37-loop*. Compared to other snake venom serine proteases, a two-residue insertion is present at position 38 in RVV-V and LVV-V. Remarkably, the segment between residues 35 and 39 only consists of serine and threonine residues, creating a very polar environment with high hydrogen bonding potential at the south-eastern boundary of the active site. Based on inhibitor docking studies, we propose a major role for the *37-loop* in the insensitivity of both FV activators towards BPTI (see below).

The *70-loop*, which is adjacent to the autolysis loop and behind the *37-loop*, is topologically similar to the calcium binding loop of trypsin-related serine proteases [65]. In FXa, which is one of the physiological activators of FV, the calcium ion is coordinated by the side chains of Asp⁷¹ and Glu⁸¹ and the carbonyl groups of Asn⁷³ and Gln⁷⁶ [66]. In RVV-V and LVV-V, these putative calcium-coordinating residues are either not present or adopt an unfavorable conformation for calcium binding. We observed that for the expression of amidolytic activity, RVV-V and LVV-V do not require a functional calcium binding site, since both FV activators retained amidase activity after treatment with the metal chelator EDTA (Table 3.1). Furthermore, it has been demonstrated that both snake venom proteases activate FV in a calcium-independent manner [17,67].

The southern edge of the active site is formed by the *148-loop*, which has the same length as in other snake venom proteases and trypsin, but shows a five-residue deletion compared to thrombin. In thrombin, this insertion loop may undergo different (auto)proteolytic attacks, so that it is also defined as the autolysis loop.

The *186-loop* of thrombin participates together with the *220-loop* in the formation of a Na⁺ binding site [68]. Both loops line a channel filled by water molecules, which connects the active site with an aperture on the surface of thrombin. Binding of Na⁺ plays a crucial role in the allosteric control of thrombin's protease activity,

as it causes a conformational transition from a slow (Na^+ -free form) to a fast (Na^+ -bound) form [68]. Two of the six ligating groups in the octahedral coordination shell of the bound Na^+ are provided by the carbonyl oxygen atoms of Lys²²⁴ and Arg^{221A} [68,69]. In our models, the orientation of the carbonyl oxygen atom of residue 224 is shifted due to the presence of a proline residue at position 225. This shift constrains the carbonyl oxygen atom of residue 224 to re-orient in a manner incompatible with Na^+ coordination similar to that seen in trypsin and other serine proteases that are not allosterically regulated by Na^+ [70]. Therefore, we conclude from both our models that RVV-V and LVV-V, in contrast to thrombin, are not allosterically regulated by Na^+ .

To the west of the active site and adjacent to the 99-loop, the 174-loop is present. In the template structure, a hydrogen bond is formed between the side chains of Tyr²¹⁵ and Glu¹⁷³. This bond pulls the side chain of Tyr²¹⁵ into the substrate binding site S3 [41]. In thrombin, the S3 subsite is narrowed by hydrogen bonding between the side chains of Trp²¹⁵ and Met¹⁸⁰. In RVV-V and LVV-V however, a glycine is present at position 215, making the S3 site less shallow. From this, it can be expected that both FV activators will have a preference for bulky residues at the P3 position. In this context, it should be noted that of the three activation cleavage sites that are present in the FV molecule, only the cleavage site around Arg¹⁵⁴⁵ presents a bulky residue (Tyr) at the P3 position. In the Arg⁷⁰⁹ and Arg¹⁰¹⁸ cleavage sites, a Gly and Ser residue are present at this position. This notion may help to clarify why both snake venom proteases preferentially cleave their substrate FV after Arg¹⁵⁴⁵.

Interactions with macromolecular serine protease inhibitors

Antithrombin

As discussed before, RVV-V and LVV-V do not present the 148-restriction-loop and the typical Tyr-Pro-Pro-Trp motif in the 60-loop (Figures 3.6 and 3.7). This could explain the observed insensitivity of RVV-V and LVV-V towards antithrombin (Table 3.1). Thrombin mutants lacking the Pro^{60B}-Pro^{60C}-Trp^{60D} motif indeed are resistant to inhibition by antithrombin. A critical role has been addressed to Trp^{60D}, which substantially contributes to the transition-state stabilization of the thrombin/antithrombin complex [71]. Furthermore, it has been demonstrated that a thrombin 148-loop deletion mutant showed a drastic decrease in association rate with antithrombin [72].

				37-loop		60-loop	
RVV-V	16	VVGGDECHINEHPFLVAL	TSTSSSTH	CGGALINPEWVLTAAHQ	-----	DRFNIR	
LVV-V	18	VVGGDECDINEHPFLVAL	TSSSSTVH	CAGTLINQEWVLTAVHQ	-----	DRKHIR	
Thrombin	16	IVEGSDAEIGMSPWQVMLE	PKSPQELI	CGASLISDRWVLTAAHQ	LLYPWDKNFTENDLL		
Factor Xa	16	IVGGQECKDGECPWQALLI	NEENEG-	ECGGTILSEFYILTAAHQ	L-----	YQAKRFK	
			70-loop		99-loop		
RVV-V	86	IKLGMHSHKNIPNED-EQ	IPVPPGK	FCLNTHKFPNGLD	DIHLIRLRPPVTYSTHIAPVSL		
LVV-V	66	IKLGMHSHKNIPNED-EQ	IPVPPRK	FCLNTHKFPNGKD	DIHLIRLRPPVKINSAHIAPISL		
Thrombin	66	VRICKHSPTFYERNIEK	ISMLEKI	THPPYNWRENLD	DIALMKLKKPVAFSDYIHFVCL		
Factor Xa	66	VRVGDPTNQEEGG-EAV	HEVEVVI	KHNFT-KETYD	DIAVLRKTPITFRMINVAPACL		
				148-loop		174-loop	
RVV-V	124	PSRSRGV-----	GSRCRIMG	WGKISTT-----	EDTYPDM	PHCTHIFIVKHKWCE	LYPWV
LVV-V	124	PSSPSSF-----	PSPCRIMG	WGKISTT-----	EETYPDM	PHCAKIFIVKHAWCE	LYPWV
Thrombin	124	PDRETAASLLQAGYKGRVTG	WGNLKETWTANV	GKGQPSV	LQVVHLPIVERPVCKI	-STRI	
Factor Xa	124	PERDWAESTLMTQKTGIVSG	EGRTHEK-----	GF-QSTF	LKMLEVFPYVDHNSCKL	-SSSF	
			186-loop			220-loop	
RVV-V	175	PADSRTICAGIL--	KGGR-	DTCHGDSGGPLICNGQIQ	-----	GIVAGG	SEPCGQHLKPA
LVV-V	175	PADSRTICAGIL--	QGGR-	DTCEGDSGGPLICNGQIQ	-----	GIVSGG	SDPCGQRLKPA
Thrombin	175	PITDNMF	CAGYKPDEGKFG	DACEGDSGGPFVMKSP	FHNNEWYQMGIVSWG	-EGCDPDGKYG	
Factor Xa	175	IITQNMFCAGI---	DTKQED	ACQGDGSGGPHVTR--	FKDTYFVTGIVSWG	-EGCARKGKYG	
RVV-V	227	VYTRKVF	DYNHWIQNIIA--	GNETVTCPP			
LVV-V	227	VYTRKVF	YTDWIQSIIA--	GNTTATCP-			
Thrombin	227	FYTHV	FRLLKWKIQKVIDQ	FGE-----			
Factor Xa	227	IYTKV	TAFLLKWKIDPSMKTR	-----			

Figure 3.7. Multiple sequence alignment of RVV-V, LVV-V, thrombin and factor Xa. Residues are numbered using the chymotrypsinogen numbering scheme [64]. Loops surrounding the active site are indicated and boxed. The alignment was created using the ICM program (Molsoft LLC).

Bovine pancreatic trypsin inhibitor (BPTI)

Although both snake venom proteases share a common overall fold with trypsin, they can be readily distinguished from trypsin by their high substrate specificity and their insensitivity to BPTI (Table 3.1). Resistance to BPTI is a common feature of snake venom proteases, but is also observed for thrombin. Thrombin's resistance to inhibition by BPTI has been ascribed to the 60-loop, which is one of the surface loops surrounding the active site cleft [64,71]. Still, other blood coagulation proteases like Activated Protein C (APC) also lack the 60-loop, but remain sensitive to inhibition by BPTI. Therefore it has been proposed that determinants other than the 60-loop are involved in the resistance mechanism. Site-directed mutagenesis studies in both APC and thrombin have shown that residue 192, which is located at the entrance of the active site, contributes substantially to BPTI-resistance [73,74]. It appeared that replacement of Glu¹⁹² by Gln is sufficient to allow effective inhibition by BPTI. Interestingly, RVV-V and LVV-V lack the 60-loop and present different residues at position 192 (a histidine in RVV-V and a glutamic acid in LVV-V). On the assumption that the underlying mechanism of BPTI resistance is the same for snake venom and blood coagulation serine proteases, one could expect only LVV-V to be insensitive to this inhibitor. However, the amidase activity of both enzymes was not affected by BPTI (Table 3.1). Our findings are thus in agreement with mutagenesis studies on TSV-PA, a plasminogen activator of *Tremesurus*

stejnegeri, which show that residue 192 does not contribute to its resistance to BPTI [75].

In an attempt to explain why both FV activators are not inhibited by BPTI, the inhibitor was docked into the RVV-V and LVV-V active site with identical position and orientation as observed for the trypsin complex [59]. As can be seen in figure 3.8, this resulted in a severe collision of the P2'-Arg and P3'-Ile of the inhibitor with the backbone and side chains of residues 39 and 40 of the snake venom FV activators. For all 10 top-scoring loop conformations that were generated during the modeling procedure, severe clashes with BPTI were observed. For this reason it is unlikely that, from the generated set of loop conformations, a loop was incorporated into the final model which adapted by chance a conformation that interferes with the inhibitor.

Figure 3.8. Interaction of RVV-V and LVV-V with bovine pancreatic trypsin inhibitor (BPTI). Structural superposition of the trypsin-BPTI complex [59] with the RVV-V and LVV-V models results in a severe collision of the P2' and P3' residues of the inhibitor with the backbone and side chains of residues 39 and 40, which are part of the 38-loop of both snake venom FV activators. The three residues on the N-terminal (P1, P2, and P3) and C-terminal side of the scissile bond (P1', P2', and P3') of BPTI are shown in orange. The van der Waals surface of these residues is also represented, and clearly shows a steric clash with the above mentioned residues in the RVV-V (shown in red) and LVV-V (shown in blue) models. Catalytic site residues are shown in yellow. The loops surrounding the active site are also indicated. The figure was created using the YASARA/WHATIF program.

In TSV-PA, the side chain of Phe¹⁹³ was documented to play a crucial role for its resistance towards BPTI [9]. This phenylalanine restricts the S2' pocket and was found to collide with the P2'-arginine of the inhibitor. Replacement of a highly conserved phenylalanine for a glycine at position 193 totally abolished resistance to BPTI. Interestingly, recent studies have attributed a key role of the 37-loop of TSV-PA in enzyme/inhibitor interactions [9,76].

Another indication for the importance of 37-loop interactions with macromolecular inhibitors is the fact that glycosylation of the 37-loop in two plasminogen activators from *Agkistrodon acutus*, AAV-SP-I and AAV-SP-II, has been reported to affect their resistance towards a number of inhibitors. The presence of a bulky glycan structure likely causes steric hindrance upon binding of both proteins to macromolecular inhibitors [41]. Together with these observations, our data suggest that the 37-loop indeed can play a key role in the interaction of serine proteases with their natural inhibitors. Furthermore, the absence of a bulky residue at position 193 in snake venom proteases does not necessarily imply sensitivity to BPTI, as both snake venom FV activators present a small glycine residue at this position but proved to be resistant towards this inhibitor.

D-Phe-Pro-Arg-chloromethyl ketone (FPRCK)

In contrast to antithrombin and BPTI, the chloromethyl ketone inhibitor D-Phe-Pro-Arg-chloromethyl ketone (FPRCK) strongly inhibited the amidase activity of both snake venom proteases (Table 3.1). Docking of this inhibitor into the active site of both enzymes reveals that the FPRCK peptidyl chain juxtaposes segment 214-216 under formation of an anti-parallel β -sheet (data not shown). The N-terminus of the inhibitor is hydrogen-bonded to the carbonyl oxygen of Gly²¹⁶ but does not form a salt bridge with the negatively charged amino acid at position 218. The Arg³ side chain of the inhibitor is accommodated in the S1-pocket in a similar geometry as observed in the FPRCK-thrombin complex [63]. The guanidyl group of the inhibitor is positioned to make favorable hydrogen bonds with Thr¹⁹⁰ or Ser²¹⁷ and Pro²²⁵. An inserted lysine side chain would fit less tightly into the specificity pocket due to the greater space and lack of a favorable hydrogen bond with residue 190 or 217, explaining the preference of both snake venom FV activators for substrates and inhibitors with an arginine at the P1 position. In the FPRCK-thrombin complex, the pyrrolidine ring of the P2-residue (Pro²) of the inhibitor is almost fully shielded from bulk water because it is encapsulated in the hydrophobic environment that is formed by the side chains of Ile¹⁷⁴, Leu⁹⁹, His⁵⁷, Tyr^{60A}, Trp^{60D} and Trp²¹⁵. The latter residue, which is highly conserved among serine proteases, is replaced by a glycine in the snake venom FV activators. In RVV-V, a hydrophobic environment is created by His⁵⁷, Leu⁹⁹, Trp¹⁷³, His¹⁹², and Val²²⁷. In LVV-V, His¹⁹² is replaced by a glutamic

acid. In both snake venom proteases, the 60-loop has undergone a large reduction and as a consequence, no counterparts for thrombin residues Tyr^{60A} and Trp^{60D} are present. However, the pyrrolidine ring of Pro at position 2 in FPRCK is still partly shielded from bulk water by the benzyl side chain of D-Phe at position 1, which is possible due to its unusual D-conformation. Furthermore, the ammonium and carbonyl group of D-Phe¹ are in favorable hydrogen-bond contacts with Gly²¹⁶. Thus, the observation that FPRCK is a good inhibitor of both snake venom proteases can be attributed to an optimal fit of all three inhibitor residues to their respective subsites.

Substrate recognition

Remarkably, RVV-V and LVV-V show an overall reduction in the length of the loops surrounding the active site, when compared to thrombin and FXa (Figure 3.7). As a result, the shape of the active site cleft of both snake venom proteases is very different from the latter two blood coagulation proteases. Whereas FXa and especially thrombin possess a deep and narrow active site pocket, both models present an elongated active site groove that spans the surface of the molecule. This is reflected in the relative size of the RVV-V and LVV-V active site (1797 Å³ and 1841 Å³, respectively), which is almost twice as big as those of thrombin (809 Å³) and FXa (1254 Å³). Still, RVV-V and LVV-V don't have a broader substrate specificity, despite the relatively better accessibility of the active site. Interestingly, a thrombin deletion mutant lacking the 60-loop still retained its specificity [71]. From this, it can be assumed that the active site environment of these FV activators only plays a minor role in substrate recognition and specificity. In analogy with thrombin, exosite interactions may play a crucial role in determining the exquisite substrate specificity of RVV-V and LVV-V.

To investigate this possibility, the electrostatic surface potentials of RVV-V and LVV-V were calculated and compared with those of thrombin. In both snake venom proteases, two positively charged surface patches are present (Figure 3.9).

Region I is located to the east of the active site and is composed of a stretch of amino acids in the first six-stranded β-barrel. This cluster of residues is located 20 Å away from the active site and carries 14 basic amino acid side chains (Table 3.2). In both FV activators, only three of these residues are electrostatically compensated by salt bridge formation with neighboring residues (Lys⁶⁷ with Glu⁸⁰, Lys⁸⁸ with Asp⁵⁹ and Arg¹⁰⁷ with Glu⁵⁰), leading to a strong positive electrostatic field outside the protein surface. Because this positively charged surface patch is topologically similar to thrombin exosite I, which has been ascribed a major role in FV activation [77-82], it is very likely that this region is involved in FV recognition by the two snake venom proteases. In addition, region I is populated with several hydrophobic

residues (Table 3.2). Some of them are relatively well solvent-exposed (Tyr³⁴, Ile⁸² and Val⁸⁴), whereas others form a hydrophobic cluster underlying the ionic residues. For thrombin, it was hypothesized that the electrostatic field of exosite I could pre-orient the enzyme for a productive interaction so that the hydrophobic stacking components can subsequently stabilize the ligand [83,84]. The presence of various solvent-exposed hydrophobic residues within the electropositive patches of the two snake venom proteases (Table 3.2) further suggests a similar steering mechanism in these enzymes.

Figure 3.9. Electrostatic surface potentials of thrombin, RVV-V and LVV-V. Molecular surfaces and electrostatic potentials were calculated using the REBEL (Rapid Exact Boundary Element) method of ICM. Proteins are represented according to thrombin's standard orientation (middle panel) and rotated 90° clockwise (left panel) or counter-clockwise (right panel) to center on the two positively charged regions that are present on the surface of these enzymes. Positively ($e > 5$ kcal/electron units) and negatively ($e < -5$ kcal/electron units) charged regions are shown in blue and red, respectively.

The second region is formed by a cluster of positively charged residues (nine in RVV-V, eight in LVV-V) located nearly 25 Å to the west of the catalytic triad (Figure 3.9, Table 3.2). Only two of these residues are involved in a salt bridge (Arg¹³⁷ with Glu²⁶ and Lys²³⁰ with Asp²³³). This results in the generation of a strong electrostatic field, although the highest surface positive field is generated by region I. Like in region I, this positively charged surface patch is populated with a number of hydrophobic residues that are relatively well solvent-exposed.

Interestingly, the second positively charged region is not topologically equivalent to thrombin exosite II, but is located more to the back of the protein (when viewing the models according to thrombin's standard orientation). The role of exosite II in FV activation by thrombin is still a matter of debate. Evidence for the involvement of thrombin exosite II (heparin binding site) in FV activation was provided by several site-directed mutagenesis studies [80,84], but contrasting data have been presented by Arocas and co-workers [82] who showed that exosite II ligands failed to inhibit FV activation even when thrombin exosite I was not present.

If thrombin exosite II is indeed crucial for cleavage at Arg⁷⁰⁹, the absence of a topologically similar positively charged region in RVV-V and LVV-V could explain their inability to cleave this peptide bond in the FV molecule. It should be emphasized however that cleavages at Arg⁷⁰⁹ and Arg¹⁵⁴⁵ appear to be facilitated by exosite I, as FV activation in the presence of the exosite I-specific (blocking) peptide Hir⁵⁴⁻⁶⁶ resulted in the accumulation of activation intermediates resulting from cleavage at Arg¹⁰¹⁸ [85]. The Arg¹⁰¹⁸ cleavage is not observed for both LVV-V and RVV-V even though it has been shown to potentiate cleavage at Arg¹⁵⁴⁵ in the case of thrombin [79]. Therefore we hypothesize that in the absence of an exosite that is functionally identical to thrombin exosite II, Arg⁷⁰⁹ and Arg¹⁰¹⁸ are not appreciably cleaved by either venom protease and that the region analogous to thrombin exosite I facilitates proteolysis at Arg¹⁵⁴⁵. Given the virtual absence of the Arg¹⁰¹⁸ cleavage, proteolysis at 1545 is however not potentiated, resulting in an overall slower cleavage at Arg¹⁵⁴⁵, as compared to thrombin.

Currently, we are further investigating the role of these two positively charged regions in RVV-V and LVV-V using a biochemical approach. Preliminary studies have shown that both snake venom FV activators have a high affinity for heparin. This observation strongly supports our hypothesis that the two positively charged regions in RVV-V and LVV-V are analogous but not identical to thrombin exosites I and II. Furthermore, this also suggests that exosite interactions may play a crucial role in determining the exquisite substrate specificity of both snake venom proteases. The very narrow substrate specificity of RVV-V and LVV-V is probably not mediated by exosite interactions alone, but rather is the result of the synergistic contribution of multiple factors. The nature of the active site residues, the non-conserved non-specific binding site, the loops surrounding the active site and ionic and hydrophobic exosite interactions probably all contribute to the enzymes' narrow substrate specificity. In light of this, it is interesting to note that a stretch of four amino acids (Phe^{95A}, Pro⁹⁶, Asn⁹⁷ and Gly⁹⁸), which seems to be characteristic for RVV-V and LVV-V and was suggested to be important for FV activation [18], is located at the apex of the 99-loop. In TSV-PA, the corresponding sequence (Lys⁹⁵, Asp⁹⁶, Asp⁹⁷, Glu⁹⁸) proved to be responsible for plasminogen activation [86].

RVV-V	LVV-V	Thrombin	RVV-V	LVV-V	Thrombin
Exosite I			Exosite II		
Central region			Central region		
Arg ⁵⁵	Arg ⁵⁵	Arg ⁷³	Lys ¹⁵⁴	Arg ¹³³	Arg ⁹³
Arg ⁸⁵	Arg ⁸⁵	Arg ⁷⁵	Lys ¹⁵⁵	Lys ¹⁵⁴	Arg ¹²⁵
Arg ¹⁰⁹	Arg ¹⁰⁹	Arg ^{77A}			Lys ²³⁵
Arg ¹¹⁰	Arg ¹¹⁰				Lys ²⁴⁰
Peripheral region			Peripheral region		
Arg ⁴⁹	Arg ⁵⁰	Arg ³⁵	Arg ¹²⁷		Arg ⁹⁷
Arg ⁵⁰	Lys ⁵²	Lys ³⁵	Arg ¹²⁹	Arg ¹³⁵	Arg ¹⁰¹
Arg ⁵²	Lys ⁵⁷	Arg ⁵⁷	Arg ¹³⁵	Arg ¹³⁷	Arg ¹⁵⁵
Lys ⁵⁷	Lys ⁷³	Lys ⁷⁰	Arg ¹³⁷	Lys ¹⁵³	Lys ¹⁵⁹
Lys ⁷³	Arg ⁷⁵	Lys ⁸¹	Lys ^{155A}	Lys ¹⁵³	Arg ¹⁷³
Arg ⁷⁵	Arg ⁸³	Lys ¹⁰⁹	Arg ¹⁸⁸	Arg ²²²	Arg ¹⁷⁵
Arg ⁸³	Arg ⁸⁷	Lys ¹¹⁰	Lys ²³⁰	Lys ²³⁰	Arg ²³⁵
Lys ⁸⁸	Lys ⁸⁸	Lys ^{149E}			
Arg ¹⁰⁷	Arg ¹⁰⁷				
Arg ^{245A}	Lys ¹¹³				
Hydrophobic region			Hydrophobic region		
Tyr ³⁴	Tyr ³⁴	Phe ³⁴	Val ¹⁵²		none
Trp ⁵¹	Trp ⁵¹	Leu ⁵⁵	Ile ¹⁵⁰	Ile ¹⁵⁰	
Ile ⁸²	Ile ⁸²	Thr ⁷⁴	Phe ¹⁵¹	Phe ¹⁵¹	
Val ⁸⁴	Val ⁸⁴	Tyr ⁷⁵	Ile ¹⁵²	Ile ¹⁵²	
		Ile ⁸²	Val ¹⁵³	Val ¹⁵³	
			Ile ¹⁵⁵	Ile ¹⁵⁵	
			Leu ¹⁵⁵	Leu ¹⁵⁵	
Hydrophobic cluster			Hydrophobic cluster		
Ile ⁵⁴	Ile ⁵⁴	Met ⁵²	none		
Ile ⁵⁵	Ile ⁵⁵	Leu ⁴⁰			
Leu ⁵⁸	Leu ⁵⁸	Trp ¹⁴¹			
Met ⁷⁰	Met ⁷⁰	Leu ¹⁵⁵			
Ile ¹⁰⁵	Ile ¹⁰⁵				
Leu ¹⁰⁸	Leu ¹⁰⁸				
Val ¹¹²	Val ¹¹²				

Table 3.2. Overview of the charged and hydrophobic residues in the exosites of RVV-V, LVV-V and thrombin.

Finally, the region between amino acids 62-65 and 73-76 in both FV activators deserves special attention. In LVV-V, both regions are composed of the same stretch of four amino acids (Arg-Asn-Ile-Lys), while in RVV-V Arg⁷³ is conservatively substituted for a lysine (Figure 3.7). Moreover, these two K/R-N-I-R regions appear to be characteristic for RVV-V and LVV-V [18,87] and could therefore play a role in FV recognition. This hypothesis is supported by the observation that the positively charged arginine and lysine residues present in these regions all contribute to the positive electrostatic potential of Region I (Figure 3.9 and Table 3.2) and that both regions are located at the apex of the reduced 60-loop and 70-loop.

3.4 Conclusions

The homology models of RVV-V and LVV-V presented here are the first three-dimensional structures of snake venom FV activators and provide insight into the mechanism by which these enzymes achieve a very high substrate specificity and resistance to inhibition by the serine protease inhibitors antithrombin and BPTI. Both models provide a basis for the rational design of experiments to study the structure-function relationship of these enzymes and the role of the two positively charged surface patches in FV recognition and activation. The study of FV activation by these snake venom activators provides further insight into the structural requirements for the activation of FV by its physiological activators.

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Chapter IV:

The role of thrombin exosites I and II in the activation of human coagulation factor V

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4.1 Summary

Human blood coagulation factor V (FV) is a plasma protein with little procoagulant activity. Limited proteolysis at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ by thrombin or factor Xa (FXa) results in the generation of activated FV, which serves as a cofactor of FXa in prothrombin activation. Both thrombin exosites I and II have been reported to be involved in FV activation, but the relative importance of these regions in the individual cleavages remains unclear. To investigate the role of each exosite in FV activation, we have used recombinant FV molecules with only one of the three activation cleavage sites available, in combination with exosite I- or exosite II-specific aptamers. In addition, structural requirements for exosite interactions located in the B domain of FV were probed using FV B domain deletion mutants and comparison with FV-activating enzymes from the venom of Russell's viper (RVV-V) and of Levant's viper (LVV-V) known to activate FV by specific cleavage at Arg¹⁵⁴⁵. Our results indicate that thrombin exosite II is not involved in cleavage at Arg⁷⁰⁹ and that both thrombin exosites are important for recognition and cleavage at Arg¹⁵⁴⁵. Efficient thrombin-catalyzed FV activation requires both the N- and C-terminal regions of the B domain, whereas only the latter is required by RVV-V and LVV-V. This indicates that proteolysis of FV by thrombin at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ shows different cleavage requirements with respect to interactions mediated by thrombin exosites and areas that surround the respective cleavage sites. In addition, interactions between exosite I of thrombin and FV are primarily responsible for the different cleavage site specificity as compared with activation by RVV-V or LVV-V.

4.2 Introduction

Human blood coagulation factor V (FV) is as a single-chain glycoprotein that consists of a mosaic domain structure composed of three homologous A-type domains, two smaller C-type domains and a large B domain (A1-A2-B-A3-C1-C2) [1]. FV, which has little or no intrinsic procoagulant cofactor activity, is activated by thrombin or factor Xa (FXa) through limited proteolysis [2]. Proteolytic activation of FV results in the removal of the FV B domain and the exposure of regions in the FV molecule that are important for the expression of its procoagulant activity [3,4]. Activated FV (FVa) is a heterodimer that consists of a heavy chain (A1-A2 domains) and a light chain (A3-C1-C2 domains) that are non-covalently associated in a calcium-dependent manner [5,6]. FVa is the essential non-enzymatic cofactor of the prothrombinase complex, which upon complex formation with FXa accelerates FXa-catalyzed prothrombin activation in the presence of calcium ions and a phospholipid membrane surface by several orders of magnitude [2,7].

Although FV can be activated by a number of proteases [8,9], thrombin is the most potent physiological activator of FV. Activation of FV by thrombin is achieved through limited proteolysis at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵, of which the cleavage after Arg¹⁵⁴⁵ is required for full expression of FVa activity [10]. Thrombin-catalyzed activation of FV follows a kinetically preferred order of bond cleavage, in which cleavage at Arg⁷⁰⁹ occurs first, followed closely by cleavage at Arg¹⁰¹⁸ and slow cleavage at Arg¹⁵⁴⁵, which results in formation of the FVa light chain [11,12].

A characteristic feature of thrombin are two distinct electropositive surface regions, termed exosite I and exosite II, that contribute to the specificity of thrombin by mediating the recognition of its substrates, inhibitors and receptors [13,14]. Although both exosites have been implicated in FV activation [15-18], their role in the recognition of the individual thrombin cleavage sites is still unclear. In this study, we have used FV cleavage site mutants in combination with specific thrombin exosite I and II inhibitors to elucidate the importance of each exosite for the individual FV activation cleavages.

Furthermore, we have used FV B domain deletion mutants to probe the structural requirements for thrombin exosite interactions located in the B domain and compared them with the structural requirements for FV activation by RVV-V and LVV-V, which are snake venom proteases that are known to activate FV by a single cleavage at Arg¹⁵⁴⁵ [19-21].

4.3 Materials and methods

Materials

Serum-free cell culture media (Optimem Glutamax) were from Invitrogen. Hepes, sodium chloride, calcium chloride, bovine brain phosphatidylserine, egg-yolk phosphatidylcholine, heparin-Sepharose, ovalbumin, bovine serum albumin and unfractionated heparin were from Sigma. 1 unit/ml unfractionated heparin contains ~5.7 µg/ml unfractionated heparin/ml [22]. Pefabloc TH was a product of Pentapharm Ltd (Basel, Switzerland). The chromogenic substrate S2238 was supplied by Chromogenix (Milan, Italy). The Micro BCA protein assay kit for protein concentration determination and the 3,3',5,5'-tetramethyl-benzidine substrate kit for ELISA were from Pierce. Polyethylene glycol 8000 was from Fluka (Buchs, Switzerland). Tris-glycine polyacrylamide gradient gels (4-12%), the Mark12 wide range unstained protein standard for SDS-PAGE and the colloidal blue staining kit were supplied by Invitrogen (Carlsbad, CA). Sulfuric acid and 2-mercaptoethanol were from Merck (Darmstadt, Germany).

Deoxyoligonucleotides GGTGGTGTGGTTGG (thrombin aptamer I) and AGTCCGTGGTAGGGCAGGTTGGGGTGACT (thrombin aptamer II) were purchased from DNA Technology AIS (Aarhus, Denmark).

Proteins

Human FV was purified essentially according to the method of Dahlbäck [23], modified as described [24]. Human thrombin was from ERL (Swansea, United Kingdom). The FV activator from the venom of *Daboia russelli* was supplied by Hematologic Technologies (Essex Junction, VT). The FV activator of *Daboia lebetina turanica* was purified as described previously [20]. Bovine FX was purified according to Fujikawa *et al.* [25]. FXa was prepared from FX after activation by RVV-X [26]. Human prothrombin was purified according to the method of Di Scipio *et al.* [27]. The monoclonal antibody HV1 specific for the human FV C2 domain was from Sigma and was biotinylated using the Pierce EZ-link sulfo-NHS-LC-Biotin kit according to the manufacturer's procedures. Monoclonal antibody 3B1 directed against the heavy chain of human FV was a kind gift from Prof. B. N. Bouma.

Protein concentration determination

Thrombin concentrations were determined using the chromogenic substrate S2238 [7]. The concentration of the snake venom FV activators RVV-V and LVV-V was measured using the Micro BCA protein assay kit (Pierce) using bovine serum albumin as a standard. FVa was quantified as described below.

FVa assay

FVa cofactor activity was measured via its ability to stimulate FXa-catalyzed thrombin generation as described [24]. Unless noted otherwise, prothrombin activation was started by adding prewarmed FV/FVa (~20 pM) to a reaction mixture containing 25 mM Hepes pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 0.5 μM prothrombin, 5 nM FXa, 40 μM phospholipid vesicles (10% brain phosphatidylserine, 90% egg-yolk phosphatidylcholine; mol/mol) and 0.5 mg/ml ovalbumin. The reversible thrombin inhibitor Pefabloc TH (1 μM) was present to avoid feedback activation of FV during the assay. Prothrombin activation was stopped after 1 min by dilution in ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 [7]. The molar FVa concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 mol of prothrombin activated/min/mole of factor Xa-Va complex [28]. Phospholipid

vesicles were prepared as described [7]. We verified that heparin and the thrombin exosite aptamers did not interfere with the FVa assay.

Recombinant FV variants

FV cDNA constructs containing different combinations of mutated and native amino acids at the three thrombin cleavage sites have been described previously [12]. The FV mutants were named according to which amino acid was present at each thrombin cleavage site, i.e. WT FV (Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵), RIQ (Arg⁷⁰⁹, Ile¹⁰¹⁸ and Gln¹⁵⁴⁵), QRQ (Gln⁷⁰⁹, Arg¹⁰¹⁸ and Gln¹⁵⁴⁵), QIR (Gln⁷⁰⁹, Ile¹⁰¹⁸ and Arg¹⁵⁴⁵), RIR (Arg⁷⁰⁹, Ile¹⁰¹⁸ and Arg¹⁵⁴⁵), QIQ (Gln⁷⁰⁹, Ile¹⁰¹⁸ and Gln¹⁵⁴⁵), and QIQQQ (Gln⁷⁰⁹, Ile¹⁰¹⁸, Gln¹⁵⁴⁵, Gln¹⁷⁶¹ and Gln¹⁷⁶⁵).

FV constructs lacking the complete B domain (FV des 709-1545) or the B domain region from residues 710 to 1477 (FV des 709-1476) were prepared as described [29]. The FV construct lacking the B domain region from residues 828 to 1499 (FV des 827-1498) was made as described [30].

Transient expression of recombinant FV

Plasmid DNA containing the various FV cDNA constructs was transfected into COS1 cells using the diethylaminoethanol-dextran method [31]. As a control, a pMT2-V vector of which the FV cDNA had been excised, through SalI cleavage and religation of the empty vector, was used to transfect cells. The proteins were collected in serum-free medium (Optimem Glutamax). 72 h after transfection, cell debris was removed by centrifugation and the medium was stored at -80 °C. Expression levels of all FV constructs were routinely estimated using the FV-specific ELISA described below. Expression levels of the recombinant FV variants were also determined after complete activation with excess thrombin (5 nM for wild type FV, FV des 827-1498 and the RIR mutant, 10 nM for the remaining FV cleavage site mutants and 50 nM thrombin for FV des 709-1476 and FV des 709-1545 (see also the Results section)), using the above functional assay. FV expression levels were estimated from the FVa cofactor activity reached upon completion of FV activation and calculated relative to the activity of WT FV. No FV(a) was detectable in the mock medium, as judged by both the ELISA and functional activity assay.

FV ELISA

FV expression levels were estimated by ELISA using the monoclonal antibody 3B1 as the primary antibody. Microtiter plates were coated overnight with 10 µg/ml of the primary antibody. The plates were blocked for 1 h at room temperature with 50

mM Hepes, 150 mM NaCl, 1% bovine serum albumin pH 7.5 (Hepes-buffered saline-BSA). A standard curve was prepared using pooled normal citrated plasma, assuming the plasma FV concentration to be 21.3 nM [32]. Standard and samples (diluted in Hepes-buffered saline-BSA) were incubated for 2 h at room temperature, and the plates were then washed three times with 0.05% Tween 20 in Hepes-buffered saline. The biotinylated secondary antibody HV1 was added (0.55 µg/ml) and incubated for 2 h at room temperature. After washing, the plates were incubated for 30 min with avidin-horseradish peroxidase (Strep ABC complex/horseradish peroxidase from DAKO, Denmark). After washing, the plates were developed for 10 min with peroxidase substrate solution (3,3',5,5'-tetramethyl-benzidine substrate kit; Pierce). The reaction was stopped by the addition of 5% (v/v) H₂SO₄ and the absorbance was measured at 490 nm. To rule out the possibility that the cell culture medium interfered with the ELISA assay, plasma-purified FV was diluted in mock medium or in buffer. No difference was detected between these diluted samples, indicating that the cell culture medium did not interfere with the FV quantitation.

Thrombin activation of FV cleavage site mutants

To determine the thrombin concentration needed to fully activate the FV cleavage site mutants, each of the recombinant FV variants (250 pM in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) was incubated with increasing concentrations of thrombin (0–50 nM) for 15 min at 37 °C and then assayed for FVa as described above.

Inhibition of thrombin-catalyzed FV activation by thrombin exosite I and II aptamers

To determine the aptamer concentrations needed for saturation of the inhibitory effect on thrombin-catalyzed activation of FV, 250 pM recombinant wild type FV (in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) was activated with 10 nM thrombin in the presence of increasing concentrations aptamer I, II, or both. At different time points (0, 1, 3, 5, 7, 10 and 15 min), FVa concentrations were measured using the assay described above.

Role of thrombin exosites I and II in FV activation

To investigate the importance of each of the two exosites of thrombin for the recognition of the individual FV activation cleavage sites, activation of the recombinant FV cleavage site mutants RIQ, QRQ and QIR (250 pM in 25 mM Hepes,

pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl_2) by thrombin (10 nM) was followed in the absence and presence of aptamer I (19 μM), aptamer II (5 μM), or both (8.2 μM aptamer I and 5 μM aptamer II). At different time points (0, 1, 3, 5, 7, 10 and 15 min), the concentration of FVa generated was measured as described above. Factor V activation rates were determined under initial steady-state second order rate conditions in which the initial rate of FV activation was linear with time and linearly proportional to the concentration of FV between 0 and 0.5 nM, as well as the concentration of thrombin present from 0.05 to 20 nM.

SDS-PAGE analysis of FV activation in the absence and presence of thrombin exosite aptamers

Time courses of activation of plasma-purified FV (75 nM in 50 mM Hepes, 150 mM NaCl, 5 mM CaCl_2 , 1 mg/ml polyethylene glycol, pH 7.5) by thrombin were monitored in the absence and presence of thrombin exosite aptamer I (10 μM), aptamer II (5 μM), or both (10 and 5 μM , respectively). Various thrombin concentrations were used such that the effect of aptamers on FV cleavage could be visualized: 0.5 nM (without aptamer and in the presence of aptamer II), 2.5 nM (aptamer I), or 20 nM (in the presence of both aptamers). At various time points, aliquots were taken from the reaction mixtures and subjected to SDS-polyacrylamide gel electrophoresis (4-12%) under reducing conditions. Simultaneously, FVa activities were determined as described above. FV fragments were visualized by colloidal blue staining.

Characterization of the FV B domain deletion mutants

The recombinant FV B domain deletion mutants and wild type FV (150 pM in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl_2) were activated with 0.5 nM thrombin, RVV-V or LVV-V. At various time points (0, 1, 3, 5, 7 and 10 min) aliquots were withdrawn and FVa generated was measured. We chose to assess the FVa cofactor activity in assays at 0.5 nM FXa to minimize the intrinsic procoagulant activity reported for recombinant B domain deleted FV forms [4,33,34]. Under our conditions, activities of unactivated FV were typically <5% for wild type FV and 17-21% for variants lacking the B domain. Mock conditioned medium gave no measurable FVa activity.

Binding of thrombin, RVV-V and LVV-V to heparin-Sepharose

Thrombin, RVV-V or LVV-V (1 ml of 5 nM) in 25 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mg/ml BSA) were applied to a 1-ml heparin-Sepharose column that was pre-

equilibrated with the same buffer. The column was washed with 4 ml of the same buffer followed by elution with a 10 ml linear gradient of 50-1000 mM NaCl. Elution fractions (1 ml) were diluted twice in compensating buffer (25 mM Hepes, pH 7.5, 0.1 mg/ml BSA and an appropriate amount of NaCl) to fix the final NaCl concentration in each elution fraction to 500 mM. The presence of FV activator was monitored by the ability of aliquots from the fractions to activate bovine FV. To this end, 50 μ l of 20 nM bovine FV in 25 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mg/ml BSA and 20 mM CaCl_2 was added to 150 μ l of elution fraction. After 10 min of incubation at 37 °C, the FVa generated was measured. During this 10 min incubation period, FVa generation was linear in time for each of the three activators, verifying a linear dependence between FVa activity generated and the amount of FV activator present. Elution profiles were represented as percentages of the amount of FVa generated in the peak fraction.

FV activation in the presence of heparin

To investigate the effect of heparin on the activation of 5 nM FV by either thrombin, RVV-V or LVV-V, FV was incubated with 0.1 nM of each activator in the presence of increasing concentrations of heparin (0-1000 units/ml) in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, and 5 mM CaCl_2 . Samples were taken at different time points during the first 2 min of activation and the amount of FVa generated was measured. From the resulting progress curves, the initial FV activation rates were calculated (nM FVa/min) and plotted as a function of the heparin concentration.

SDS-PAGE analysis of FV activation in the presence of heparin

To identify fragment generation during FV activation in the presence of heparin, 75 nM plasma-purified human FV (in 50 mM Hepes, 150 mM NaCl, 5 mM CaCl_2 , 1 mg/ml polyethylene glycol, with or without 10 units/ml heparin, pH 7.5) was activated with 1 nM thrombin or 5 nM snake venom activator. At various time points, aliquots were removed and subjected to SDS-polyacrylamide gel electrophoresis (4-12%) under reducing conditions. Simultaneously, FVa activities were determined as described above. FV fragments were visualized by colloidal blue staining.

4.4 Results

Role of thrombin exosites I and II in FV activation

To examine the role of thrombin exosites in FV activation, a panel of FV cleavage site mutants was expressed in COS1 cells. The expression levels of the FV variants, as determined by ELISA, were between 0.6 and 1 nM. Thrombin titration was performed to determine the concentration required for full activation of the different mutants (Figure 4.1).

Figure 4.1. Activation of FV cleavage site mutants by thrombin. Recombinant FV cleavage site mutants (250 pM of each: ●, WTFV; ▽, RIR; ◆, QIR; △, RIQ; ▲, QRQ; ◇, QIQ; ▼, QIQQQ) were activated with increasing thrombin concentrations (0.1-50 nM). After 15 min at 37 °C, FVa cofactor activity was measured as described under "Materials and Methods". The data were fit by nonlinear regression with the program GraphPad Prism.

All of the mutants, except the QIR mutant, reached a maximal and stable level of cofactor activity after 15 min of activation with 5 nM thrombin. A control experiment, with excess thrombin (50 nM) or RVV-V (25 nM), verified that the QIR variant could be completely activated to a stable FVa activity level, corresponding to an activity:antigen ratio that was similar to that of wild type FV (not shown). As expected, incubation of the QIQ and QIQQQ mutants did not result in an increase in FVa cofactor activity, which illustrates that both FV variants are completely resistant to thrombin activation. Full cofactor activity of wild type FV and the RIR variant were observed at low thrombin concentration (typically 1–2 nM). Furthermore, cleavage at Arg⁷⁰⁹ was more sensitive to thrombin than cleavage at Arg¹⁵⁴⁵ as illustrated by the fact that the RIQ mutant reached maximum activity with 1 nM thrombin, whereas the QIR mutant required >50 nM for full activation within 15 min. Finally, complete activation of the QRQ mutant was observed at 2.5 nM

thrombin, but only a slight increase (~2-fold) in FVa cofactor activity was observed between the fully activated and non-activated forms.

Aptamer titrations were performed to determine the concentration required to inhibit thrombin-catalyzed activation of wild type FV (Figure 4.2). The aptamers used are single-stranded DNA oligonucleotides, which have been shown to be specific for thrombin exosite I (aptamer HD1 [35-39]) or exosite II (aptamer HD22 [40]). Aptamer I almost completely inhibited FV activation at a concentration of 19 μ M. Maximal inhibitory activity of thrombin exosite II aptamer was observed at 5 μ M, but this aptamer inhibited the activation of wild type FV only partially.

Figure 4.2. Effect of exosite aptamers I and II on activation of wild type FV by thrombin. Recombinant WT FV (250 pM) was activated with thrombin (10 nM) in the presence of varying concentrations of aptamer I ($\square$, 0 μ M; $\triangle$, 1 μ M; ∇ , 5 μ M; $\diamond$, 10 μ M; $\circ$, 19 μ M), aptamer II ($\square$, 0 μ M; $\triangle$, 1 μ M; ∇ , 5 μ M; $\diamond$, 9 μ M), or both aptamers I and II ($\square$, 0 + 0 μ M; $\triangle$, 1 + 1 μ M; ∇ , 5 + 5 μ M; $\diamond$, 5 + 8.2 μ M). At the time points indicated aliquots were taken from the activation mixture and the FVa cofactor activity was measured as described under "Materials and Methods" and expressed as a percentage of the FVa cofactor activity of fully activated WT FV. The data were fit by nonlinear regression with the program GraphPad Prism.

The importance of thrombin exosites I and II for the recognition of the individual FV activation cleavage sites was investigated by monitoring the time course of activation of FV cleavage site mutants with only one thrombin cleavage site available. These time courses were recorded in the absence and presence of aptamer I (19 μ M), aptamer II (5 μ M), or both (8.2 μ M aptamer I and 5 μ M aptamer II). Thrombin-catalyzed cleavage at Arg⁷⁰⁹ (mutant RIQ) was almost completely blocked in the presence of exosite I aptamer, whereas thrombin exosite II aptamer showed a minor effect (Figure 4.3, *left panel*). Although activation of FV mutant QRQ by thrombin-catalyzed cleavage at Arg¹⁰¹⁸ resulted in only a 2-fold increase of FVa cofactor activity in prothrombin activation, inhibition of activation of this mutant was observed by exosite I aptamer with only a marginal effect by thrombin exosite II aptamer (Figure 4.3, *middle panel*). Activation of FV variant QIR, which can only be cleaved by thrombin at Arg¹⁵⁴⁵, was significantly inhibited by both aptamer

I and II. With both aptamers simultaneously present, thrombin-catalyzed activation was virtually completely inhibited (Figure 4.3, *right panel*).

Figure 4.3. Time course of activation of FV cleavage site mutants RIQ, QRQ, and QIR in the presence of thrombin exosite aptamers I and II. Recombinant FV cleavage site mutants RIQ, QRQ and QIR (250 pM of each: ●, no aptamer; ▽, aptamer I; ◇, aptamer II; ○, aptamer I+II) were activated with thrombin (10 nM) in the absence and presence of thrombin exosite aptamer I (19 μ M), aptamer II (5 μ M), or both (8.2 μ M aptamer I and 5 μ M aptamer II). At the time points indicated aliquots were taken from the activation mixture. FVa activity was measured as described under "Materials and Methods" and expressed as a percentage of the FVa cofactor activity of the fully activated FV variant. The *error bars* represent the standard deviation of three experiments. The data were fit by nonlinear regression with the program GraphPad Prism.

Because the kinetics of the individual cleavage sites are not independent of one another [12,29,45] and therefore the results with the mutants cannot be interpreted with certainty as reflecting the kinetics of cleavage of wild type FV, we employed SDS-PAGE analysis and compared cleavage reactions of plasma-purified FV by thrombin in the absence and presence of thrombin aptamers (Figure 4.4).

Figure 4.4 (A). Activation of plasma-purified FV in the absence or presence of thrombin exosite aptamers. Time courses of activation of plasma-purified FV (75 nM) in the absence or presence of thrombin exosite aptamers (○, no aptamer; ◇, aptamer I, 10 μ M; ▽, aptamer II, 5 μ M; □, aptamer I and II, 10 and 5 μ M respectively). Thrombin concentrations used were: 0.5 nM (no aptamer and aptamer II), 2.5 nM (aptamer I), or 20 nM (aptamer I+II). At the time points indicated, the aliquots were taken from the activation mixture and diluted 1/250 in ice-cold HNBSA/Ca buffer (Hepes-buffered saline containing 5 mg/ml BSA and 5 mM CaCl_2) and the FVa activity was measured as described under "Materials and Methods". Time courses of activation were fit by nonlinear regression with the program GraphPad Prism.

Figure 4.4 (B-E). Activation of plasma-purified FV in the absence or presence of thrombin exosite aptamers. (B-E), From the activation mixtures described in Fig. 4.4A, aliquots were withdrawn at the indicated time points and subjected to SDS-PAGE under reducing conditions. The proteins were visualized by colloidal blue staining.

Thrombin aptamer I (Figure 4.4C) decreased the rate of disappearance of single chain FV (330 kDa) and delayed the appearance of the 280-kDa intermediate and the heavy chain (105 kDa), which result from cleavage of single chain FV at Arg⁷⁰⁹. In addition, the formation of the FVa light chain (71/74 kDa), which is formed after cleavage at Arg¹⁵⁴⁵, was considerably delayed in the presence of aptamer I. Inhibition of cleavage at Arg⁷⁰⁹ and Arg¹⁵⁴⁵ resulted in the accumulation of 220- and 150-kDa intermediates originating from single cleavage at Arg¹⁰¹⁸ in single chain FV. Thrombin aptamer II (Figure 4.4D) decreased the rate of disappearance of single chain FV and formation of the FVa light chain but had no major effect on the rate of formation of the 280-kDa intermediate and the heavy chain. In the presence of both aptamers (Figure 4.4E), FV cleavage was almost completely blocked, and under the conditions chosen, only the 220- and 150-kDa intermediates were formed during the first 45 min of activation. In summary, the data obtained with wild type FV also show that thrombin exosite I is essential for cleavage at Arg⁷⁰⁹ and Arg¹⁵⁴⁵, whereas exosite II is particularly important for cleavage at Arg¹⁵⁴⁵.

FV cleavage requirements for activation by thrombin, RVV-V and LVV-V

To evaluate the structural elements in the substrate FV B domain that are important for FV activation, we expressed a number of FV B domain deletion mutants in COS1 cells. FV expression levels were determined using a quantitative FV ELISA and a functional assay (Table 4.1).

FV cleavage site mutants			
Construct	Activity (nM)	Antigen (nM)	Activity/antigen
WT-FV	0.60	0.65	0.92
QRQ	0.07	0.69	0.11
QIQQQ	0.05	0.97	0.06
QIR	0.47	0.57	0.81
RIQ	0.20	0.58	0.33
QIQ	0.06	0.66	0.09
RIR	0.65	0.74	0.88
FV B domain deletion mutants			
Construct	Activity (nM)	Antigen (nM)	Activity/antigen
WT-FV	0.517	0.53	0.98
FV des 709-1476	0.331	0.86	0.38
FV des 709-1545	0.463	0.78	0.59
FV des 827-1498	0.491	0.54	0.91

Table 4.1. FV concentrations. FV concentrations were determined by an ELISA as described under "Materials and Methods". FVa activities were determined using the FVa assay as described under "Materials and Methods" using the kinetic parameters as determined for plasma-purified FV [28]. The values are the averages of three experiments, and the standard deviations were <5% for all of the FV variants. As a parameter of specific activity, the ratio of FV activity to antigen was calculated.

The activity:antigen ratio for B domain deleted mutant FV des 827-1498 was the same as the ratio obtained for the wild type construct, whereas the activity of FV des 709-1545 and FV des 709-1476 were somewhat decreased.

The FV cleavage requirements for thrombin, RVV-V and LVV-V (0.5 nM) were investigated by following the time course of activation of wild type FV and the B domain deletion mutants (Fig. 4.5).

Figure 4.5. Activation of recombinant WT FV and FV B domain deletion mutants by thrombin, RVV-V and LVV-V. Recombinant wild type FV or FV B domain deletion mutants (150 pM of each: $\square$, wild type; ∇ , FV des 709-1545; Δ , FV des 709-1476; $\diamond$, FV des 827-1499) were activated with 0.5 nM thrombin, RVV-V or LVV-V. At the time intervals indicated, the aliquots were taken from the activation mixture and FVa cofactor activity was determined in the prothrombinase-based FVa assay described under "Materials and Methods". The error bars represent the standard deviations of four experiments.

Although all of the B domain deletion constructs could be activated by incubation with excess activator (Table 4.1) no increase in cofactor activity was observed for the FV des 709-1545 and FV des 709-1476 mutants upon incubation with 0.5 nM thrombin, whereas FV des 827-1498 was activated by thrombin at a rate similar to that observed for wild type FV (Figure 4.5, *left panel*). Upon incubation with RVV-V or LVV-V, no increase in cofactor activity was observed for the FV des 709-1545 mutant, indicating that this mutant could also not be activated by the snake venom FV activators (Figure 4.5, *middle and right panels*). FV des 709-1476 and FV des 827-1498 were rapidly activated by RVV-V and LVV-V and appear to be even more rapidly activated than wild type FV.

The C-terminal region of the FV B domain is enriched in acidic amino acids and also contains several sulfated tyrosine residues that have been proposed to be important for interaction with the positively charged exosites of thrombin [29,44]. The observation that RVV-V and LVV-V require the C-terminal region of the B domain for efficient activation of FV indicates that this acidic region is also of vital importance for the recognition of the Arg¹⁵⁴⁵ cleavage site by the venom FV activators.

Binding of thrombin, RVV-V and LVV-V to heparin-Sepharose

Structural models of RVV-V and LVV-V show the presence of two positively charged surface regions that may play a role in FV recognition and activation [20]. By analogy to thrombin, these positively charged surface regions in RVV-V and LVV-V may represent potential exosites for the binding to macromolecular ligands other than FV [13,14]. One such potential ligand is the thrombin exosite II ligand heparin. To determine whether the snake venom activators bound heparin, both activators were subjected to heparin-Sepharose affinity chromatography. Both RVV-V and LVV-V bound to heparin-Sepharose and eluted at ~500 mM NaCl, whereas thrombin eluted at ~600 mM NaCl (Figure 4.6). This indicated that RVV-V and LVV-V can bind heparin, although with a somewhat lower affinity than thrombin.

Figure 4.6. NaCl gradient elution profiles of RVV-V, LVV-V, and thrombin from heparin-Sepharose. The FV activators from *D. russelli* (RVV-V), *D. lebetina* (LVV-V) and thrombin were subjected to heparin-Sepharose affinity chromatography as described under "Materials and Methods". Peak activities of RVV-V ($\diamond$) and LVV-V (∇) co-eluted around 500 mM NaCl, thrombin ($\circ$) at 600 mM NaCl. The closed circles represent the NaCl gradient.

FV activation in the presence of heparin

To further assess the importance of thrombin exosite II for FV activation, we compared the effect of heparin on thrombin-catalyzed activation of FV with RVV-V and LVV-V-catalyzed activation. Figure 4.7 shows initial rates of FV activation in the presence of increasing concentrations of heparin. Low concentrations of heparin (<5 units/ml) accelerated FV activation by approximately 3-fold (RVV-V and thrombin) to 4-fold (LVV-V) with an optimum enhancement at 5 units/ml. At higher heparin concentrations (up to 1000 units/ml), the rates of FV activation gradually declined, demonstrating an inhibitory effect on FV activation by all three activators. The observation that heparin has an optimal concentration for acceleration of FV activation, with the activities decreasing at concentrations above and below this

value, suggests that heparin serves as a template to which both enzyme and FV bind.

Figure 4.7. FV activation in the presence of heparin. The activation of 5 nM purified human FV by thrombin, RVV-V and LVV-V (0.1 nM) was monitored as a function of increasing concentrations of heparin (0-1000 units/ml). One unit of heparin was defined as 5.7 $\mu\text{g/ml}$ [22]. The initial rates of FV activation were measured as described under "Materials and Methods" and plotted as a function of the heparin concentration. The symbols are as follows: thrombin ($\circ$), RVV-V ($\diamond$) and LVV-V (∇). The inset figure shows the first part of the curve.

SDS-PAGE analysis of FV activation in the presence of heparin

To assess whether heparin alters the cleavage pattern of FV activation and/or influences cleavages other than the cleavage at Arg¹⁵⁴⁵ by thrombin, RVV-V or LVV-V, time courses of activation were followed in the absence and presence of heparin (Figure 4.8A).

Figure 4.8 (A). Time courses of FV activation by RVV-V, LVV-V, and thrombin in the absence and presence of heparin. Time course of factor V activation by thrombin ($\circ$), RVV-V ($\diamond$) and LVV-V (∇) in the absence (*open symbols*) or presence (*closed symbols*) of 10 units/ml heparin. Factor V (75 nM) was activated with 1 nM thrombin or 5 nM snake venom factor V activator in the absence or presence of 10 units/ml heparin. After the indicated time intervals, the aliquots were taken and diluted 1/500 in ice-cold HNBSA/Ca buffer (Hepes-buffered saline containing 5 mg/ml BSA) and the FVa activity was measured as described under "Materials and Methods".

At various times, aliquots from the activation mixture were taken and subjected to SDS-PAGE to characterize the FV fragments formed during FV activation (Figure 4.8B). For the thrombin-catalyzed reaction, cleavage of the 330- and 280-kDa bands and corresponding formation of the light (71/74 kDa) and heavy chain (105 kDa) were accelerated ~2-fold by the presence of heparin. In case of RVV-V and LVV-V, cleavage of the 330-kDa band in the presence of heparin was accelerated 5- to 6-fold and coincides with a faster formation of the 290-kDa heavy chain and the 71/74-kDa light chain typically observed upon cleavage of FV at Arg¹⁵⁴⁵. Notably, upon prolonged activation with excess snake venom factor V activator in the presence of heparin, the FVa heavy chain (290 kDa) was further cleaved into fragments with apparent masses of 150 and 175 kDa (Figure 4.8B, lane 120+) through cleavage at as yet unidentified cleavage sites.

Figure 4.8 (B). Time courses of FV activation by RVV-V, LVV-V, and thrombin in the absence and presence of heparin. From the activation mixtures described in Fig. 4.8A, aliquots were withdrawn at 0, 1, 3, 5, 7, 10, 20, 45, 60 and 90 min and subjected to SDS-PAGE under reducing conditions. An aliquot of fully activated FV (after 120 min of incubation with 30 nM snake venom FV activator or 5 nM thrombin, indicated as 120+) was also taken for comparison. The proteins were visualized by colloidal blue staining.

Role of the sodium binding loop of thrombin in FV activation

We have investigated the role of the sodium binding site of thrombin in the recognition of the individual thrombin cleavage sites at positions 709 and 1545. The initial activation rates of the FV cleavage site mutants RIQ and QIR by thrombin were increased (2- to 4-fold) in the sodium concentration range tested (20-200 mM Na⁺; ionic strength was kept constant with choline chloride). In a control experiment in which the FV variant QIR was activated with the snake venom FV activator RVV-V (which does not contain the Na⁺ binding site), the FV activation rate remained unchanged over the entire Na⁺ concentration range tested (data not shown). This demonstrates that the effect of sodium on the thrombin-catalyzed cleavage at position 1545 is mediated through its binding to thrombin and not to FV.

4.5 Discussion

Anion-binding exosites I and II of thrombin have been demonstrated to be involved in the activation of factor V [15-18]. However, the importance of each exosite in the recognition of the individual thrombin cleavage sites in FV is still unclear. Using recombinant FV variants that harbor only one of the three thrombin cleavage sites in combination with aptamers that specifically bind to either thrombin exosite I or II, we have studied the role of thrombin exosite I and II in FV activation. The data obtained with the RIQ mutant in combination with aptamers specific for thrombin exosite I and/or II show that thrombin exosite I, but not exosite II, plays a critical role in the recognition of the Arg⁷⁰⁹ cleavage site and consequently in the initiation of FV activation.

This observation is supported further by SDS-PAGE analysis of cleavage of plasma-purified FV by thrombin in the absence and presence of thrombin aptamers. Cleavage at Arg⁷⁰⁹ and formation of the FVa heavy chain were significantly delayed in the presence of aptamer I, but not in the presence of aptamer II. These data are in agreement with results reported by others who have shown that residues Arg⁷³ and Tyr⁷⁶ (chymotrypsinogen numbering) in exosite I are essential for the recognition and cleavage of FV at Arg⁷⁰⁹ [41]. Furthermore, fluorescence binding studies using active site labeled thrombin have demonstrated that exosite I is predominantly responsible for the interaction with a site on the FV/FVa heavy chain preceding Arg⁷⁰⁹ [42].

Our results also yield information concerning the other cleavages associated with FV activation. We found that thrombin exosite I and II are both involved in the recognition and cleavage at Arg¹⁵⁴⁵. The observation by Dharmawardana and colleagues [16] that FV activation by thrombin in the presence of the exosite I-

specific peptide hirudin⁵⁴⁻⁶⁵ showed the accumulation of activation intermediates resulting from cleavage at Arg¹⁰¹⁸ supports our finding that exosite I is important for cleavage at Arg¹⁵⁴⁵.

Although it is widely accepted that thrombin exosite I is involved in FV activation, the role of exosite II in this process has been less clear. Because thrombin exosite II ligands (prothrombin fragment 2 and heparin) did not inhibit FV activation, even when thrombin exosite I was not functional, Arocas *et al.* [17] suggested that thrombin exosite II is not directly involved in FV recognition by thrombin. Others, however, have proposed exosite II to be involved in FV activation because mutations in exosite II residues showed a reduced capacity to cleave and activate FV [15-41]. Here, we demonstrate using recombinant FV cleavage site mutants that thrombin exosite II is involved in the cleavage at Arg¹⁵⁴⁵, but not in cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸ (Figure 4.3, *left and right panels*). This result is confirmed by SDS-PAGE analysis of cleavage of plasma-purified FV by thrombin in the presence of aptamers. We observed that blocking of exosite II has a major effect on the formation of the FVa light chain but not on formation of the heavy chain (Figure 4.4D).

Because it has been demonstrated that thrombin activation of FV requires an extensive interaction interface [41], we also investigated whether in addition to thrombin exosites I and II, the sodium binding site of thrombin is also involved in FV activation. The initial rates of FV activation of the cleavage site mutants RIQ and QIR by thrombin appeared to be increased (2- to 4-fold) between 20 and 200 mM Na⁺. These results confirm and extend earlier mutagenesis data reported by Myles *et al.* [41] showing that the sodium binding loop of thrombin is involved in FV activation. From our results, it can be concluded that the effect of sodium binding is similar for all cleavages involved. Thus, in contrast to the finding that exosites I and II differentially stimulate the different FV cleavages, the thrombin sodium binding loop is involved to a similar extent in all FV cleavages.

Discrete regions in the B domain have been reported to maintain FV in an inactive procofactor state [34]. These inhibitory constraints are removed after limited proteolysis in the B domain, a process that is mediated by electrostatic interactions between highly acidic regions in the FV molecule and positively charged regions (exosites) in the FV-activating enzyme [1,29,43]. To investigate the structural requirements for exosite interactions located in the B domain of FV, we expressed FV variants in COS1 cells that lack the complete B domain (FV des 709-1545), the N-terminal and central region of the B domain (FV des 709-1476), or only the central region of the B domain (FV des 827-1498). Although FV des 709-1545 and FV des 709-1476 could be fully activated after prolonged incubation with excess thrombin (as described in the Materials and Methods section; data not shown), no substantial activation of these mutants was observed at low levels of thrombin

(Figure 4.4A). On the other hand, activation of FV des 827-1498 was comparable with wild type FV. This indicates that both the N- and C-terminal region of the FV B domain are required for efficient activation by thrombin and thus that the sequences 710-826 and 1499-1544 contain sites that contribute to optimal activation of FV by thrombin. These results seem to contradict earlier studies showing the importance of an acidic region preceding Arg⁷⁰⁹ for thrombin-catalyzed FV activation. This region is not present in the B domain but resides within the FVa heavy chain. A likely explanation is that the region between residues 709-827 in the B domain contains sequences that are part of the thrombin recognition site. We can, however, not exclude the possibility that the deletion of a large part of the B domain introduces local structural changes in the acidic region preceding the Arg⁷⁰⁹ cleavage site that hamper the interaction between thrombin and this part of the FV molecule.

Similar to the Arg⁷⁰⁹ cleavage site, the Arg¹⁵⁴⁵ cleavage site is preceded by an acidic region that shows sequence homology to hirudin⁵⁴⁻⁶⁵ and that contains three putative sulfated tyrosine residues [1-43]. In agreement with our findings, this region has been proposed as a possible thrombin-binding site important for cleavage at Arg¹⁵⁴⁵ [29-43]. However, the acidic region preceding the Arg¹⁵⁴⁵ cleavage site seems not to be solely responsible for efficient cleavage by thrombin at Arg¹⁵⁴⁵, as indicated by the observation that mutant FV des 709-1476 was not substantially activated by thrombin. Local structural changes in the region preceding the Arg¹⁵⁴⁵ cleavage site that might be introduced by the deletion can be excluded, because mutant FV des 709-1476 was efficiently activated by RVV-V and LVV-V. This again illustrates that the initial cleavage at Arg⁷⁰⁹, which coincides with the liberation of the FVa heavy chain, facilitates cleavage at Arg¹⁵⁴⁵. The facilitating effect of thrombin cleavage at Arg⁷⁰⁹ (and also at Arg¹⁰¹⁸) on cleavage at Arg¹⁵⁴⁵ has been demonstrated earlier using FV cleavage site mutants [12,29,45].

In contrast to thrombin, the presence of the region preceding the Arg¹⁵⁴⁵ cleavage site seems to be sufficient for efficient FV activation by RVV-V and LVV-V. Remarkably, FV variants FV des 709-1476 and FV des 827-1498 were more rapidly activated by RVV-V and LVV-V than wild type FV. It is possible that access to the Arg¹⁵⁴⁵ cleavage site in these mutants is less restricted than in WT FV because of the absence of a large part of the FV B domain. Alternatively, deletion of the central part of the B domain may cause conformational changes in the region around Arg¹⁵⁴⁵ that result in an increased affinity of RVV-V and LVV-V for this cleavage site. It is likely that FV recognition by RVV-V and LVV-V, like thrombin, is also mediated by exosite-driven interactions, given the requirement of the acidic region that precedes the Arg¹⁵⁴⁵ cleavage site for efficient activation by both proteases. Three-dimensional structural models of RVV-V and LVV-V reveal the presence of two positively charged surface regions on opposite sides of their active site that may be

involved in the recognition of the Arg¹⁵⁴⁵ cleavage site [20]. Nonetheless, the thrombin exosite aptamers did not inhibit FV activation by RVV-V and LVV-V (data not shown). This not only suggests that the thrombin exosites are distinctly different from the positively charged surface regions in RVV-V and LVV-V, but it also indicates that the inhibition of thrombin-dependent FV activation by the aptamers is due to the binding to thrombin and not due to interaction with FV.

The high degree of overall sequence identity (30%) of RVV-V and LVV-V with thrombin indicates evolutionary conservation of structure and function. In search of a suitable anionic ligand for the positively charged surface regions of RVV-V and LVV-V, we subjected both enzymes to heparin affinity chromatography. Both enzymes bound with high affinity to heparin, which is a thrombin exosite II ligand [46,47] that is of clinical importance both because of its common use as an antithrombotic drug but also because heparin resembles the endogenous glycosaminoglycans that line the inner wall of our vasculature. Because exosite II is also involved in FV activation, we have studied the effect of heparin on FV activation by thrombin, RVV-V and LVV-V. Heparin was found to stimulate FV activation by thrombin and the two snake venom FV activators 3- to 4-fold at the optimal concentration (5 units/ml). Our results confirm and extend earlier reports concerning the effects of heparin on thrombin-catalyzed FV activation [15-17].

The observation that heparin does not inhibit FV activation by thrombin was reported earlier by Esmon and Lollar [15]. However, the fact that a stimulating effect of heparin on FV activation was not observed can be explained by the higher FV/heparin ratio used in this study [15]. A similar accelerating effect of heparin on FV activation by thrombin was found by Arocas *et al.* [17], who suggested that the effect of heparin could result from an allosteric modification of the active site of thrombin promoting FV cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸. However, we have found that heparin did not accelerate chromogenic substrate hydrolysis by thrombin (data not shown). This likely suggests that together with the observation that heparin has an optimal concentration for acceleration of FV activation and the fact that this stimulating effect is not observed at higher concentrations, heparin serves as a template to which both enzyme and substrate bind. Moreover, neither low molecular mass heparin (~6000 Da) nor pentasaccharide accelerated FV activation (data not shown), which supports further our idea that heparin accelerates FV activation by acting as a template.

Time courses of FV cleavage show that in the thrombin-catalyzed reaction, cleavage of the 330- and 220-kDa FV fragments was accelerated by heparin. The accelerated cleavage of the 330-kDa fragment indicates that heparin facilitates the initial cleavage at Arg⁷⁰⁹, whereas the accelerated cleavage of the 220-kDa intermediate and the simultaneous generation of the 71/74-kDa FVa light chain point to an enhancement of the cleavage at Arg¹⁵⁴⁵. Furthermore, activation of FV

by RVV-V and LVV-V in the presence of heparin resulted in the accelerated cleavage at Arg¹⁵⁴⁵ and the formation of the heavy (290 kDa) and light chain (71/74 kDa).

In conclusion, this work provides new information on the role of the exosites of thrombin for FV recognition and activation and on the regions in the FV B domain that are important for the interaction with thrombin. We propose that exosite I of thrombin plays a dominant role in the activation of FV; cleavage at Arg⁷⁰⁹ precedes those at Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ and requires the interaction between exosite I of thrombin and regions surrounding the scissile bond at Arg⁷⁰⁹ in FV. Exosites I and II appear to be equally important for the proteolytic cleavage at Arg¹⁵⁴⁵ in FV.

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Chapter V:

Design of protein-membrane interaction inhibitors by virtual ligand screening, proof of concept with the C2 domain of factor V

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5.1 Summary

Most orally bioavailable drugs on the market are competitive inhibitors of catalytic sites, but a significant number of targets remain undrugged, because their molecular functions are believed to be inaccessible to drug-like molecules. This observation specifically applies to the development of small-molecule inhibitors of macromolecular interactions such as protein-membrane interactions that have been essentially neglected thus far. Nonetheless, many proteins containing a membrane-targeting domain play a crucial role in health and disease, and the inhibition of such interactions therefore represents a very promising therapeutic strategy. In this study, we demonstrate the use of combined *in silico* structure-based virtual ligand screening and surface plasmon resonance experiments to identify compounds that specifically disrupt protein-membrane interactions. Computational analysis of several membrane-binding domains revealed they all contain a druggable pocket within their membrane-binding region. We applied our screening protocol to the second discoidin domain of coagulation factor V and screened >300,000 drug-like compounds *in silico* against two known crystal structure forms. For each C2 domain structure, the top 500 molecules predicted as likely factor V-membrane inhibitors were evaluated *in vitro*. Seven drug-like hits were identified, indicating that therapeutic targets that bind transiently to the membrane surface can be investigated cost-effectively, and that inhibitors of protein-membrane interactions can be designed.

5.2 Introduction

The availability of thousands of genes potentially involved in disease has stimulated interest in the discovery of new drug targets [1]. However, many such targets are underexploited because their molecular functions are believed to be inaccessible to small drug-like molecules and because the lead discovery costs are estimated to be too high, anywhere from \$500,000 to \$1,000,000 for screening 1 million compounds via high-throughput screening (HTS) experiments [2,3]. Along the same line of reasoning, the discovery of drug-like molecules acting outside catalytic sites is still considered an unattainable goal by many research scientists. However, with the advent of high-throughput technologies, we are witnessing a paradigm shift in drug discovery research. Potent inhibitors of protein-protein and protein-DNA interactions can be found, but the costs usually remain outrageous [4]. Consequently, relatively few lead discovery campaigns against such targets have been performed, and even fewer studies have used *in silico* directed approaches, precluding cost-efficient discovery of active drug-like molecules against these macromolecular interactions. Although small non-peptide inhibitors

against macromolecular interactions are emerging, many cellular processes influencing the health and disease states depend on yet another kind of interaction, protein-membrane interactions. This interaction class has been largely neglected for conceptual and technical reasons, even though efficient and cost-effective protocols for the design of small inhibitors would represent a valuable new therapeutic approach for many disease indications. Indeed, with the availability of complete genome sequences for several different organisms and with structural genomics initiatives further supported by progress in homology modeling, an increasing number of potentially important therapeutic proteins that interact with the membrane surface are likely to be identified, indicating further that fast, inexpensive and accurate protocols to target this molecular mechanism have to be developed.

Despite their wide and successful applications, HTS approaches often remain very costly for hit/lead identification purposes. Therefore, *in silico* techniques should be applied wherever possible prior and complementary to HTS experiments. For instance, if the 3D structure of a membrane-binding target is known, a rational approach to identify inhibitors is to use structure-based virtual ligand screening (SB-VLS) methods [5-9]. However, it is important to note that SB-VLS methods are also expensive, because they usually require costly computer farms and several commercial software licenses [10,11]. In addition to the 3D structure of the target and a fast and accurate computational protocol, there is at least one other prerequisite for successful SB-VLS studies, the knowledge of the ligand-binding site. This is generally not known in detail for proteins interacting with the membrane surface, but binding site prediction methods can be applied to assist the identification of the most promising regions [12].

Next to the use of *in silico* experiments, appropriate *in vitro* protocols are required for the identification and validation of membrane-binding inhibitors. Traditionally, membrane-binding property assays are carried out by using different techniques, ranging from microtiter-plate based assays (ELISA-like) to direct binding experiments that make use of, for instance, surface plasmon resonance (SPR). The immobilization of a well defined phospholipid membrane surface and the stability and reproducibility of binding, along with a true quantitative and direct binding measurement character of the assay system, are of major importance for assay outcomes. We therefore suggest that the right functional assays coupled with SPR experiments appear to be an optimal combination for the identification of leads inhibiting protein-membrane interactions. Indeed, SPR is ideally suited for the identification of small molecular inhibitors (molecular mass >350 Da) in direct binding assays. Further, the use of SPR with liposomes captured to an L1-chip represents a general experimental approach to investigate inhibition of membrane binding at physiological temperature [13,14]. The method is extremely robust and

reproducible and requires only minute amounts of the test compounds and the target protein. Although the SPR throughput is modest, it perfectly complements SB-VLS, because the number of molecules to be tested after *in silico* screening computations is usually small. Indeed, in our opinion, the combination of SB-VLS with SPR screening represents a generic approach enabling cost-effective identifications and developments of compounds that affect protein-membrane interactions.

In the present study, we investigated five proteins with known 3D structure that bind transiently to the membrane and performed a theoretical prediction of druggable pockets. We found that all these proteins possess a druggable pocket within the membrane-binding region. For our proof of concept, we selected the second discoidin domain (C2 domain) of coagulation factor V (FV) as a representative domain displaying calcium-independent membrane-binding properties [15]. We used our hierarchical SB-VLS protocol [16], followed by functional and SPR-based assays, to identify and characterize membrane-binding inhibitors. We thereby demonstrate that membrane-binding inhibitors can be identified cost-effectively and that this approach can be implemented and efficiently operated.

5.3 Materials and methods

Computational procedure

Numerous membrane-binding domains from the RCSB Protein Data Bank [17] were investigated by using ICM (Molsoft, San Diego, CA). Five representative structures were selected to illustrate our study: the "tubby protein" [18] (PDB ID code 1I7E, resolution 1.95 Å), a PX domain [19] (PDB ID code 1H6H, resolution 1.70 Å), the plasma β_2 -glycoprotein I [20] (PDB ID code 1C1Z, resolution 2.87 Å), cyclooxygenase [21] (PDB ID code 1DIY, resolution 3.00 Å) and the C2 domain of coagulation FV [22] (PDB ID code 1CZT, resolution 1.87 Å). Binding pocket predictions were carried out with the ICM PocketFinder utility [23] and with Q-SiteFinder [24]. For each structure, we analyzed pockets with a high druggability index (i.e., those with a pocket of appropriate size and chemical nature to bind a drug-like molecule). The best-ranked binding cavities were found at the expected protein-membrane interface.

We used an efficient multistep procedure that is both time- and cost-effective [16,25] and studied the 500,000-molecule ChemBridge compound collection. Absorption, distribution, metabolism, and excretion/tox filtering was performed with Filter (OpenEye Scientific Software, Santa Fe, NM) and FAF-Drugs [26]. The remaining 304,000 molecules were docked by using the rigid-body docking

package FRED (OpenEye Scientific Software) into the closed (PDB ID code 1CZV) and open crystal (PDB ID code 1CZT or 1CZS) forms of the FV C2 domain. Up to 50 conformers were generated for each ligand with OMEGA (OpenEye Scientific Software). The top 60,000 unique molecules (Gaussian docking function) after FRED docking in the open and closed forms were selected for flexible docking with Surflex version 1.33 and the scoring function of version 1.31 [27]. The top 2,000 docked poses were analyzed by using PyMOL (DeLano, San Carlos, CA), ICM (Molsoft) and Cerius2 (Accelrys, San Diego, CA). The hit compounds inhibiting FV-membrane binding were also redocked and investigated with LigandFit [28] to identify consensus poses. Protonation state definitions for the FV C2 domains, open and closed forms, were computed by using the PCE server [29] and the addition of hydrogen atoms was performed accordingly with InsightII (Accelrys). The binding pocket was defined theoretically by using Surflex, Q-SiteFinder and ICM PocketFinder. All water molecules were removed from the structures before docking. The hit rate was computed as the number of actives found divided by the number of molecules screened x 100.

Proteins and reagents

Human FVa and the FVa light chain (LC) were purified from human plasma as described [30]. The recombinant FV C2 domain was obtained as follows. Phospholipid vesicles containing dioleoyl phosphatidylserine (PS) and dioleoyl phosphatidylcholine (PC) were prepared as described [30]. Small molecules were purchased from ChemBridge (San Diego, CA). All other coagulation proteins were purchased through Kordia Lab Supplies (Leiden, The Netherlands).

Cloning, expression and purification of the recombinant FV C2 domain

The pMT2-V expression vector (American Type Culture Collection no. 40515) containing the full-length human FV cDNA was used as a template for the construction of a PCR fragment containing the coding region of the hFV C2 domain (hFVC2), using the following forward and reverse primers: (FW: 5'-CTGGTCCCCCGGGGATGTTCCACACCCCTGGGTAT-3'; RV: 5'-TAGGATTGCCGTCAAGTTTGGCGCG-3'). After digestion with SmaI and SalI, the hFVC2 fragment was purified (Gel Extraction Kit, Qiagen, Valencia, CA) and subcloned into the pet43.1.a expression vector (Novagen, Madison, WI). The resulting pet43.1.a-C2 plasmid was introduced into *Escherichia coli* DH5 α cells and positive clones were identified by DNA sequencing. The recombinant plasmid was then introduced into *E. coli* [RosettaGami(DE3)pLysS; Novagen] for expression of a NusA-hFV C2 fusion protein.

Bacteria were grown at 37 °C in LB medium with 100 µg/ml carbenicillin, 30 µg/ml kanamycin, 34 µg/ml chloramphenicol and 12.5 µg/ml tetracycline. At OD₆₀₀~0.6, cultures were induced with 1 mM isopropyl β-D-thiogalactoside and incubated overnight at room temperature (RT). Cells were pelleted, resuspended in cell lysis buffer [50 mM Tris, 10 mM NaCl, 0.1% (vol/vol) TritonX-100, 2.5% (vol/vol) glycerol, 1 µM PMSF, 10 mM benzamidine, 1 mg/ml lysozyme, 0.1 units/ml benzonase (Novagen), 1 mM MgCl₂, pH 8.5], incubated for 1 h at RT, and then sonicated twice (MSE Scientific Instruments, Beun de Ronde, The Netherlands) at 16 µm amplitude for 2 min. The cell debris were removed by centrifugation and the supernatant containing the recombinant NusA-C2 fusion protein was applied to an anion exchange column (Q-Sepharose-FF, GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM Tris, 10 mM NaCl, 2.5% glycerol, pH 8.5. After extensive washing with the same buffer, bound proteins were eluted with a linear gradient from 10 to 1000 mM NaCl. Peak fractions were analyzed for C2 antigen by ELISA and SDS-PAGE. Fractions from a major peak that eluted at 0.2 mM NaCl were pooled and applied to a Ni-NTA affinity chromatography column (HisTrap FF, GE Healthcare) equilibrated in 50 mM Tris, 10 mM imidazole, 300 mM NaCl, 2.5% glycerol, pH 8.0. After extensive washing with the same buffer, bound proteins were eluted with a linear gradient from 10 to 1000 mM imidazole. Pooled fractions containing the NusA-C2 fusion protein were concentrated (Macrosep 3K centrifugal device, Pall Corporation, New York, NY) and applied to a PD10 column (GE Healthcare) to exchange the buffer to 50 mM Tris, 100 mM NaCl, pH 8.0. The fusion protein was treated overnight at RT with 250 nM thrombin to cleave off the fusion tag. After 4-fold dilution in 50 mM Tris, 10 mM NaCl, 2.5% glycerol, pH 8.0, the protein suspension was loaded onto a Mono S column (GE Healthcare) in the same buffer. After washing, the bound native (untagged) hFV C2 domain was eluted with a linear gradient from 10 to 1000 mM NaCl. Fractions containing hFV C2 were concentrated and applied to a Superdex 200 column (GE Healthcare) equilibrated in 50 mM Hepes, 100 mM NaCl, 1 mg/ml PEG, pH 7.4. Peak fractions containing hFV C2 were identified by ELISA and SDS-PAGE. Peptide mass fingerprinting using MALDI-TOF MS was performed to further verify the identity of the purified protein. The concentration of purified hFV C2 was determined by measuring the absorbance at 280 nm.

In vitro screening of the top-scoring molecules

The 509 top-scoring molecules for the open and closed conformation of the FV C2 domain structure were screened in a functional assay, essentially as described [30]. In brief, compounds were diluted from 10 mM stocks in DMSO to mixes of four compounds each at 2.5 mM per compound in DMSO. Next, an 100 µM

concentration of each compound mixture was incubated for 5 min at 37 °C with 20 pM FVa₁, 0.5 μM prothrombin, 2 mM CaCl₂ in 25 mM Hepes, 150 mM NaCl, 0.5 mg/ml ovalbumin, pH 7.5. Three minutes after the addition of phospholipid vesicles (5 μM 10:90 dioleoyl PS/dioleoyl PC, mol/mol), prothrombin activation was started by the addition of FXa to a final concentration of 0.5 nM. After 2 and 4 min, aliquots were drawn from the reaction mixture and rates of prothrombin activation were determined by using the chromogenic substrate S2238. Compound mixtures showing >95% inhibition were deconvoluted to identify the inhibitory individual compound(s) from the initial mixture. The screen resulted in nine compounds that inhibit prothrombin activation in the assay >99% at a concentration of 100 μM. The IC₅₀ values of these hit compounds were determined by titration of variable concentrations of compounds (0.1-100 μM) in the functional assay described above. None of the inhibitors was found to inhibit prothrombin activation in the absence of phospholipids and/or FVa and also did not inhibit chromogenic substrate conversion by FXa or thrombin.

Surface plasmon resonance experiments

Experiments were performed on a Biacore T100 instrument (GE Healthcare) using a L1 sensor chip and PBS with 5% DMSO as running buffer. Phospholipid vesicles (500 μM 20:80 PS:PC in 50 mM Hepes, 150 mM NaCl, pH 7.6) were injected at a flow rate of 10 μl/min for 3 min resulting in capture levels of ~5,000 RU. Captured vesicles were conditioned with a 30 second pulse of 50 mM NaOH. The resulting baseline was stable at 5,000 RU and allowed for >50 measurements. Vesicles were stripped off the chip with isopropanol:50 mM NaOH (40:60 vol/vol). A flowcell not covered with liposomes was used as reference cell. All experiments were performed at 37 °C.

Injection of 200 nM C2 or 50 nM LC for 2 min resulted in binding signals of 400 and 1,000 RU, respectively. Both binding curves exhibited saturation toward the end of the injection phase. Inhibition of membrane binding was analyzed by using 2-fold serial dilutions of compounds in a range from 0.1 to 100 μM. Samples were prepared fresh in LoBind protein tubes (Eppendorf, Hamburg, Germany) and measured within 1 h. Blank subtracted binding levels were normalized, plotted against their corresponding compound concentrations and fitted to the following equation by using GraphPad (San Diego, CA) Prism:

$$y = \frac{1}{1 + 10^{(\log c_{50} - \log c) \cdot h}}$$

with y representing the relative binding signal, c the concentration of the compound, c₅₀ the concentration at which half-maximal inhibition is reached and h the Hill coefficient.

5.4 Results and discussion

Membrane-targeting domains and theoretical prediction of druggable pockets

Structural analysis of proteins that bind to the membrane in a reversible manner via a membrane-targeting domain shows that these membrane-binding regions possess specific chemical and topological properties (e.g. a cationic patch surrounding aromatic and aliphatic clusters), whereas their overall fold varies, from all α through α/β to all β structures [31]. Proteins or protein modules of therapeutic importance with a known 3D structure that belong to this membrane-binding class include the "tubby protein" [18], the PX domain [19], the plasma β_2 -glycoprotein I [20], cyclooxygenase [21] and coagulation FV [22]. We investigated these five proteins and performed a theoretical prediction of druggable pockets [2,23,24]. We found that the membrane-binding domains of all of the above-mentioned proteins not only display patches of basic and aliphatic/aromatic residues at the membrane-binding interface but, in the same region, also possess a cavity that could be potentially targeted by drugs (Figure 5.1).

Figure 5.1. Relative orientation of the predicted druggable pockets with respect to the membrane-binding surface. Five membrane-binding domains are oriented toward the membrane surface on the basis of previously reported biophysical and mutation studies. From left to right, the membrane binding domains of coagulation FV [22], plasma β_2 -glycoprotein I [20], cyclooxygenase [21], PX domain [19] and the "tubby protein" [18] are displayed. A color gradient is applied from the N-terminal region (blue) to the C-terminal region (red). The predicted druggable pockets are shown in magenta. This figure was prepared with ICM. The proteins are scaled differently to facilitate the reading.

These cavities are likely to play a major role in membrane binding and could participate in (stereo)-specific interactions with the phospholipid head groups. Interestingly, the sizes of the predicted binding pocket envelopes for our five

membrane-binding proteins within the membrane-binding region range from 200 to 600 Å³, values commonly computed for proteins co-crystallized with small drug-like ligands [32]. The pertinence of the pocket prediction algorithms used here has been reported [12,32,33] and is further validated in the case of FV. The C2 domain has been co-crystallized with the phenyl mercury molecule (a covalent bond is formed; PDB ID code 1CZS, open crystal form) [22] and the region surrounding this small compound has been proposed to form a key binding groove for specific interaction with acidic phospholipids. This site was readily predicted with both the ICM PocketFinder utility [23] and with Q-SiteFinder [24].

Combined SB-VLS-SPR screen to identify FV-membrane interaction inhibitors

The C2 domain of coagulation factor V

As proof of concept, we selected the C2 domain of coagulation FV as a representative protein domain involved in calcium-independent membrane binding. This domain is closely related, structurally and functionally, to the C2 domain of factor VIII (FVIII, a protein involved in hemophilia A) [34]. Although this domain is named C2, this region of FV or FVIII is structurally unrelated to the so-called C2 domain of for instance cytosolic phospholipase A2. FV and FVIII are homologous molecules sharing the domain architecture A1-A2-B-A3-C1-C2. The binding of coagulation FV (via its C2 domain) to activated membranes of circulating platelets is essential for its functions in coagulation. Upon activation by thrombin, activated FV (FVa) acts as a cofactor of activated factor X (FXa) in the prothrombinase complex, which converts prothrombin to thrombin on an appropriate phospholipid surface [15,35]. Excess thrombin production can lead to thrombotic events, suggesting that small inhibitors of FV-membrane interactions could be the starting point for the development of a novel class of antithrombotic drugs. The FV C2 domain comprises a distorted jelly-roll β -barrel motif consisting of eight major antiparallel strands arranged in two β -sheets. Several loops, presenting hydrophobic/aromatic residues, facilitate immersion into the membrane. Specific interactions with phosphatidylserine (PS) head groups are expected to occur in the groove enclosed by these membrane-binding loops [22]. This zone is surrounded by several basic residues, which facilitate the formation of an encounter complex with the negatively charged membrane phosphate groups via electrostatic steering, and anchor the module into the membrane bilayer [22,36-38]. The FV membrane-binding loops can assume an open or closed conformation [22], and it is believed (but not proven) that the closed form has low membrane affinity, whereas the open form is suitable for membrane interaction. In the open

crystal form, the exposed indoles of Trp²⁰⁶³ and Trp²⁰⁶⁴ contribute to the immersion of the module by interaction with the apolar membrane core (see also Figure 5.4A, page 130). The nearby-predicted druggable pocket is lined with polar, basic, aliphatic and aromatic side chains (Ser²¹⁸³, Gln²⁰⁸⁵, Lys²⁰⁶⁰, Trp²⁰⁶⁸, Met²¹²⁰, Ser²¹¹⁵, Leu²¹¹⁶ and Arg²⁰⁸⁰) which are ideally arranged for interactions with lipid head groups.

SB-VLS and SPR experiments on FV

SB-VLS methods help to prioritize large compound collections before experimental testing and are complementary and often superior to massive experimental HTS [39]. However, these computational approaches also suffer from limitations [9]. We applied our validated multistep SB-VLS protocol [16,25] to both the open and closed C2 crystal forms and selected the best 509 molecules for each receptor form (i.e., we decided to screen ~1,000 compounds in total). These 1,018 molecules were tested in groups of four compounds (each at a final concentration of 100 μ M) for their ability to inhibit prothrombin activation. Compound mixtures showing >95% inhibition of prothrombin activation were deconvoluted to determine the activity of the individual compounds. Nine molecules were identified that inhibited the prothrombinase assay by >99% at a concentration of 100 μ M (Table 5.1).

Compound	Chembridge ID	IC ₅₀ Ptase, μ M	IC ₅₀ FVa LC, μ M	IC ₅₀ FVa C2, μ M	Surflex ranking	Comments
001C07	7364519	18.6 $\pm$ 1.89	2.50 $\pm$ 0.18	3.51 $\pm$ 0.57	Open form, position 502	FV-specific
007H10	6305867	62.9 $\pm$ 2.56	4.80 $\pm$ 0.89	5.53 $\pm$ 1.04	Open form, position 73	Acts on FV and FVIII
006H08	6043266	9.19 $\pm$ 0.94	8.56 $\pm$ 1.14	6.71 $\pm$ 1.66	Open form, position 305	FV-specific
001D08	5843746	8.95 $\pm$ 1.02	8.55 $\pm$ 0.95	7.40 $\pm$ 1.05	Open form, position 187	Acts on FV and FVIII
005B10	7688319	32.4 $\pm$ 5.1	15.71 $\pm$ 2.26	14.00 $\pm$ 1.88	Open form, position 476	Acts on FV and FVIII
007D08	7971347	40.57 $\pm$ 2.42	14.07 $\pm$ 2.54	16.40 $\pm$ 1.38	Open form, position 86	FV-specific
008A07	6446853	38.05 $\pm$ 3.07	14.07 $\pm$ 2.54	21.92 $\pm$ 2.43	Closed form, position 13	Acts on FV and FVIII
001B03	5169083	3.8 $\pm$ 0.61	ND	ND	Open form, position 84	Binds to phospholipids
010G06	5870804	1.81 $\pm$ 0.17	ND	ND	Closed form, position 115	Binds to phospholipids

Table 5.1. IC₅₀ values for the identified hits. The IC₅₀ values represent the mean of two independent experiments with their corresponding 95% confidence intervals.

The IC₅₀ of these molecules was determined by varying their concentrations in the functional assay (Figure 5.2A). Control experiments showed that none of the nine hit compounds inhibited chromogenic substrate conversion by FXa or thrombin. However, the two most active small molecules (001B03 and 010G06; Table 5.1) also

inhibited prothrombin activation in the absence of FVa and/or phospholipids, indicating that the inhibitory effect of these compounds was not due to the impediment of the FVa-lipid interaction. Therefore, both molecules were excluded from further evaluation. To confirm that the remaining seven compounds inhibited association between the FV C2 domain and the membrane, we performed SPR experiments by using the isolated FVa light chain (FVa LC; this part of the molecule contains the A3 domain and two discoidin domains) and a recombinant FV C2 domain. The compounds were tested for their ability to inhibit the binding of FVa LC or FV C2 to an immobilized phospholipid bilayer containing 20:80 PS:PC (Figure 5.2B and C, respectively, and Table 5.1). We further verified that compounds failing to inhibit prothrombin activation (Figure 5.2B and C) were unable to impede membrane binding of the FVa LC and the FV C2 domain.

Figure 5.2. Titration curves for FV membrane-binding inhibitors identified by our multistep VLS procedure and *in vitro* screening. IC₅₀ values of hit compounds were determined by using a functional assay (A) or SPR analysis by using FVa LC (B) or FV C2 domain (C), as described in *Materials and Methods*. ▲, 010G06; ●, 001B03; △, 001C07; ▼, 007H10; ○, 006H08; □, 001D08; ▽, 005B10; ◇, 007D08; ■, 008A07; ◆, 007A09 (Neg. control).

The direct interaction of the hit compounds with FVa LC or the FV C2 domain was additionally confirmed by SPR analysis (data not shown). SPR control experiments were performed to show that none of the seven hit compounds significantly bound to the membrane (typically <25 resonance units (RU)), excluding the possibility that penetration and subsequent disruption/distortion of the lipid bilayer by these molecules were responsible for their inhibitory effect. Moreover, none of the seven hit compounds inhibited the calcium-dependent membrane-binding activity of the human vitamin K-dependent proteins, prothrombin and FXa. Furthermore, four of the seven hit compounds also inhibited membrane binding of the structurally related blood coagulation FVIII (Table 5.1), two of which (molecules 5B10 and 8A07) presented with an IC₅₀ of 7.8 and 8.9 μM, respectively, for the inhibition of FVIII-membrane interaction, as measured by SPR.

Although the small molecules described here show reasonable inhibition of FV C2 membrane binding and FVa cofactor activity in the activation of prothrombin by FXa, the compounds in their current form, at 100 μ M final concentration, do not inhibit FVa procoagulant activity in a plasma-based assay system (data not shown). A likely explanation for this observation is that the active concentration of a given compound is markedly decreased when bound to plasma proteins (particularly albumin, α_1 -acid glycoprotein and lipoproteins). To verify this hypothesis, we tested the most potent compound (001C07) at a concentration of 50 μ M in the functional assay in the presence of increasing albumin concentrations (0.5–80 mg/ml). As albumin concentration increases, the inhibitory activity of the compound gradually decreases to ~25% at an albumin concentration of 40 mg/ml, which approaches the plasma albumin concentration (Figure 5.3). The binding of hit compounds to albumin was further confirmed by SPR analysis (data not shown). Optimization procedures could be carried out to reduce affinity for albumin as shown for some cyclooxygenase-2-inhibitors [40] but are beyond the scope of the present investigation.

Figure 5.3. Effect of albumin on the inhibitory activity of compound 001C07. The inhibitory activity of compound 001C07 (50 μ M) on FVa cofactor activity in the activation of prothrombin by FXa was measured in the presence of varying human albumin concentrations (0.5–80 mg/ml). Error bars represent the standard deviation of the mean of three independent experiments.

In silico structural analysis of the docked poses revealed that the active molecules were all partially buried in the PS-binding groove. Critical interactions with residues located in this cavity were identified. A consensus pose obtained by Surflex and LigandFit is shown in figure 5.4. In general, the compounds display favorable contacts with the side chains of FV C2 residues Gln²⁰⁸⁵, Lys²⁰⁶⁰, Trp²⁰⁶⁸, Ser²¹¹⁵,

Leu²¹¹⁶, Trp²⁰⁶³, Trp²⁰⁶⁴, Arg²⁰⁸⁰ or Lys²⁰⁶¹. The compounds found by using the closed crystal form could also be docked in the open form, although with a different orientation. Further investigation of the precise interaction would require site-directed mutagenesis and/or NMR or X-ray crystallography experiments.

Figure 5.4. Membrane-binding region of the FV C2 domain. (A) Ribbon plot of the C2 domain open form highlighting the membrane binding loops and displaying the amino acid side chains of the key membrane-binding players (*Left*). (B) Proposed docked pose for compound 001C07 (*Upper Right*, same orientation as in A). The small molecule after Surflex and LigandFit consensus docking occupies the phospholipids binding groove thereby impeding membrane interaction. Hydrophobic-aromatic interactions occur with W2063, W2064, and L2116 region. Hydrogen bonds between the side chain of K2060, N2089, and Q2182 and the compound carboxylate group are predicted, whereas the fluorobenzene group shows a favorable interaction with the side chains of either W2064 or R2080. (C) The domain was rotated to have a view from the membrane side (*Lower Right*). This figure was prepared with PyMol.

SB-VLS-SPR generic platform to design protein-membrane interaction inhibitors

To the best of our knowledge, lead discovery campaigns against membrane-targeting domains that include virtual ligand screening have not been carried out, and only one study has reported inhibition of FVIII-membrane interaction [41]. In that report, a traditional HTS lead discovery approach was applied, leading to the initial identification of 10 best hits disrupting FVIII C2 domain-membrane interactions after screening 10,000 molecules from the ChemBridge collection. These 10 hits were grouped into three categories: weak inhibitors (five compounds with $IC_{50} > 20 \mu M$), moderate inhibitors (two compounds with IC_{50} between 10 and 20 μM) and strong inhibitors (three compounds with $IC_{50} < 10 \mu M$). The hit rate was

0.1, whereas we achieved a hit rate of 1.2 when using the FV C2 domain open crystal form (six inhibitors found after screening 500 molecules, four compounds with $IC_{50} < 10 \mu M$, and two compounds with $IC_{50} < 20 \mu M$). We obtained a hit rate of 0.7 when testing the 1,018 molecules (i.e., on the open and closed crystal structures). This value is still much better than when using HTS alone as in the case of FVIII.

After completion of our study, control *in silico* experiments were performed on the FVIII C2 domain to further validate our approach. We retrieved the X-ray structure of the FVIII C2 domain and applied the same VLS protocol as was used for FV, including the FVIII inhibitors of ref. 35 in our 300,000 ChemBridge absorption, distribution, metabolism and excretion/tox filtered compound collection. Because the FVIII loops important for membrane binding are in an open conformation [34], we performed rigid-body docking and flexible docking on this X-ray structure. We identified five compounds out of the 10 found by HTS in the top 1,000 Surflex list. In addition, we also found three molecules identified in the present study that cross-react with FVIII (Table 5.1). Thus, we obtained an overall hit rate for FVIII of at least 0.8. These results indicate that for FVIII, our strategy is more efficient than the HTS-only approach. In fact, typical hit rates from SB-VLS are usually in the range of 1-5% (and more), depending on the knowledge about the binding pocket, its overall shape and its physicochemical nature, compared with common hit rates from HTS of $\leq 0.1\%$ [42]. Higher hit rates than the ones calculated here using SB-VLS approaches can be obtained when some active molecules are already known (and when the binding pocket is well defined), but in such situations, the use of SB-VLS approaches in combination with ligand-based methods is highly recommended [43]. In the present study, we initially ignored the data on FVIII to evaluate the strengths and weaknesses of SB-VLS approaches. The advantages of using hierarchical SB-VLS protocols with rigid-body docking before flexible ligand docking is that the computations run significantly faster (thus can be carried out on only one workstation in ~2 weeks) than full flexible ligand docking of the entire compound collection (~8 weeks on the same single workstation) while performing better or equally well [10,16,25]. The hierarchical approach used here is also very interesting when several 3D structures of the receptor are available, because again, the computations can be performed on a few workstations in parallel.

Along with appropriate *in silico* protocols, the use of suitable experimental procedures is crucial to identify protein-protein or membrane-binding inhibitors. For example, when studying protein-protein interactions, ligand immobilization and regeneration are frequently a central problem. Whenever possible, capture assays are preferred to exclude ligand heterogeneity; however, proper capture reagents are not generally available. Protein-membrane interactions differ in this respect, because both native and artificial liposomes [13,14] can be efficiently

captured onto the surface of L1 sensorchips. The analyte (i.e., the membrane-binding protein) is used in its unmodified native form. Chemical modification and immobilization can obstruct the binding site, restrict conformational flexibility, and thus impair membrane binding. However, SPR enables real-time kinetics resolution even for fast and/or weak interactions. Highly reproducible binding data are generated because of precise control of experimental parameters. Captured liposomes are not affected by 5% DMSO, a property that is extremely useful in the early phase of drug development where the binding affinities of the compounds are low, and solubility is often a problem. Inhibition, but also stabilization of membrane binding due to the interaction of the analyte with a test compound, can be analyzed quantitatively. The assay is thus truly generic, because no specific detection reagents or biological assays are required, the only requirement being the membrane binding of the analyte. Consumption of the test compounds is minimal, and consumption of analyte is very favorable. For example, 1 mg of the C2 domain or the LC is sufficient to screen ~10,000 test compounds. Importantly, the lipid-binding properties of the test compound can be determined at the same time. Moreover, the binding of a drug to human serum albumin can be analyzed by SPR-based experiments [13,44]. These assays can thus also be used to generate early absorption, distribution, metabolism and excretion data and to confirm the specificity and mode of action of the test compounds. A major disadvantage of the SPR assays is its modest throughput of ~100 samples per day. However, this limitation can be elegantly overcome by combination with virtual screening approaches.

5.5 Conclusion

Experimental screening of difficult targets is time-consuming and cost-intensive. Therapeutic targets that bind transiently to the membrane have been neglected and remain among the most difficult challenges in contemporary drug discovery. The nature of protein-membrane interaction is not fully understood, success stories are extremely rare, and generic approaches are missing. Protein-membrane interactions are, however, crucial in many biological processes because they localize key molecular factors on specific cell surfaces. In the present study, we combined SB-VLS experiments with *in vitro* assays and found seven active molecules that were able to disrupt FV membrane-binding activity in a timely and cost-effective fashion. These molecules are promising leads for the development of *in vitro* tools for haemostasis research and for the design of a novel class of anticoagulant drugs. It is remarkable that small molecules can impede membrane binding, because the binding interface between the membrane-binding domain and the phospholipids is relatively large, with immersion of the proteins several

angstroms deep into the membrane bilayers. However, just as in the case of protein-protein interaction [45], the bulk of the binding energy appears to derive from contacts with just a small number of amino acid residues. We conclude that hierarchical virtual screening approaches in combination with SPR technology are an efficient and generally applicable approach for routinely identifying membrane-binding inhibitors. This strategy could therefore be applied to other relevant membrane-binding proteins in search of the next generation of therapeutics.

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Chapter VI:

Identification of surface epitopes of human coagulation factor Va that are important for interaction with activated protein C and heparin

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6.1 Summary

Inactivation of factor Va (FVa) by activated protein C (APC) is a key reaction in the down-regulation of thrombin formation. FVa inactivation by APC is correlated with a loss of FXa cofactor activity as a result of three proteolytic cleavages in the FVa heavy chain at Arg³⁰⁶, Arg⁵⁰⁶ and Arg⁶⁷⁹. Recently, we have shown that heparin specifically inhibits the APC-mediated cleavage at Arg⁵⁰⁶ and stimulates cleavage at Arg³⁰⁶. Three-dimensional molecular models of APC docked at the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites in FVa have identified several FVa amino acids that may be important for FVa inactivation by APC in the absence and presence of heparin. Mutagenesis of Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ to Ala, resulted in an increased inactivation rate by APC at Arg³⁰⁶, which indicates the importance of these residues in the FVa-APC interaction. No heparin-mediated stimulation of Arg³⁰⁶ cleavage was observed for these mutants and stimulation by protein S was similar to that of wild type FVa. With this, we have now demonstrated that a cluster of basic residues in FVa comprising Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ are required for the heparin-mediated stimulation of cleavage at Arg³⁰⁶ by APC. Furthermore, mutations that were introduced near the Arg⁵⁰⁶ cleavage site had a significant but modest effect on the rate of APC-catalyzed FVa inactivation, suggesting an extended interaction surface between the FVa Arg⁵⁰⁶ site and APC.

6.2 Introduction

The protein C pathway provides a major anticoagulant mechanism for the regulation of haemostasis by nullification of the procoagulant activities of FVIIIa and FVa, the cofactors in the tenase and prothrombinase complexes, respectively. Protein C is a vitamin K-dependent protein composed of a heavy and a light chain that are connected through a single disulfide bridge [1]. The light chain is composed of an N-terminal γ -carboxyglutamic acid (gla)-rich domain and two epidermal growth factor-like domains. The heavy chain contains a short (12 amino acid) signal peptide and the serine protease (SP, peptidase) domain.

Previously, we and others have demonstrated that three surface loops (37, 70 and 148, chymotrypsinogen numbering) in the SP domain of APC form an extended FVa binding site [2-4]. Furthermore, a cluster of basic residues located on loops 37, 60 and 70 form a positively charged exosite on the surface of APC important for its interaction with thrombomodulin, protein C inhibitor, α_1 -antitrypsin and heparin [2,5-7].

APC is able to cleave FVa, the non-enzymatic cofactor of FXa in the prothrombinase complex, at least at five peptide bonds, of which those at Arg³⁰⁶ and Arg⁵⁰⁶ are considered most important for the inactivation of FVa [8-10]. FVa is

a multidomain protein which consists of a 71/74 kDa light chain (A3, C1 and C2 domains) and a 105 kDa heavy chain (A1 and A2 domains) that are non-covalently associated. All APC cleavage sites are located in the FVa heavy chain [8]. In the presence of negatively charged phospholipids, the cleavages at Arg⁵⁰⁶ and Arg³⁰⁶ are primarily responsible for inactivation of FVa [11]. Cleavage of Arg⁵⁰⁶ is kinetically favoured over the Arg³⁰⁶ cleavage (up to 20-fold rate difference) and results in a FVa inactivation intermediate with partial cofactor activity, whereas cleavage at Arg³⁰⁶ fully inactivates FVa [11,12]. In the presence of its cofactor protein S however, APC cleaves the Arg³⁰⁶ peptide bond with a rate that is approximately similar to that of cleavage at Arg⁵⁰⁶ [13].

Heparin is a naturally occurring sulphated glycosaminoglycan that can be found in several organs and tissues of the human body, notably on the surface of endothelial cells that form the inner lining of our vasculature. Unfractionated heparin (UFH) and low molecular weight heparin (LMWH) are widely used as effective pharmacotherapeutics for the prevention and treatment of thrombosis. The anticoagulant functions of heparin are diverse, and include stimulation of the inhibition of various coagulation factors (thrombin, FIXa, FXa, FXIa and FXIIa) by antithrombin as well as inhibition of platelet functions [14-17]. Apart from its anticoagulant functions, heparin also has anti-inflammatory properties as it modulates the production and release of pro-inflammatory factors [18].

Beside its anticoagulant and anti-inflammatory functions, heparin also regulates APC activity by stimulating the inhibition of APC by the serpin protein C inhibitor (PCI) [2,19,20]. In addition, it has been shown that heparin alters the pathway of APC-catalyzed FVa inactivation by inhibition of the Arg⁵⁰⁶ cleavage and stimulation of the Arg³⁰⁶ cleavage in FVa [9]. This latter inhibition has been suggested to contribute to the less favourable effects of APC in heparin-cotreated sepsis patients, as were studied in the PROWESS trial [18,21]. Three-dimensional models of APC docked at the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites in FVa suggest that heparin prevents optimal docking of APC at Arg⁵⁰⁶ and stimulates cleavage at Arg³⁰⁶ by bridging electropositive regions in FVa and APC [9].

In this study, we have used site-directed mutagenesis to show that FVa residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ are important for the heparin-mediated stimulation of FVa cleavage at Arg³⁰⁶ by APC. In addition, we observed minor but significant effects of mutations that were introduced near the Arg⁵⁰⁶ cleavage site on the APC-catalyzed FVa inactivation. The most prominent effect was observed for the FVa mutants with mutations in an acidic cluster spanning from residues 659 to 663, which is likely part of an extended FVa:APC binding interface.

6.3 Materials and methods

Materials

Serum free cell culture media (Optimem Glutamax) were from Gibco BRL. Hepes, sodium chloride, calcium chloride and bovine serum albumin were from Sigma (St. Louis, MO, USA). Unfractionated heparin (UFH) was obtained from Leo (Ballerup, Denmark); 1 IU/ml UFH contains ~5.7 µg/ml UFH/ml [22]. The chromogenic substrates S2238 and S2366 were supplied by Chromogenix (Milan, Italy). Phospholipid vesicles were prepared as described [23]. Synthetic lipids used were 1,2 dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2 dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and these were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA).

Proteins

Human α -thrombin and protein S were from ERL (Swansea, United Kingdom). Hirudin was purchased from Kordia Laboratory Supplies (Leiden, The Netherlands). Bovine FX was purified according to Fujikawa *et al.* [24]. Human FXa was prepared from FX after activation by RVV-X [24,25]. Human prothrombin was purified according to the method of Di Scipio *et al.* [26]. Monoclonal antibody 3B1 directed against the heavy chain of human FV was a kind gift from Prof. B. N. Bouma. The anti-human protein C polyclonal antibodies Dako A0370 and Dako P0374 were obtained from Dako Company (Germany).

Site-directed mutagenesis

FV cDNA constructs containing either combined mutations at the Arg⁵⁰⁶ and Arg⁶⁷⁹ APC cleavage sites (FV R506Q/R679Q, abbreviated as RQQ) or at the Arg³⁰⁶ and Arg⁶⁷⁹ (FV R306Q/R679Q, abbreviated as QRQ) were prepared as described, using the pMT2-V vector as template [27]. Amino acid substitutions (Table 6.1 and 6.2) were introduced near the Arg³⁰⁶ or Arg⁵⁰⁶ cleavage sites by PCR-based site-directed mutagenesis of the expression vectors encoding the RQQ and QRQ mutants by use of the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, USA). For reasons of clarity, recombinant FV molecules given in Table 6.1 and 6.2 are identified only by their mutations other than the R306Q, R506Q or R679Q that were present in the template that was used in their construction.

Transient expression of recombinant FV variants

Plasmid DNA containing the various FV cDNA constructs was transfected into COS-1 cells using the DEAE-dextran method [28]. As a control, a pMT2-V vector of which the FV cDNA had been excised, was used to transfect cells. Proteins were collected in serum-free medium (Optimem Glutamax) 72 h after transfection. Cell debris was removed by centrifugation and the media containing the recombinant proteins were concentrated ~10-fold using Vivaspin concentrators having a molecular weight cut-off of 100,000 (Vivascience, Hannover, Germany). Expression levels of all FV variants were determined using a FVa activity assay and FV antigen was determined with the FV-specific ELISA described below.

FV ELISA

FV expression levels were determined by a FV-specific enzyme-linked immunosorbent assay, essentially as described [29]. Briefly, microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were coated overnight with the monoclonal antibody 3B1. The plates were blocked for 1 h at room temperature with 50 mM Hepes pH 7.5, 150 mM NaCl, 1% bovine serum albumin (Hepes-buffered saline, HBS-BSA). A standard curve was prepared using different dilutions of pooled normal citrated plasma, assuming a plasma FV concentration of 21.3 nM [30]. Standard and samples (diluted in HBS-BSA) were incubated for 2 h at room temperature and the plates were then washed three times with 0.05% Tween-20 in HBS. The biotinylated monoclonal antibody HV1 (0.1 µg/ml), was used as secondary antibody. After 2h, avidin-horseradish peroxidase (Strep ABC complex/HRP from DAKO, Denmark) was added for 30 min and plates were developed for 10 min with peroxidase substrate solution (TMB substrate kit, Pierce). The reaction was stopped and the absorbance was measured at 490 nm.

To exclude the possibility that the cell culture medium interfered with the ELISA assay, plasma-purified FV and plasma were diluted in mock medium or in buffer. No difference was detected between these diluted samples, indicating that the cell culture medium did not interfere with the FV quantitation.

FVa assay

Expression levels of the various FV variants were also determined with a functional assay. Recombinant FV proteins were incubated with 2 nM α -thrombin for 20 min at 37 °C. FVa cofactor activity was determined by measuring the rate of FXa-catalyzed prothrombin activation as described [11]. Typically, reaction conditions for the prothrombinase assay were: 0-20 pM FVa, 5 nM FXa, 0.5 µM prothrombin,

40 μ M phospholipid vesicles (10% DOPS/ 90% DOPC; mol/mol) and 0.5 mg/ml ovalbumin in 25 mM Hepes pH 7.5, 150 mM NaCl and 3 mM CaCl_2 . Thrombin generation was allowed to continue for 2 min at 37 °C before the reaction was stopped with ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 [31]. The molar factor Va concentration in the assay mixture was calculated as described [32].

In control experiments, equal antigenic amounts of plasma-purified FVa and recombinant wild type FVa in conditioned medium or in buffer gave the same rates of prothrombin activation, indicating that conditioned medium did not interfere with FVa functional activity. Moreover, no FV(a) was detectable in the conditioned medium, as judged by both ELISA and functional activity assay.

Expression and purification of recombinant human protein C variant K37S/K38Q/K39Q/K62N/K63D

Recombinant human protein C variant K37S/K38Q/K39Q/K62N/K63D was expressed after PCR-based site-directed mutagenesis of the eukaryotic expression vector pGT-hyg (Eli Lilly). The recombinant protein was expressed in 293 cells (CRL-1573; ATCC), purified and activated as described [2,6]. Protein C was quantified by measurement of absorbance at 280 nm and by a chromogenic assay as described [2].

APC-catalyzed inactivation of FVa in the absence and presence of UFH and protein S

Time courses of FVa inactivation by APC were determined in the absence and presence of UFH and protein S by following the loss of FXa cofactor activity of FVa in the prothrombinase complex as a function of time. To this end, 20 pM of the activated FV variants was incubated with 40 μ M phospholipid vesicles (10% DOPS/ 90% DOPC; mol/mol) at 37 °C in the absence or presence of 25 U/ml UFH or 150 nM protein S in 25 mM Hepes pH 7.5, 150 mM NaCl and 3 mM CaCl_2 . Next, three aliquots of 115 μ l were taken from the reaction mixture to determine the FVa activity in the absence of APC in triplicate. Then, APC was added to the reaction mixture and the mixture was subsequently separated into several 115 μ l aliquots, one for each time point measured. For the QRQ and RQQ variants, final APC concentrations of 0.085 nM and 0.75 nM were used, respectively. At different time points after the addition of APC, the residual FVa activity was measured in the prothrombinase assay described above, with minor modifications. Prewarmed FXa (0.5 nM for the QRQ variants; 5 nM for the RQQ variants, unless otherwise

indicated) and prothrombin (1 μ M) were added to the FVa aliquots and thrombin generation was allowed to continue during 2 min after which the reaction was stopped by >40-fold dilution in ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 [31]. We verified that the formation of thrombin was linearly dependent on the FVa concentration present in the prothrombinase mixture and that heparin (25 U/ml) did not interfere with the FVa assay. The stability of the various FVa variants was assessed by following the loss of FVa activity as a function of time in a reaction mixture to which no APC had been added.

Analysis of kinetic data

We have previously shown that the loss of FVa cofactor activity during the APC-catalyzed inactivation of membrane-bound wild type FVa can proceed via two pathways [11]:

Initial cleavage at Arg⁵⁰⁶ (with a corresponding rate constant of k_{506}) results in a reaction intermediate (FVa_{int}) with partial cofactor activity, which must be further cleaved at Arg³⁰⁶ (k_{306}) to completely abolish FVa cofactor activity (pathway I). Alternatively, initial cleavage at Arg³⁰⁶ (k'_{306}) results in complete loss of FVa cofactor activity (pathway II).

For calculation of the pseudo first-order rate constant for cleavage at Arg⁵⁰⁶ in the FVa mutants, time curves obtained for the FVa QRQ variants were fit to the following equation (equation 1):

$$Va_t = Va_0 \cdot e^{-(k_{506} + k_1) \cdot t} + A \cdot Va_0 \cdot \frac{k_{506} \cdot e^{-(k_2 \cdot t)}}{(k_{506} + k_1 - k_2)} \cdot (1 - e^{-(k_{506} + k_1 - k_2) \cdot t})$$

in which Va_t is the FVa cofactor activity determined at time t , Va_0 is the cofactor activity before APC is added, A is the fraction of remaining procoagulant cofactor activity of the FVa variant cleaved at position 506, k_{506} is the observed pseudo first-order rate constant for cleavage at position 506 and k_1 and k_2 are the first-order rate constants of spontaneous inactivation of intact FVa and of the FVa intermediate, respectively. The value for k_1 was determined by following the loss of

FVa activity as a function of time in a reaction mixture that did not contain APC. The stability of the FVa intermediate (k_2) was assessed in a separate experiment in which time courses of APC-catalyzed inactivation were monitored as follows. Wild type recombinant FVa was inactivated with APC and at the beginning of the observed second slow phase, the reaction volume was divided into two portions, each transferred into a new tube. To one reaction mixture, anti-APC polyclonal antibodies were added that completely block APC-catalyzed FVa inactivation and to the other an equal volume of buffer was added. Loss of FVa was monitored in both samples and the resulting time courses of FVa inactivation were compared and found to be superimposable. This observation further rules out the possibility that APC-mediated cleavages at alternative cleavage sites contribute to the observed loss of FVa activity. The time courses were fit to a biphasic exponential from which the value for k_2 was derived.

Time curves of inactivation of the FVa RQQ variants were fit to a single exponential that describes the loss of FVa cofactor activity resulting from a single cleavage at Arg³⁰⁶,

$$Va_t = Va_0 \cdot e^{-(k'_{306} + k_1) \cdot t} \quad (\text{equation 2})$$

in which Va_t and Va_0 are the FVa cofactor activities determined at time t and before APC addition respectively, k'_{306} is the observed pseudo first-order rate constant of cleavage at position 306 and k_1 is the first-order rate constant of spontaneous inactivation of intact FVa.

6.4 Results

Expression of FV RQQ and FV QRQ variants

Three-dimensional models of APC/heparin complexes and of APC alone docked at either the Arg³⁰⁶ and the Arg⁵⁰⁶ cleavage sites in FVa provided a molecular basis for the experimentally observed heparin-mediated inhibition of Arg⁵⁰⁶ cleavage and stimulation of the Arg³⁰⁶ cleavage in FVa by APC [9]. On the basis of these molecular models, several charged FVa residues located near the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites were identified that may play a role in the interactions between FVa, APC and heparin. To investigate whether these FVa residues are indeed important for the recognition of the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites by APC in the absence and/or presence of heparin, we have constructed a panel of FV mutants in which the charge of these surface residues was neutralized or reversed (Table 6.1 and 6.2). To facilitate the kinetic analysis of APC-mediated cleavage at these sites, we

have used FV variants that have only the Arg³⁰⁶ or the Arg⁵⁰⁶ cleavage site available for proteolysis by APC (Figure 6.1).

Figure 6.1. Location of the mutations that were introduced near the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage sites in the FVa variants. FVa is shown in grey ribbon and the side chains of mutated residues are shown as a colored ball-and-stick representation. The Arg³⁰⁶ and Arg⁵⁰⁶ APC cleavage sites are colored in red. Residues that were mutated near (<25Å) the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage site (see also Table 6.1 and 6.2) are shown in green and magenta, respectively. The coordinate files of the FVa model were provided by Dr. B. Villoutreix. The putative C-terminal region of the FVa heavy chain, for which no template structure is available, is indicated as a cylinder, positioned as was proposed by Orban and co-workers [42]. The figure was created with the YASARA/WhatIf Twinset program.

After transfection of COS-1 cells, conditioned media containing the FV mutants were collected, centrifuged, concentrated and stored at -80 °C. The activity to antigen ratio of the various FV variants was determined using a quantitative FV ELISA and a functional assay (Table 6.1 and 6.2). Expression levels for the different recombinant FV forms, as determined by FV ELISA, were all approximately equal (~100 pM), indicating that no major structural abnormalities were introduced by mutation of the wild type amino acid sequence. Remarkably, the activity to antigen ratio of FV mutants D577N-D578N and D513N-D577N-D578N was ~50-fold lower than that of FV QRQ. Both FV variants express a reduced affinity for FXa as a result of the replacement of Asp⁵⁷⁷ and Asp⁵⁷⁸ for an asparagine residue [33].

APC-catalyzed inactivation of QRQ FVa variants

The importance of the amino acids listed in Table 6.1 for APC-catalyzed cleavage at Arg⁵⁰⁶ were evaluated by following time courses of inactivation of FVa variants that can only be cleaved by APC at this single peptide bond. In these experiments, loss of FVa cofactor activity, as a result of cleavage at Arg⁵⁰⁶ by APC, was followed in a prothrombinase assay. Single cleavage at Arg⁵⁰⁶ in FVa results in the formation of FVa_{int} which exhibits ~40% partial FVa cofactor activity [11] at high FXa and negligible cofactor activity at low FXa concentrations. We have performed the FVa assay at a lower FXa concentration (0.5 nM) than routinely used (5 nM FXa) as a result of which the FVa_{int} activity is <20% of initial FVa activity.

FV Mutant	FV Mutation	Activity/ antigen	wtAPC k ₅₀₆ (M ⁻¹ s ⁻¹)	APC mutant k ₅₀₆ (M ⁻¹ s ⁻¹)
QRQ	R306Q/R679Q	0.82	1	1
1	D513N	1.09	1.83	7.71
2	D577N-D578N	0.01	0.86	1.25
3	D513N-D577N-D578N	0.02	1.21	1.08
4	E323Q	1.25	0.99	0.78
5	E372Q-D373N-E374Q	1.19	0.63	0.88
6	E323Q-E372Q-D373N-E374Q	0.74	0.68	0.90
7	D659N-D660N	0.56	0.50	1.47
8	D661N-E662Q-D663N	0.87	0.52	1.52
9	D659N-D660N-D661N-E662Q-D663N	1.20	0.49	1.57

Table 6.1. Activity to antigen ratio and relative rate constants (k₅₀₆) for the APC-catalyzed inactivation of FV QRQ variants by wild type APC and mutant APC (K37S/K38Q/K39Q/K62N/K63D). FV concentrations were determined by an ELISA and a functional assay as described in "Materials and methods". As a parameter of specific activity, the ratio of FV activity to antigen was calculated. Values are the average of 3 experiments and standard deviations were <5% for all of the FV variants. Rate constants (M⁻¹s⁻¹) for inactivation of FVa variants that can only be cleaved at Arg⁵⁰⁶ by recombinant wild type APC (0.085 nM) or mutant APC (0.56 nM) were obtained by fitting time courses of inactivation to an integral time course equation as described in "Materials and methods". Rate constants given are means from 2 experiments and standard deviations were <10% for all FVa variants. All rate constants are expressed relative to the rate constants for cleavage of R306Q/R679Q (QRQ), which were 6.25 x 10⁷ M⁻¹s⁻¹ and 4.98 x 10⁶ M⁻¹s⁻¹ for wild type and mutant APC respectively.

Note that since FV mutants D577N-D578N and D513N-D577N-D578N had a low activity to antigen ratio, their activity loss could not be followed under the same experimental conditions as used for the other FVa QRQ variants. To this end, final concentrations of 100 pM FVa and 5 nM FXa were used in experiments in which these mutants were investigated.

We verified that under the experimental conditions used, FVa inactivations were determined under pseudo first-order rate conditions in which the initial rate of FVa inactivation was linearly proportional to the concentration of FVa and APC.

Apparent second-order rate constants for APC-mediated cleavage at Arg⁵⁰⁶ were derived from fitting the obtained time courses to equation 1 using non-linear least squares regression analysis. FVa mutant QRQ, which can only be cleaved at Arg⁵⁰⁶ and does not contain any other amino acid substitutions, was inactivated at a rate of $6.25 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Table 6.1).

For the FVa QRQ variants that contain additional mutations around the Arg⁵⁰⁶ cleavage site, the rate constants for cleavage at Arg⁵⁰⁶ (k_{506}) were determined likewise and the resulting rates were expressed relative to the inactivation rate of QRQ (Figure 6.2 and Table 6.1). Substitution of residues Glu³⁷², Asp³⁷³ and Glu³⁷⁴ (mutant 5 and 6) resulted in a reduction of about 40% of the inactivation rate compared to QRQ. For the FVa variants with mutations in the basic cluster between residues 659 to 663, the cleavage rate at Arg⁵⁰⁶ was reduced to 50% of that of QRQ. Replacement of residues Glu³²³, Asp⁵⁷⁷ or Asp⁵⁷⁸ had no effect on the cleavage rate at Arg⁵⁰⁶, whereas the replacement of Asp⁵¹³ for a neutral asparagine residue remarkably resulted in an approximate 2-fold increase in k_{506} . As described above, reaction conditions for studying the APC-catalyzed inactivation of mutants D577N-D578N and D513N-D577N-D578N were different from those of the other FV variants, which explains the different shape of the inactivation curve for FV variants D577N-D578N and D513N-D577N-D578N.

Figure 6.2. Inactivation of FVa QRQ variants by APC. Time courses of inactivation of FVa QRQ variants (20 pM) by APC (0.085 nM) were performed as described in "Materials and methods". FVa cofactor activity was determined at 0.5 nM FXa, except for FVa mutants D577N-D578N and D513N-D577N-D578N, which were assayed at a final concentration of 100 pM FVa and 5 nM FXa (see "Results" section). The figure represents the average of 2 experiments and standard deviations were <10% for all of the FVa variants. ● QRQ, ◇ D513N, ○ D577N-D578N, ▲ D513N-D577N-D578N, Δ E323Q, * E372Q-D373N-E374Q, ◆ E323Q-E372Q-D373N-E374Q, + D659N-D660N, ■ D661N-E662Q-D663N, × D659N-D660N-D661N-E662Q-D663N.

Inactivation of FVa variants R306QR679Q by APC mutant K37S/K38Q/K39Q/K62N/K63D

To further probe the importance of negatively charged residues in FVa that surround the Arg⁵⁰⁶ cleavage site, we monitored time courses of inactivation of FVa QRQ variants by APC mutant K37S/K38Q/K39Q/K62N/K63D (Figure 6.3 and Table 6.1), a mutant that has reduced activity towards the Arg⁵⁰⁶ cleavage site and that shows no detectable binding to heparin. Compared to the inactivation by wild type APC, FVa QRQ was inactivated at a ~15-fold slower rate by APC mutant K37S/K38Q/K39Q/K62N/K63D (Table 6.1). All FVa QRQ variants were inactivated by the APC mutant at a rate similar to QRQ, except for FVa D513N, for which a ~8-fold increase in inactivation rate was observed (Table 6.1 and Figure 6.3).

Figure 6.3. Inactivation of FVa QRQ variants by APC mutant K37S/K38Q/K39Q/K62N/K63D. Time courses of inactivation of FVa QRQ variants (20 pM) by APC mutant K37S/K38Q/K39Q/K62N/K63D (0.56 nM) were determined as described in "Materials and methods". FVa cofactor activity was determined at 0.5 nM FXa, except for FVa mutants D577N-D578N and D513N-D577N-D578N, which were assayed at a final concentration of 100 pM FVa and 5 nM FXa (see "Results" section). The figure represents the average of 2 experiments and standard deviations were <10% for all of the FVa variants. ● QRQ, ◇ D513N, ○ D577N-D578N, ▲ D513N-D577N-D578N, Δ E323Q, * E372Q-D373N-E374Q, ◆ E323Q-E372Q-D373N-E374Q, ⊕ D659N-D660N, ■ D661N-E662Q-D663N, × D659N-D660N-D661N-E662Q-D663N.

Effect of heparin and protein S on the APC-catalyzed inactivation of FVa variants QRQ

To evaluate the effect of heparin and protein S on the APC-mediated cleavage at Arg⁵⁰⁶, time courses of inactivation of the FVa R306QR679Q variants were monitored in the presence of 25 U/ml heparin or 150 nM protein S. Apparent second-order rate constants for APC-mediated cleavage at Arg⁵⁰⁶ in the presence

of heparin or protein S were derived from fitting time courses of inactivation to the same equation that was used to determine k_{506} in the absence of heparin or protein S (equation 1). We verified that heparin and protein S did not alter the cofactor activity and stability of the FVa intermediate (k_2). For each FVa QRQ variant, the Arg^{506} cleavage rate in the presence of heparin or protein S was expressed relative to the Arg^{506} cleavage rate of the mutant in the absence of heparin or protein S (Figure 6.4). The inactivation of QRQ, which does not contain amino acid substitutions in the Arg^{506} region, was 3- to 4-fold inhibited by heparin but was unaffected by protein S. Heparin and protein S had a similar effect on the inactivation of the FVa variants, indicating that these mutations have no influence on the heparin-mediated inhibition of cleavage at Arg^{506} and also that they do not notably contribute to protein S cofactor activity in the APC-catalyzed cleavage at Arg^{506} in FVa.

Figure 6.4. Effect of heparin and protein S on the APC-catalyzed inactivation of FVa QRQ variants. Time courses of inactivation of FVa QRQ variants (20 pM) by APC (0.085 nM) were performed in the absence or presence of UFH (25 U/ml; open bars) or protein S (150 nM; shaded bars). Data were fit to an equation describing the loss of FVa cofactor activity as a result of Arg^{506} cleavage by APC (equation 1 in "Materials and methods"). Rates for cleavage at Arg^{506} (k_{506}) in the presence of heparin or protein S were expressed relative to the inactivation rate of the FVa mutant in the absence of heparin or protein S. Values are the average of 2 experiments and standard deviations were less than 10% for all FVa variants.

Effect of heparin and protein S on the APC-catalyzed inactivation of FVa RQQ variants

A three-dimensional model of APC docked at the Arg^{306} cleavage site in FVa indicated that heparin could bridge FVa and APC, thus providing a structural basis

for the heparin-mediated stimulation of the Arg³⁰⁶ cleavage in FVa by APC [9]. The FVa residues that were proposed to contact heparin when APC is positioned at Arg³⁰⁶ are part of a basic cluster in the A2 domain of FVa and involve residues Lys³²⁰, Arg³²¹, Arg⁴⁰⁰ and Arg⁵⁰¹ (See also Figure 6.1). To investigate the role of these residues in the APC-catalyzed cleavage at Arg³⁰⁶, we have constructed a number of FV variants in which these positively charged amino acids were replaced by neutral (Ala) or negatively charged (Glu) residues (Table 6.2). Time courses of inactivation of the activated mutants by APC were monitored in the absence and presence of heparin (25 U/ml) or protein S (150 nM), from which second-order rate constants for cleavage at Arg³⁰⁶ were determined (equation 2). FVa mutant RQQ, which can only be cleaved at Arg³⁰⁶ and which does not contain any further amino acid substitutions in this region, was inactivated with a rate of $1.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (Table 6.2). In the absence of heparin or protein S, the inactivation rate (k_{306}) of FVa variants K320A-R321A, R400A and K320E-R321E was 4- to 7-fold increased compared to RQQ. All other FVa variants were inactivated at Arg³⁰⁶ with a rate similar to RQQ.

FV Mutant	FV Mutation	Activity/ antigen	$k_{306} (\text{M}^{-1}\text{s}^{-1})$
RQQ	R506QR679Q	0.87	1
10	K320A-R321A	1.09	7.20
11	R400A	0.77	6.87
12	K320A-R321A-R400A	0.84	0.83
13	K320E-R321E	1.27	3.83
14	K320E-R321E-R400E	0.35	0.72
15	K320E-R321E-R400E-R506E	0.35	0.89
16	K320A-R321A-R400A-R501A	0.32	0.96
17	K320E-R321E-R400E-R501E	0.29	0.88

Table 6.2. Activity to antigen ratio and relative rate constants (k_{306}) for the APC-catalyzed inactivation of FV RQQ variants. FV concentrations were determined by an ELISA and a functional assay as described in "Materials and methods". As a parameter of specific activity, the ratio of FV activity to antigen was calculated. Values are the average of 3 experiments and standard deviations were <5% for all of the FV variants. Relative rate constants for inactivation of FVa variants that can only be cleaved at Arg³⁰⁶ by recombinant wild type APC (0.75 nM) were obtained by fitting time courses of inactivation to an integral time course equation as described in "Materials and methods". Rate constants given are means from 2 experiments and standard deviations were <10% for all FVa variants. Rate constants are expressed relative to the rate constants for cleavage of R506Q/R679Q (RQQ), which was $1.12 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$.

Effect of heparin and protein S on the APC-catalyzed inactivation of FVa RQQ variants

In the presence of heparin, the inactivation of RQQ was 3- to 4-fold stimulated (Figure 6.5). However, none of the FVa RQQ variants showed a heparin-mediated stimulation of cleavage at Arg³⁰⁶, but even a mild inhibition (~2-fold) (Figure 6.5). Because the stimulating effect of heparin on the inactivation of FVa mutants K320A-R321A and R400A was completely abolished, we conclude that residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ constitute a heparin binding region in FVa that is important for the accelerated cleavage at Arg³⁰⁶ by APC in the presence of heparin.

Protein S stimulated APC-mediated cleavage at Arg³⁰⁶ for all FVa RQQ variants (Figure 6.5). The stimulating effect of protein S was similar for all FV variants, and slightly lower (1.5-2~fold) compared to the effect of protein S on RQQ. This indicates that the mutated residues play only a minor role in the cofactor activity of protein S in the cleavage at Arg³⁰⁶ by APC.

Figure 6.5. Effect of heparin and protein S on the APC-catalyzed inactivation of FVa RQQ variants. Time courses of inactivation of FVa RQQ variants (20 pM) by APC (0.75 nM) were determined in the absence or presence of UFH (25 U/ml; open bars) or protein S (150 nM; shaded bars). Data were fit to an equation describing the loss of FVa cofactor activity as a result of Arg³⁰⁶ cleavage by APC (equation 2 in "Materials and methods"). Rates for cleavage at Arg³⁰⁶ (k_{306}) in the presence of heparin and protein S were expressed relative to the inactivation rate of the FVa mutant in the absence of heparin or protein S. Values are the average of 2 experiments and standard deviations were less than 10% for all FVa variants.

6.5 Discussion

We have recently shown that heparin alters the APC-catalyzed inactivation of FVa by specifically inhibiting cleavage at Arg⁵⁰⁶ and stimulating cleavage at Arg³⁰⁶ [9]. On the basis of the available kinetic data and three-dimensional molecular models of the APC/heparin complex and of APC alone docked at the Arg³⁰⁶ and Arg⁵⁰⁶ site in FVa, several amino acid residues were identified in FVa that may be important for the FVa:APC interaction and its modification by heparin. To investigate the role of these residues in APC-catalyzed FVa inactivation, we have replaced these amino acids by neutral or charge-reversed residues by site-directed mutagenesis (Table 6.1 and 6.2). Since all target amino acid residues are solvent-exposed and most of the introduced residues can be considered as conservative, we expected that these mutations had no adverse effects on protein folding and thus, that the effects of the substitution would be limited to the interaction under study. The interaction surfaces between FVa and APC are likely not limited to single amino acid contacts and therefore we chose to produce both single as well as multiple mutations in order to probe the FVa:APC interaction.

Upon calculation of activity/antigen ratios for the different FV variants we noticed a remarkable ratio for the FV mutants D577N-D578N and D513N-D577N-D578N. Whereas ratios for all mutants were within a 2-3 fold range, the activity/antigen ratio in this case was ~50-fold lower than that of FV QRQ. We ascribe this discrepancy to a reduced affinity for FXa as a result of the replacement of Asp⁵⁷⁷ and Asp⁵⁷⁸ for an asparagine residue [33]. Several data in literature, both structural [34] and functional [35], agree with a role for Asp⁵⁷⁷ and Asp⁵⁷⁸ in FVa-FXa interaction. In addition, FVa sequences between residues 311-325 [36] and 493-506 [37] have also earlier been reported to interact with FXa, which may explain the observed lower activity:antigen ratio of FVa variants bearing combined mutations of these residues (Table 6.1).

Analysis of the time courses of APC-catalyzed inactivation of the FVa variants that can only be cleaved at Arg⁵⁰⁶ showed only minor effects of the mutations that were introduced near the Arg⁵⁰⁶ cleavage site. Significant differences were however noted for several of the mutants tested, which suggests that there is an extended interaction surface between FVa and APC. The most prominent effect was observed for the FVa mutants with mutations in an acidic cluster spanning from residues 659 to 663, for which a ~2.5 fold reduction in the second-order rate constant of inactivation (k_{506}) was observed. A possible role of this cluster of negatively charged residues has been proposed by Pellequer *et al.*, who suggested that this cluster could interact with APC residues Arg⁷⁴ and Arg⁷⁵, in addition to Arg^{149D} and Arg¹⁵¹ from the autolysis loop from APC [38].

None of the introduced mutations had an effect on the heparin-mediated inhibition of Arg⁵⁰⁶ cleavage by APC. Together with the observation that heparin does not inhibit FVa inactivation by APC variants with a defective heparin binding site [9], these data suggest that the inhibition of the Arg⁵⁰⁶ cleavage is not mediated by a direct interaction of heparin with FVa. Heparin binds to three conserved surface loops in APC (loops 37, 60 and 70) that contain a cluster of basic residues. This positively charged exosite partially overlaps with a FVa binding region (loops 37 and 70) crucial for cleavage at Arg⁵⁰⁶ by APC [2,3,20]. Our data indicate that binding of heparin to APC likely prevents appropriate docking of APC at Arg⁵⁰⁶ due to steric hindrance by the heparin chain, which had also prior been observed in *in silico* docking studies [9]. In contrast to the Arg⁵⁰⁶ cleavage site, there are no negatively charged residues present in the close vicinity of the Arg³⁰⁶ site. This suggests that cleavage at Arg³⁰⁶ by APC is not merely driven by an exosite-mediated interaction, based on mostly electrostatic forces, given the fact that a cluster of net positively charged surface loops on the SP domain of APC have been shown to be required for interaction of APC with its substrates. However, inspection of the APC/heparin and APC models docked at the FV Arg³⁰⁶ cleavage site suggested that although direct contacts between FVa and APC are sparse, heparin could bridge the heparin-binding region of APC and a positively charged region comprising residues Lys³²⁰, Arg³²¹, Arg⁴⁰⁰ and Arg⁵⁰¹ near the Arg³⁰⁶ cleavage site [9]. To investigate whether the predictions based on the theoretical model were correct and to obtain detailed information on the role of a basic cluster of surface exposed residues in the APC-mediated cleavage at Arg³⁰⁶, several FVa variants that can only be cleaved at Arg³⁰⁶ were made in which these positively charged amino acids were replaced by neutral or negatively charged residues. Remarkably, the rate of Arg³⁰⁶ cleavage by APC of FVa mutants K320A-R321A, R400A and K320E-R321E was 4- to 7-fold increased compared to FVa RQQ. Residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ are located on the C-terminus of the Arg³⁰⁶ loop and are involved in hydrogen bonds and salt bridges with surrounding residues Glu³¹⁵, Met³¹⁹, Glu³²³, Ile⁴⁵⁹ and Glu⁴⁶¹. Because the distance between the N- and C-terminus of the Arg³⁰⁶ loop is much larger than that of the Arg⁵⁰⁶ loop, while both loops involve about the same number of residues, it was proposed that the structure of the Arg³⁰⁶ loop may be more constrained. Local melting of the Arg³⁰⁶ loop therefore could be required for proper presentation of the Arg³⁰⁶ loop to the active site of APC, which in turn could contribute to the slower cleavage rate at Arg³⁰⁶ compared with Arg⁵⁰⁶ [38]. Because replacement of residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ for Ala disrupts hydrogen bonding and substitution by Glu residues introduces local electrostatic repulsion, these mutations may result in a less constrained Arg³⁰⁶ loop which hypothetically can explain the observed increase of the rate of cleavage at Arg³⁰⁶ in these FVa mutants. More experimental data is

needed to confirm this hypothesis. Our data are in agreement with a reported global involvement of the area around Ile³¹¹-Phe³²⁵ in the FVa-APC interaction as was recently shown by the inhibitory activities of a peptide, comprising residues 311-325 of FVa, in the inactivation of FVa by APC [39]. One might expect that the inactivation rate of the other FVa variants carrying mutations at Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ would also be increased, but this was not the case. An explanation for this observation cannot be provided at this point however, other than that local differences in the conformation of the Arg³⁰⁶ loop structure as a result of different combinations of mutations in these FVa variants may explain this apparent discrepancy. In addition to the arguments given here for the relatively slow cleavage at Arg³⁰⁶, as compared to Arg⁵⁰⁶, it is noteworthy that a number of new structures for FVa [34,40-43] present both dominant APC cleavage sites (i.e. Arg³⁰⁶ and Arg⁵⁰⁶) at a distance to the membrane of ~80 Å, which would argue against an earlier hypothesis that the position of both cleavage sites with respect to the membrane, and thus with respect to the APC active site is different, causing the 20-fold difference in cleavage rate [44]. More experimental data is needed however to verify the relative positions of Arg³⁰⁶ and Arg⁵⁰⁶ in human FVa. The fact however, that mutagenesis can increase the rate of cleavage at Arg³⁰⁶ strongly suggests that differences in cleavage between Arg³⁰⁶ and Arg⁵⁰⁶ are likely to be caused by differences in the local interactions independent of the cleavage site:membrane distance. The stimulating effect of protein S, mainly on Arg³⁰⁶, would then be explained by improved contacts between residues probed in this study in the Arg³⁰⁶ region (see also below) and the SP domain of APC.

In the presence of heparin, the rate of inactivation of FVa RQQ was almost 4-fold increased. Replacement of amino acid residue Arg⁴⁰⁰ or Lys³²⁰ and Arg³²¹ nullified the heparin-mediated stimulation of cleavage at Arg³⁰⁶, indicating that these amino acid residues are pivotal for the FVa-heparin interaction. When no bridging between APC and FVa with heparin can occur, due to mutation of the Arg⁴⁰⁰-Lys³²⁰-Arg³²¹ mutants, heparin even mildly (~2-fold) inhibited the cleavage at Arg³⁰⁶. Similar to the heparin-mediated inhibition of cleavage at Arg⁵⁰⁶, the inhibition of cleavage at Arg³⁰⁶ by APC can likely be explained by an overall dominant steric hindrance of the APC-bound heparin chain, thus preventing a correct positioning of the APC serine protease domain at the Arg³⁰⁶ cleavage site.

All FV variants that can only be cleaved at Arg³⁰⁶ were stimulated by protein S (3- to 5-fold), although to a lesser extent when compared to FVa RQQ, which was about 8-fold stimulated. Possibly, the positively charged region in FVa comprising residues Lys³²⁰, Arg³²¹, Arg⁴⁰⁰ and Arg⁵⁰¹ are part of, or close to a proposed protein S binding site [37], which is very close (15-20 Å) and that comprises FVa residues 493-506. However, since protein S did not protect the inactivation of the FVa mutants that can only be cleaved at Arg⁵⁰⁶, a protein S binding site different from

the 493-506 region is likely present in FVa. In line with this, protein S does not ablate the protective effect of FXa on the APC-catalyzed cleavage at Arg⁵⁰⁶ in FVa [13].

In conclusion, we have demonstrated that an extended region around the Arg⁵⁰⁶ cleavage site contributes to the proposed electrostatic interaction between the 37, 60 and 70-loop of APC and the region around Arg⁵⁰⁶ in FVa. Furthermore, we have shown that the observed inhibition of Arg⁵⁰⁶ by heparin cannot be nullified by a mere removal of negative surface charges around the Arg⁵⁰⁶ site. Finally, we identified an electropositive region in FVa involving residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ that forms an heparin-binding region in the FVa heavy chain and that facilitates the heparin-mediated stimulation of Arg⁵⁰⁶ cleavage by APC.

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FVa epitopes important for the interaction with APC and heparin

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Chapter VII:

General discussion and conclusions

7.1 FV, a protein with pro- and anticoagulant properties

Together with thrombin, FV is the only coagulation factor that is directly implicated both in procoagulant and anticoagulant pathways. Both proteins are therefore central regulators of clot formation, with FV being particularly important in the early phase of coagulation when only limited amounts of thrombin are formed.

Under physiological conditions (i.e., when anticoagulant forces prevail), activated protein C (APC) can convert circulating FV to an anticoagulant protein (FVac) that participates in the downregulation of FVIIIa activity. When the local concentration of procoagulant proteins is high however, FV and FVac are converted to a procoagulant protein (FVa) that serves as a cofactor of FXa in the prothrombinase complex and enhances thrombin formation by several orders of magnitude. Considering the essential role of FVa in the procoagulant pathway, it is not surprising that downregulation of thrombin formation partly proceeds via inactivation of FVa (and also FVIIIa) by the anticoagulant protein C system.

The research presented in this thesis focuses on the structure-function relationship of FV, particularly with regard to its activation by thrombin, its membrane-binding properties and the inactivation of the activated cofactor FVa by APC.

7.2 FV activation by thrombin and the snake venom serine proteases from *Daboia russelli* and *Daboia lebetina*

In the first part of this thesis (chapter III and IV) we have investigated the proteolytic mechanisms underlying the conversion of the inactive procofactor FV to the active cofactor FVa. The physiological activators of FV, (meizo)thrombin and FXa, activate FV via three sequential cleavages in the B domain at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵. It is believed that FV activation results in the exposure of FXa binding sites in the FV molecule that are necessary for expression of its procoagulant activity. It is however not clear how these binding regions are expressed following proteolysis. One possibility implies that the FV B domain sterically blocks functional FXa binding sites that become accessible after limited proteolysis and removal of the B domain. Evidence against this hypothesis comes from recombinant FV studies, which demonstrate that release of the B domain is not required for expression of FVa cofactor activity [1-3]. In addition, these studies show that formation of the light chain, resulting from a single cleavage at Arg¹⁵⁴⁵, is sufficient for the expression of full FVa cofactor activity. Both observations are confirmed by FV activation studies employing a protease from Russell's viper venom (RVV-V) that specifically activates FV by a single cleavage at Arg¹⁵⁴⁵ and yields a fully functional FVa molecule [4,5]. The high specificity of RVV-V is no exception, as snake venom proteases that affect haemostasis are in general highly selective.

Because of this tight specificity and their resistance to physiological inhibitors, venom proteases have been extensively used for the design of several routine diagnostic laboratory tests and even for therapeutic applications. In addition, snake venom proteins that interfere with haemostasis are potentially useful tools to study the mechanisms of action, the regulation and the structure-function relationships of coagulation factors since they are very similar in structure to human coagulation factors but at the same time display very different proteolytic activities.

In order to obtain more information about the mechanism of FV activation, we have predicted the three-dimensional (3D) structures of two FV activators that are present in the venom of Russell's viper and Levant's viper.

7.2.1 Structural models of the FV activators from the venom of Russell's viper and Levant's viper

Despite their widespread use in the field of thrombosis and haemostasis, little or no structural data on snake venom proteases that affect haemostasis are currently available. In the absence of an experimentally determined protein structure, computational methods can be used to obtain useful information about a protein's 3D structure. A widely used technique for the prediction of the 3D structure of a protein is homology modeling. The method is based on the observation that the 3D structure is conserved more than the amino acid sequence and that small or medium changes in sequence typically result in only small changes in the 3D structure [6]. Thus, it is possible to predict the 3D structure of a given protein based on its alignment to one or more proteins with known 3D structure that share sequence identity (typically > 30-40%) with the studied protein. Accordingly, we have generated homology models of the FV-activating enzymes from the venom of Russell's and Levant's viper, RVV-V and LVV-V, based on the crystal structure of a plasminogen activating enzyme from *Agkistrodon acutus*. These 3D models display several structural features that may account for the peculiar substrate specificity of RVV-V and LVV-V, which will be discussed in the following paragraphs.

Active site: non-specific substrate binding site and S3 subsite

An intriguing characteristic of RVV-V and LVV-V is the non-conserved 'non-specific substrate binding site', which is formed by residues 214-216 (chymotrypsinogen numbering). The Ser²¹⁴-Trp²¹⁵-Gly²¹⁶ tripeptide is strictly conserved among serine proteases, reflecting its structural and functional importance. The backbone of the Ser²¹⁴-Trp²¹⁵-Gly²¹⁶ segment creates the opening to the primary specificity pocket and is also crucial for secondary binding. Upon binding, the backbone of this

tripeptide forms a β -sheet with the peptide backbone of substrates and inhibitors [7]. Although there is no experimental evidence that the non-specific substrate binding site of RVV-V and LVV-V adopts a conformation that is different from that observed in other serine proteases, it can be anticipated that mutations in this functionally important region may have consequences for enzyme catalysis.

Only a few serine proteases that lack the conserved region between residues 214 and 216 have been reported in literature, most of which show a low activity towards small chromogenic substrates or macromolecular substrates [8-10]. The high-resolution crystal structures that are available for these proteases reveal major conformational changes in the 214-216 tripeptide that are not compatible with productive peptide substrate binding [8-10]. In analogy, an unusual conformation of the non-specific substrate binding site may account for the observed low amidase activity of RVV-V and LVV-V and for their unique specificity for the Arg¹⁵⁴⁵ cleavage site in FV. In the absence of structural or biochemical data that support this hypothesis however, the structure of the 214-216 tripeptide in RVV-V and LVV-V was predicted based on the crystallographic coordinates of the corresponding region in the template structure.

Analysis of the active site environment of RVV-V and LVV-V may provide additional clues for the specificity of both snake venom proteases for the Arg¹⁵⁴⁵ cleavage in FV. Serine proteases that belong to the chymotrypsin family show limited substrate selectivity for residues at the C-terminal side of the scissile bond (prime side), implicating that the amino acids on the N-terminus of the scissile bond are the major determinants for specificity [11]. From this point of view it is interesting to note that the P3 residue (Tyr) is the only N-terminal residue in the Arg¹⁵⁴⁵ cleavage site that considerably differs from the corresponding residues in the two other FV activation cleavage sites (Table 7.1).

FV cleavage site	P3	P2	P1	P'1	P'2	P'3
709	Gly	Ile	Arg	Ser	Phe	Arg
1018	Ser	Pro	Arg	Thr	Phe	His
1545	Tyr	Leu	Arg	Ser	Asn	Asn

Table 7.1 Amino acid sequences of the thrombin and FXa activation cleavage sites in FV. Residues surrounding the Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵ cleavage sites are numbered according to the nomenclature of Schechter and Berger [70]. Amino acids at the N-terminal side of the scissile bond are numbered P3, P2, P1 and those at the C-terminal side are numbered P1', P2', P3'.

In thrombin and FXa, the S3 substrate binding site is quite narrow as a result of hydrogen bond formation between the side chains of Trp²¹⁵ and Met¹⁸⁰. Because Trp²¹⁵, which is part of the non-specific substrate binding site, is replaced by a glycine in RVV-V and LVV-V, it can be expected that both FV activators preferentially cleave substrates with a bulky residue at the P3 position, as is tyrosine. This preference may contribute to the selectivity of RVV-V and LVV-V for the Arg¹⁵⁴⁵ cleavage site in FV.

Surface loops surrounding the active site

It is well documented that the substrate specificity of serine proteases is only partly determined by the active site environment and that unique surface loops that surround the active site cleft play a more important role in substrate recognition and specificity [12]. It can thus be expected that differences between the loops of physiological and snake venom FV activators may account for their distinct substrate specificity. Comparison of the RVV-V and LVV-V models with the thrombin structure reveals an overall reduction of the size of the loops that surround the active site, in particular the 60- and 148-loop. Mutagenesis and structural studies have demonstrated that the 60- and 148-loop block access of many macromolecular substrates and inhibitors to the active site of thrombin [13,14]. The large reduction of both loops in RVV-V and LVV-V and the resulting increased accessibility of their active site may explain why both proteases can readily cleave the Arg¹⁵⁴⁵ peptide bond, whereas thrombin requires preceding cleavages at Arg⁷⁰⁹ and Arg¹⁰¹⁸ for efficient cleavage at Arg¹⁵⁴⁵. Using FV cleavage site mutants, we and others have demonstrated that the Arg¹⁵⁴⁵ cleavage site is less susceptible to thrombin cleavage than the Arg⁷⁰⁹ and Arg¹⁰¹⁸ cleavage sites (chapter IV and reference [2]). In addition, it has been shown that the preceding cleavages at Arg⁷⁰⁹ and Arg¹⁰¹⁸ by thrombin facilitate subsequent cleavage at Arg¹⁵⁴⁵ [2,15]. These data suggest that in intact FV, the Arg¹⁵⁴⁵ peptide bond is hardly accessible for the deep and narrow active site of thrombin and that this inhibitory constraint is relieved by the initial cleavages at Arg⁷⁰⁹ and Arg¹⁰¹⁸. The lack of large insertion loops that block the entrance to the active site may explain why RVV-V and LVV-V cleave intact FV at Arg¹⁵⁴⁵ more efficiently than thrombin (Figure 7.1.)

Further, the presence of a K/R-N-I-R sequence at the apex of the 60- and 70-loop of RVV-V and LVV-V may be worth mentioning. Because this motif is not found in other snake venom serine proteases and appears to be characteristic for RVV-V and LVV-V [16,17], both K/R-N-I-R regions could be important for FV recognition. The observation that the arginine and lysine residues in both sequences are all

located in a highly positively charged region that overlaps with thrombin exosite I further strengthens this hypothesis.

Figure 7.1 Activation of FV cleavage site mutants by thrombin (A) and RVV-V (B). Recombinant FV cleavage site mutants (250 pM of each; ○ wild type FV; △ RIR; ◇ QIR) were activated with increasing thrombin or RVV-V concentrations (0.1-50 nM). After 15 min activation at 37 °C, FVa cofactor activity was measured in a prothrombinase assay as described [71].

Electropositive exosites

Electrostatic surface potential calculations reveal the presence of two positively charged surface patches on opposite sides of the active site of RVV-V and LVV-V. It has been well documented that two electropositive regions on the surface of thrombin are important for the interaction with its various macromolecular substrates and ligands. In particular, a crucial role has been ascribed to thrombin exosite I, which has been proposed to enhance the rate of complex formation with complementary electrostatic fields generated by its substrates or ligands [18]. This 'electrostatic steering' effect is important to overcome the restricted accessibility of the active site of thrombin and leads to the productive formation of an enzyme-ligand complex via electrostatic interactions between basic exosite and acidic ligand residues. This phenomenon, known as 'ionic tethering', also serves to optimally align complementary hydrophobic residues in the enzyme and the ligand, which finally results in short-range hydrophobic contacts that stabilize the complex [18-20]. It is likely that RVV-V and LVV-V use a similar strategy for the recognition and cleavage of the Arg¹⁵⁴⁵ peptide bond, given the presence of two positively charged surface patches that are populated with several hydrophobic residues. The observation that the acidic hirudin-like sequence in the B domain of FV is necessary for FV activation by RVV-V and LVV-V further supports this hypothesis, although the currently available data do not provide information about which of the two (or both) electropositive surface patches contribute to FV

activation. However, one of the two electropositive regions in RVV-V and LVV-V topologically overlaps with thrombin exosite I, suggesting that this region might be involved in FV recognition and cleavage at Arg¹⁵⁴⁵ by the snake venom FV activators.

7.2.2 The role of thrombin exosites in FV activation

Mutagenesis and structural studies have provided a lot of information about the molecular basis of the interactions between thrombin and its various substrates, ligands and inhibitors. A unique feature of thrombin are two electropositive surface regions, termed exosite I and exosite II, that are located on opposite sides of the active site. Both exosites have been implicated in FV activation, but the relative importance of these regions in the individual thrombin-catalyzed FV cleavages is still a matter of debate.

Most approaches aimed at understanding FV activation are principally based on the functional assessment of FVa cofactor activity and its correlated SDS-PAGE analysis of FV cleavage reactions catalyzed by thrombin exosite mutants or, alternatively, by wild type thrombin in the absence and presence of thrombin exosite ligands. Interpretation of these data is often not straightforward because different FV cleavage intermediates with different cofactor activities are generated during the reaction and also because the kinetics of the three activation cleavages are not independent of one another [15]. For this reason, we have monitored the thrombin-catalyzed activation of recombinant FV mutants that can only be cleaved at one of the three activation cleavage sites (chapter IV). To investigate the importance of each of thrombin's exosites for the recognition of the individual FV cleavage sites, all activation reactions were performed in the presence and absence of DNA aptamers that specifically bind to either thrombin exosite I or II [21,22]. Because these experiments merely provide information about the importance of thrombin exosites for cleavage at the individual cleavage sites in single chain FV, we also performed SDS-PAGE analysis of the activation of plasma-purified FV by thrombin in the absence and presence of both aptamers.

Overall, our results show that thrombin exosite I is essential for cleavage at both Arg⁷⁰⁹ and Arg¹⁵⁴⁵. This finding is consistent with the observation that two acidic sequences that are homologous to the exosite I-ligand hirudin⁵⁴⁻⁶⁵ are present upstream of these two cleavage sites [23]. A surprising number of thrombin substrates and inhibitors contain acidic regions with sulfated tyrosine residues that are important for the interaction with exosite I [24-26]. Because the acidic sequences that precede the Arg⁷⁰⁹ and Arg¹⁵⁴⁵ cleavage sites also contain one or more sulfated tyrosine residues, it was proposed that both regions could interact directly with exosite I during FV activation. This thought is supported by

recombinant FV studies which show that non-sulfated recombinant FV is slowly cleaved by thrombin at Arg⁷⁰⁹ [27].

It is generally recognized that thrombin exosite I is involved in FV activation, but the role of exosite II in this process is still a matter of debate. Kinetic and SDS-PAGE analysis of the activation of FV cleavage mutants and plasma-derived FV by thrombin in the presence of aptamer II indicate that thrombin exosite II is particularly important for the recognition of the Arg¹⁵⁴⁵ cleavage site. In the absence of a 3D structure of full length FV, it is difficult to imagine how the exosites of thrombin mutually contribute to FV activation at Arg¹⁵⁴⁵. Although it is theoretically possible that FV interacts with the exosites of two different thrombin molecules, similar to the mechanism by which thrombin binds to platelet glycoprotein Ib α [28], it is more likely that both exosites recognize two distinct electronegative regions on the surface of FV. Accordingly, FV activation at Arg¹⁵⁴⁵ by thrombin may involve interactions of exosite I with the hirudin-like sequence upstream from the Arg¹⁵⁴⁵ cleavage site, whereas the residues at the prime side of the Arg¹⁵⁴⁵ scissile bond may extend into exosite II. In this context, the presence of a highly acidic sequence in the N-terminal part of the FVa light chain (residues 1559-1584) is worth mentioning [23]. This electronegative sequence is fully solvent-exposed in the 3D FVa structures described in literature [29,30], supporting the idea that this region may be involved in exosite-mediated interactions with thrombin. However, no interaction of the FVa light chain with thrombin was detected neither in affinity chromatography studies in which the binding of FV activation products to active-site-immobilized thrombin was quantified, nor in fluorescence binding studies employing active-site-labeled thrombin [31,32]. This suggests that the acidic region in the light chain is not involved in FV activation by thrombin, although it cannot be ruled out that thrombin interaction sites were differently expressed in the isolated fragments and therefore lost their affinity for thrombin. It is interesting to note that, in the same studies, also no interaction between thrombin and the fragment of the FV B domain containing the acidic hirudin-like sequence could be detected [31,32].

To evaluate the importance of the acidic sequence that precedes the Arg¹⁵⁴⁵ cleavage site and to probe other regions in the B domain that may be essential for FV activation, we have followed activation time courses of FV mutants in which different parts of the B domain were deleted. These experiments indicate that, in contrast to RVV-V and LVV-V, thrombin requires both the N- and C-terminal region of the FV B domain for efficient activation. Although the region between residues 710 and 826 does not contain a hirudin-like sequence like the regions preceding the cleavage sites at Arg⁷⁰⁹ and Arg¹⁵⁴⁵, the B domain region may contain sequences that are part of the thrombin recognition site. It is interesting to note that despite the overall poor conservation of the B domain, the sequence

between residue 709 and 750 is highly conserved among species of different origin, indicating that this region is important for FV function. In addition, this region contains a short electronegative cluster consisting of four successive glutamic acid residues that may be involved in exosite-mediated interactions during FV activation [23].

Our finding that deletion mutant FV des 709-1476 was efficiently activated by RVV-V and LVV-V, but required prolonged incubation with excess thrombin to get fully activated by thrombin is in line with the data obtained with the FV cleavage site mutant that can only be cleaved at Arg¹⁵⁴⁵ (QIR). Compared to wild type FV and FV RIR, this mutant was slowly activated by thrombin, but was activated by RVV-V and LVV-V at a similar rate as other FV variants containing an arginine at position 1545. Together, these data not only suggest that FV recognition by RVV-V and LVV-V is mediated by exosite-driven interactions that minimally require the C-terminal region of the B domain, but also confirm earlier observations that thrombin cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸ facilitates cleavage at Arg¹⁵⁴⁵ [2].

Because thrombin and the snake venom FV activators share the characteristic serine protease fold and both apparently employ positively charged surface sites for substrate recognition, it is likely that these properties were adapted from a common ancestor. The evolutionary history of serine proteases can be traced back based on the presence of discrete sequence markers [7]. The first report of such a sequence marker was the observation that active site serine residues in a variety of enzyme families are encoded by both AGY and TCN codons (Brenner, 1988)¹. Because the transition from AGY to TCN codons is extremely rare since it requires double-nucleotide shifts, it was reasoned that proteases that utilize different active site serine codons belong to a different lineage.

Other evolutionary markers that were found in members of the trypsin family include Ser¹⁹⁵ (AGY/TCN dichotomy) and residue 225 (Pro/Tyr dichotomy) [7]. Snake venom serine proteases have kept the primordial evolutionary markers (Ser¹⁹⁵:TCN/Ser²¹⁴:TCN/Pro²²⁵) and therefore belong to the most primordial lineage [33]. It was proposed that the snake venom serine proteases have diverged from glandular kallikrein, a member of the trypsin/kallikrein gene family [34] (Fig. 7.2). Furthermore, comparison of cDNAs encoding snake venom serine proteases indicates that these proteases have acquired their specificity via accelerated evolution [35]. With respect to this, it is interesting to note that RVV-V and LVV-V only cleave the peptide bond in FV that results in the generation of full procoagulant cofactor activity [1-3]. The accelerated acquisition of a set of venom enzymes with diverse biological functions was likely advantageous for the snake in

¹ A=adenine; T=thymine, C=cytosine; G=Guanine; Y=C or T; N=any base

terms of defence against predators and catching and digestion of their prey. Thrombin, on the other hand, evolved from the complement factors C1r, C1s or MASP-2 that belong to the modern $\text{Ser}^{195}:\text{AGY}/\text{Ser}^{214}:\text{TCN}/\text{Tyr}^{225}$ lineage and was most likely the ancestral protein for all other vitamin K-dependent proteases [7]. Considering its evolutionary history (Figure 7.2), it is not surprising that thrombin can perform many physiological functions in haemostasis, developmental processes and inflammation.

Figure 7.2 Phylogenetic marker-based evolutionary pathways of thrombin and the snake venom FV activators RVV-V and LVV-V.

Sequence dichotomies used to categorize chymotrypsin-like proteases were TCN or AGY usage for the highly conserved active site residues Ser^{195} and Ser^{214} and Pro or Tyr usage for residue 225. The primitiveness of TCN compared with AGY and of Pro compared with Tyr were used to establish the relative ages of the lineages [7]. Thus, $\text{Ser}^{195}:\text{TCN}/\text{Ser}^{214}:\text{TCN}/\text{Pro}^{225}$ is the most primordial marker configuration and $\text{Ser}^{195}:\text{AGY}/\text{Ser}^{214}:\text{AGY}/\text{Tyr}^{225}$ is the most modern.

Although thrombin, RVV-V and LVV-V originate from the same primordial lineage, it thus appears that they have developed their FV-activating activity independently from each other. As a consequence, their molecular basis of FV recognition is different, which is reflected in the observation that the 60 loop, which is strongly reduced in RVV-V and LVV-V, in the case of thrombin is involved in FV activation [36]. Moreover, experimental and theoretical data support the idea that RVV-V and LVV-V recognize FV by one or more positively charged surface sites, but the nature of this interaction appears to differ from that observed for thrombin because exosite I-dependent thrombin inhibitors did not inhibit FV activation by RVV-V and LVV-V.

7.3 Identification of small-molecule inhibitors of FV membrane binding via structure-based VLS

Thromboembolic diseases are the leading cause of death in the Western world [37]. Limitations of existing anticoagulants have led to the development of a wide range of novel drugs that target specific steps in coagulation. Many of these candidate drugs block the procoagulant effects of thrombin, either by inactivating the enzyme itself or by preventing its generation [38]. Accordingly, molecules that prevent the incorporation of FVa in the prothrombinase complex may represent an attractive class of novel anticoagulant drugs since prothrombin activation by FXa is very slow in the absence of its cofactor FVa [39]. We decided to target the FV C2 domain because this module has been shown to be critical for FV(a) membrane binding and prothrombinase complex assembly [40,41].

A commonly used approach in the early stage of drug development employs experimental screening of large chemical libraries in order to identify bioactive molecules against the target protein. Although high-throughput screening (HTS) has become a dominant approach in early drug discovery programs, such a procedure is generally very expensive, time-consuming, labor-intensive and requires a significant amount of biological material [42]. A more rational and economic strategy for the discovery of hit molecules involves the integration of computational (*in silico*) and experimental screening methods. *In silico* screening of chemical libraries allows the creation of smaller sublibraries that are enriched in potential hit molecules for the selected target protein. Such a hierarchical approach does not only drastically reduce the total number of molecules that have to be tested experimentally, but can also help improve hit rates [43]. Therefore, we have combined computational and experimental screening methods to identify small-molecule inhibitors of FV membrane binding (chapter V).

The Chembridge compound collection, a database of ~500,000 commercially available small-molecules, was used as input for the virtual ligand screening (VLS) procedure. After ADME/tox filtering and 3D conformer generation, the resulting 3D compound library was screened via structure-based VLS methods. In a first rigid-body docking step, the 3D conformers were docked to the phosphatidylserine binding pocket of the open and closed conformation of the human FV C2 domain. The docked compounds were evaluated using a scoring function based on shape complementarity with the binding pocket and the top-scoring 60,000 molecules were subjected to a flexible docking step. The top-scoring 500 compounds for each C2 conformation were then tested for their ability to inhibit prothrombin activation in a prothrombinase assay *in vitro*. Active molecules were further characterized by surface plasmon resonance (SPR), yielding 7 hit compounds that inhibit the interaction of the FV C2 domain with the phospholipid membrane.

Although the 7 identified hit compounds efficiently blocked FV C2 domain binding to immobilized phospholipids and also showed good inhibition of FVa cofactor activity in the activation of prothrombin by FXa, we did not observe inhibition of thrombin generation in a plasma-based assay system. Similar to this observation, small-molecule inhibitors of FVIII membrane binding that were identified via HTS also showed poor inhibition of FVIIIa procoagulant activity in whole plasma [44]. It was proposed that competitive binding of von Willebrand Factor (vWF) with the FVIII inhibitors could explain these results because FVIII binding to vWF and phospholipids involves an overlapping epitope in the FVIII C2 domain [45]. The lack of an anticoagulant effect of the FV compounds in plasma is likely caused by other factors, since no plasma equivalent of vWF has been described for FV. Because plasma protein binding is a major factor that influences drug distribution and availability [46], we investigated whether the *in vitro* activity of the hit compounds is influenced by human serum albumin (HSA). Albumin is the most abundant protein in plasma and is one of the major determinants of drug disposition. HSA abrogated the inhibitory effect of hit compounds in the prothrombinase assay in a concentration-dependent manner, which strongly suggests that excessive binding to plasma proteins accounts for the poor activity of the small-molecules in the plasma-based assay system.

While a degree of albumin binding may be desirable in the solubilization and distribution of drugs, overcoming the binding affinity for albumin is a major challenge in drug development as it drastically lowers the availability of the drug in its free, bioactive form. In recent years, several high-throughput methods have been developed to study the albumin-binding properties of candidate small-molecule drugs. These techniques make it feasible to screen large compound

collections for small-molecules with desirable albumin-binding properties and to develop structure-activity relationships for albumin binding [47,48]. Such information can be combined with structural information obtained by crystallographic studies to develop computational models for *in silico* prediction of drug binding to HSA [49]. However, these methods are still in their infancy and require further optimization and validation before they are sufficiently robust for the rational design of compounds with appropriate ADME properties. An alternative approach to significantly reduce albumin binding of small-molecules while maintaining a high-affinity interaction with the target protein involves the combined use of structure-activity relationships and structural data obtained via NMR or crystallographic studies, as recently demonstrated for a cyclooxygenase-2 (COX-2) inhibitor [50].

The four most potent compounds share some structural and physicochemical features like a central furane ring and a pyrimidine-like structure (pyrazolidinedione), which are both flanked by a carboxylated, halogenated or nitrated phenyl group (Figure 7.3).

Figure 7.3 Structural similarity of hit compounds that inhibit FV membrane binding.

Chemical structures of the 4 most active small-molecules are shown with their corresponding IC_{50} values for inhibition of FV C2 membrane binding. Shared structural elements are colored (furane ring, red; benzoic acid, blue; nitrophenyl, green; halogenated phenyl ring, purple; azole, yellow).

These structures may guide chemical library searches to find structurally related molecules which then can be tested again *in vitro*, from which structure-activity relationships can be established. Such information can then be applied to define a pharmacophore, which is a reduced 3D representation of a few key structural elements of the molecule, such as hydrogen bond donors and acceptors, ionic groups and lipophilic centers. Such a simplified description allows rapid searching of 3D compound libraries to identify molecules with complementary structural elements to the target pharmacophore, which eventually will lead to new molecules with improved affinity and specificity for the target protein.

In silico structural analysis of the (re-)docked hit compounds revealed that all molecules were partially buried in the proposed phosphatidylserine binding pocket. In general, the compounds showed favorable interactions with the side chains of Lys²⁰⁶⁰, Lys²⁰⁶¹, Trp²⁰⁶³, Trp²⁰⁶⁴, Asp²⁰⁶⁶, Trp²⁰⁶⁸, Arg²⁰⁸⁰, Gln²⁰⁸⁵, Ser²¹¹⁵ and Leu²¹¹⁶. A consensus pose for the most active compound describes hydrophobic and aromatic interactions with Trp²⁰⁶³, Trp²⁰⁶⁴ and Leu²¹¹⁶ (Figure 7.4).

Figure 7.4 Proposed binding mode of compound 001C07 in the membrane-binding pocket of the FV C2 domain. The backbone of the open form of the FV C2 domain is shown in grey ribbon, the bound compound in red. Side chains of FVa residues in contact with the compound are shown in cyan. Residues discussed in the text are labeled. Predicted hydrogen bonds between the side chain of Lys²⁰⁶⁰, Asn²⁰⁸⁹ and Gln²¹⁸² are depicted in purple dotted lines.

These three residues are located at the apex of two hairpin loops at the bottom of the C2 domain and have been shown to be critical for FV membrane binding [41,51]. Moreover, several other interesting contacts with the FV C2 domain are made via Lys²⁰⁶⁰, Asp²⁰⁶⁶ and Arg²⁰⁸⁰. The importance of these residues for FV membrane binding was demonstrated earlier via alanine scanning mutagenesis [51], molecular dynamic studies of FV C2 membrane binding [52] and epitope mapping of inhibitory FV C2-specific antibodies [53]. It has been suggested that Lys²⁰⁶⁰ and Arg²⁰⁸⁰ are involved in specific interactions with phospholipid head groups, with Lys²⁰⁶⁰ compensating the negative charge of the phosphatidylserine phosphate group [40,52]. Although the proposed binding mode of the compound rationalizes its inhibitory activity, an explicit structural explanation for its action mechanism would require site-directed mutagenesis and NMR or crystallographic experiments.

One may wonder how many active compounds would have been identified when no VLS had been performed prior to the experimental screening. An unambiguous answer to this issue could only be given after randomly picking another 1000 molecules from the Chembridge compound collection and testing them *in vitro*. Since this would be a very costly strategy, we instead decided to screen the Chembridge compound collection for inhibitors of the FVIII C2 domain and to compare the *in silico* results with those obtained in a recently performed HTS study on FVIII [44]. Of the 10 hits that were identified in the latter study, 5 molecules were retrieved in the top-scoring 1000 molecules of the VLS procedure, together with 3 molecules that cross-reacted with FVIII in our FV study. In the case of FVIII, combining VLS and HTS thus increased the hit rate at least 8-fold (0.8 versus 0.1%). VLS thus appears to be an effective strategy to access a large chemical space without the commitment in time, material and infrastructure that is typical of HTS approaches.

It should be noted that even higher hit rates would have been obtained when the data from the FVIII HTS study had been considered for the design of the VLS protocol applied in the FV study. However, we deliberately decided to ignore the data from the FVIII HTS study to evaluate the usefulness of structure-based VLS in the discovery of small-molecule inhibitors of protein-membrane interactions. Membrane-binding regions of peripheral proteins like FV and FVIII are generally considered as poorly druggable because they are highly flexible and as such often lack a well defined cavity with a topology and physicochemical properties that are compatible with tight small-molecule binding [54]. Our results however show that proteins that bind transiently to the membrane can be successfully targeted via combined *in silico* and *in vitro* approaches, suggesting that a similar approach can be applied to other therapeutically relevant membrane-binding proteins.

7.4 Interaction of FVa with APC and heparin

Inactivation of FVa by APC occurs preferably at a phospholipid surface and involves multiple cleavages in the FVa heavy chain, of which those at Arg³⁰⁶ and Arg⁵⁰⁶ are considered most important [55]. It has been shown that heparin alters the pathway of APC-catalyzed FVa inactivation by stimulation of the Arg³⁰⁶ cleavage and inhibition of the Arg⁵⁰⁶ cleavage [56]. Several charged amino acids that might be important for the interaction of FVa with APC and heparin were identified on the basis of kinetic data and *in silico* docking studies. To investigate the role of these charged amino acids in the inactivation of FVa by APC, a number of recombinant FVa variants were expressed in which these residues were replaced by neutral or charge-reversed residues by site-directed mutagenesis. We have used FVa mutants with only one of the three APC cleavage sites available to facilitate the kinetic analysis of the APC-mediated cleavage at Arg³⁰⁶ and Arg⁵⁰⁶. The importance of the mutated residues for FVa inactivation by APC was then evaluated by following time courses of FVa inactivation in the absence and presence of heparin and protein S.

Several of the mutations that were introduced near the Arg⁵⁰⁶ cleavage site had a small but significant effect on the FVa inactivation rate at Arg⁵⁰⁶.

A first region comprises the electronegative cluster between FVa residues 659 and 663. Acidic residues 659-663 are not present in our 3D homology model because it was only possible to reliably predict the structure of the FVa A2 domain up to residue 656, since the C-terminal region of the FVa A2 domain (residues 664-709) is missing in the ceruloplasmin template structure. In a model proposed by Pellequer *et al.*, the structure of the FVa A2 domain was predicted up to residue 663 and it was suggested that the electronegative cluster between FVa residues 659-663 could interact with APC residues Arg⁷⁴, Arg⁷⁵ and Arg^{149D} and Arg¹⁵¹ from the autolysis loop of APC [57].

In addition to residues 659-663, the replacement of the negatively charged surface residues Glu³⁷², Asp³⁷³ and Glu³⁷⁴ by neutral amino acids (Gln or Asn) was found to slightly (~2-fold) decrease the APC-catalyzed cleavage at Arg⁵⁰⁶. It can be predicted that these residues, which are all located in the FVa A2 domain, interact with a cluster of basic residues located on the autolysis loop of APC [56]. This loop is part of an electropositive exosite on the surface of APC that has been reported to be important for its interaction with various substrates and inhibitors [58-61]. Together with the 37- and 70-loops of APC, the autolysis loop is particularly important for the recognition of the Arg⁵⁰⁶ cleavage site by APC, but plays only a minor role in the interaction with the Arg³⁰⁶ cleavage site [62,63]. The observation that the introduced mutations only mildly affected the rate at which FVa was inactivated at Arg⁵⁰⁶ by APC may reflect the extended nature of the binding

interface between FVa and APC. It will be interesting to identify the additional FVa residues that are part of the extended FVa:APC interaction surface, particularly those involved in the interaction with the 37- and 70-loop of APC.

None of the introduced mutations had an effect on the heparin-mediated inhibition of Arg⁵⁰⁶ cleavage by APC. Heparin did also not inhibit FVa inactivation at Arg⁵⁰⁶ by APC variant K37S/K38Q/K39Q/K62N/K63D, which is defective in heparin binding [56]. These data together indicate that heparin binding to the 37- and 60-loop of APC prevents appropriate docking of APC at Arg⁵⁰⁶, which might be due to charge compensation of the electropositive field created by the exosite of APC and/or steric hindrance by the negatively charged heparin chain [56].

The absence of negatively charged residues in the close vicinity of the Arg³⁰⁶ site suggests that FVa inactivation at Arg³⁰⁶ is not mediated by exosite-driven interactions, which is in agreement with the observation that mutations in the electropositive exosite of APC impair FVa inactivation at Arg⁵⁰⁶, but have only a minor effect on the cleavage at Arg³⁰⁶ [62,63]. However, *in silico* docking studies suggest that heparin can bridge the heparin-binding region of APC and a positively charged region comprising residues Lys³²⁰, Arg³²¹, Arg⁴⁰⁰ and Arg⁵⁰¹ near the Arg³⁰⁶ cleavage site [56]. In order to investigate the importance of these residues for the heparin-mediated stimulation of FVa inactivation at Arg³⁰⁶ by APC, we have monitored inactivation time courses of FVa variants that can only be cleaved by APC at Arg³⁰⁶. Whereas inactivation of the wild type variant was 3- to 4-fold stimulated by heparin, no such heparin-mediated acceleration of FVa cleavage at Arg³⁰⁶ was observed for FVa variants in which Lys³²⁰, Arg³²¹ and/or Arg⁴⁰⁰ were substituted for neutral or negatively charged residues. It thus appears that Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ comprise a heparin binding region in FVa that is essential for the heparin-mediated stimulation of FVa cleavage at Arg³⁰⁶ by APC. However, our data do not indicate that this cluster of positively charged residues plays a direct role in FVa inactivation at Arg³⁰⁶ in the absence of heparin. It can be anticipated that the electropositive field created by these FVa residues would not allow favorable contacts with the positively charged exosite of APC. In fact, the 3D model of APC docked at Arg³⁰⁶ predicts only a few strong contacts between the electropositive Arg³⁰⁶ loop and the electronegative active site cleft of APC [56]. Moreover, the electropositive loops that surround the active site of APC are relatively remote from the molecular surface of FVa and are directed towards neutral regions in FVa, which can be explained by the size of the Arg³⁰⁶ loop and the absence of electronegative surface regions near the Arg³⁰⁶ cleavage site.

Although heparin alters the pathway of FVa inactivation by APC *in vitro*, the physiological relevance of the heparin-mediated modulation of FVa inactivation

remains unclear. Many heparin-like structures like dermatan sulfate, heparin sulfate and chondroitin sulfate are present on the surface of vascular endothelial cells. This network of glycosaminoglycans, known as the glycocalyx, plays a major role in the regulation of many biological processes, including haemostasis [64]. Little is known about the composition of the glycocalyx, but it can be expected that the local concentration of the various glycosaminoglycans that line the vascular wall is well below the concentration required for *in vitro* modulation of APC-catalyzed FVa inactivation (i.e., 2 U/ml for half-maximal inhibition of FVa cleavage at Arg⁵⁰⁶).

Nevertheless, the reported *in vitro* effect of heparin on APC-catalyzed FVa inactivation may also have significance *in vivo*, particularly in patients receiving heparin for the treatment of chronic or acute cardiovascular disorders. Typical heparin levels in patients receiving antithrombotic therapy vary between 0.3 and 0.7 U/ml, but levels between 2 and 5 U/ml can be achieved during cardiopulmonary bypass [65].

Of special interest are the results of two recently conducted phase III clinical trials for the treatment of human sepsis, in which the use of recombinant APC as an adjunctive therapy for the treatment of severe sepsis was investigated [66, 67]. Although it was found that administration of recombinant human APC had a significant beneficial effect on mortality outcome in severe sepsis patients, the incidence of serious bleeding was higher in the APC-treated group than in the placebo group. This less favorable effect of recombinant APC in heparin-cotreated sepsis patients was partly attributed to heparin-mediated stimulation of FVa inactivation by APC [68].

7.5. Summarizing remarks

The research presented in this thesis focused on the structure-function relationship of FV with special attention to its activation by thrombin, its membrane-binding properties and the inactivation of FVa by APC.

Structural requirements for FV activation by thrombin were investigated using recombinant FV mutants that can only be cleaved at one of the three activation cleavage sites. In combination with DNA aptamers that specifically bind to one of the two exosites of thrombin, we have demonstrated that particularly thrombin exosite I is important for thrombin-catalyzed FV activation. In addition, we have shown that the N- and C-terminal region of the FV B domain contains sequences that are important for recognition and cleavage of the Arg⁷⁰⁹ and Arg¹⁵⁴⁵ sites by thrombin.

Furthermore, we have employed structure-based virtual ligand screening and high-throughput screening to identify small-molecule inhibitors of FV membrane binding. The structures of the identified compounds may assist in the rational

design of FV inhibitors with improved properties and can guide chemical library searches for membrane-binding inhibitors of the closely related coagulation factor VIII.

Finally, we have demonstrated that an electropositive region in the FVa heavy chain consisting of residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ forms a heparin-binding region that facilitates the heparin-mediated stimulation of Arg³⁰⁶ cleavage by APC. In addition, we have shown that an extended electronegative region around the Arg⁵⁰⁶ cleavage site contributes to the proposed electrostatic interaction with APC.

After the recent elucidation of the crystal structure of B-domainless FVIII [69], it can be expected that the crystal structure of complete human FV(a) will also become available. Such structural information can improve the reliability and accuracy of docking predictions, thus guiding the design of site-directed mutagenesis experiments which would further increase our understanding of the structure-function relationship of FV.

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Summary

The coagulation cascade entails a series of enzymatic reactions in which coagulation factors, which circulate as inactive precursor molecules, are converted to active proteins that then catalyze the next reaction in the cascade. These activation reactions occur preferably on a negatively charged phospholipid surface, typically provided by the platelet membrane. This complex series of reactions is triggered by tissue factor, a subendothelial protein that is exposed together with the platelet activator collagen when the endothelial lining of the vascular wall is damaged.

An essential step in the coagulation cascade is the formation of the prothrombinase complex, which is a membrane-bound macromolecular complex that consists of the serine protease FXa and its non-enzymatic cofactor FVa. The prothrombinase complex effectively converts prothrombin to thrombin, which in turn converts soluble fibrinogen into fibrin polymers that stabilize the primary platelet plug. Under optimal conditions (i.e. in the presence of calcium ions and a negatively charged phospholipid surface), FVa accelerates FXa-catalyzed prothrombin activation over 100,000-fold compared with FXa alone. Since FVa plays such an important role in thrombin generation, proteolytic inactivation of FVa by activated protein C (APC) is a major physiological mechanism that regulates thrombin formation. Strict regulation of thrombin formation during haemostasis is a prerequisite to prevent inappropriate thrombus formation.

The research presented in this thesis focuses on the structure-function relationship of coagulation factor V, particularly regarding the regulation of its activities through interaction with thrombin, negatively charged phospholipids, and APC.

Chapter I provides an overview of the structure and functions of FV, with special emphasis on the activation of FV, the interaction of FV(a) with negatively charged phospholipid membranes and the inactivation of FVa by APC.

Chapter II describes inherited defects in FV that are considered as risk factors for thrombosis. Particular attention is given to the molecular mechanism by which FV polymorphisms contribute to APC resistance. Interactions with acquired risk factors that are known to synergistically increase the thrombotic risk that is related to FV genetic defects are also discussed.

In chapters III and IV, the proteolytic mechanism underlying the conversion of the inactive procofactor FV to the active cofactor FVa was studied. (Meizo)thrombin and FXa are the major physiological activators of FV, but several other proteases of different origin have been shown to activate FV. Of special interest are FV activators that are present in the venom of snakes belonging to the *Viperidae* family. In contrast to the physiological FV activators (meizo)thrombin and FXa,

which activate FV via three sequential cleavages in the B domain at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵, these snake venom proteases convert FV to a fully functional FVa molecule through a single cleavage at Arg¹⁵⁴⁵. Because of their specificity and their resistance to physiological inhibitors, snake venom FV activators are used in diagnostic tests for quantification of plasma FV levels and for screening of defects in the anticoagulant protein C pathway. In chapter III, we have predicted the three-dimensional structures of the FV activators that are present in the venom of Russell's and Levant's viper (RVV-V and LVV-V, respectively) via homology modeling. Both protein structures reveal several structural features that may account for the remarkable substrate specificity of RVV-V and LVV-V. Notably, two positively charged surface regions are present on opposite sides of the active site which we propose to form the exosites for FV interaction, similar to the two electropositive exosites in thrombin.

Both exosites of thrombin have been implicated in FV activation, but the relative importance of each exosite in the individual thrombin-catalyzed FV cleavages is unclear. In chapter IV, recombinant FV variants that can only be cleaved at one of the three activation cleavage sites were used in combination with thrombin exosite I- or exosite II-specific DNA aptamers to assess the contribution of each exosite to the individual thrombin-catalyzed activation cleavages in FV. Kinetic and SDS-PAGE analysis of the activation of FV cleavage mutants and plasma-derived FV by thrombin in the presence of aptamers indicate that thrombin exosite I is essential for cleavage at both Arg⁷⁰⁹ and Arg¹⁵⁴⁵, whereas thrombin exosite II appears to be particularly important for cleavage at Arg¹⁵⁴⁵. Moreover, activation studies with FV B domain deletion mutants show that an electronegative region in the B domain that precedes the Arg¹⁵⁴⁵ cleavage site is required for efficient FV activation by thrombin, RVV-V and LVV-V. Although recognition of the Arg¹⁵⁴⁵ cleavage site by RVV-V and LVV-V thus appears to be mediated by exosite-driven interactions, the nature of this interaction is likely different from that observed for thrombin because thrombin exosite-inhibitors did not inhibit FV activation by RVV-V and LVV-V.

Because thrombin generation by the prothrombinase complex is a crucial step in haemostasis, it is an attractive target for anticoagulant therapy. In chapter V, structure-based virtual ligand screening and high-throughput screening methods were applied to discover small-molecules that block the interaction of FV(a) with the phospholipid membrane and thus prevent prothrombinase complex assembly. Several small-molecules were identified that inhibit FXa-catalyzed prothrombin activation in a purified system. Direct binding experiments employing surface plasmon resonance with purified recombinant C2 domain and plasma-purified FVa light chain further confirmed that the identified compounds inhibit the interaction of the FV C2 domain with negatively charged phospholipids. These molecules may

serve as lead compounds for the development of a novel class of anticoagulant drugs and/or can be applied in structure-function studies on FV.

The activity of the prothrombinase complex is physiologically regulated by the protein C pathway via the proteolytic inactivation of FVa by APC. It has been shown that heparin alters the pathway of APC-catalyzed FVa inactivation by stimulation of the Arg³⁰⁶ cleavage and inhibition of the Arg⁵⁰⁶ cleavage. Several charged surface residues near the Arg³⁰⁶ and Arg⁵⁰⁶ cleavage site that might be important for the interaction of FVa with APC and heparin have been identified through computational docking studies. In chapter VI, the role of these charged amino acids in the inactivation of FVa by APC was investigated via a site-directed mutagenesis approach. Analysis of inactivation time courses of recombinant FV variants in which these residues were replaced by neutral or charge-reversed residues demonstrates that an extended region around the Arg⁵⁰⁶ cleavage site in FVa is involved in the interaction with APC. Furthermore, FVa residues Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰ were found to be indispensable for the heparin-mediated stimulation of Arg³⁰⁶ cleavage by APC. These results suggest that heparin accelerates the APC-catalyzed inactivation of FVa at Arg³⁰⁶ by bridging the electropositive exosite of APC and the electropositive region in the FVa heavy chain comprising Lys³²⁰, Arg³²¹ and Arg⁴⁰⁰.

Finally, chapter VII summarizes and discusses the main results and conclusions of the data that are presented in this thesis.

Samenvatting

Het bloedstollingsproces bestaat uit een reeks opeenvolgende enzymatische reacties waarbij stoffactoren, die in een inactieve vorm in het bloed circuleren, worden geactiveerd. Deze activeringsreacties vinden bij voorkeur plaats op een negatief geladen fosfolipidenoppervlak, zoals dat van geactiveerde bloedplaatjes. De stolling wordt geïnitieerd door weefselfactor, een eiwit dat zich in de vaatwand bevindt en pas na beschadiging van de wand met het bloed in contact komt. Hierdoor wordt de eerste stoffactor van de cascade omgezet in een actieve vorm, die dan op zijn beurt de volgende factor activeert. Zo ontstaat een hele waterval van reacties waarbij de ene stoffactor na de andere wordt geactiveerd. Uiteindelijk wordt het oplosbare eiwit *fibrinogeen* door trombine omgezet in het onoplosbare *fibrine*. Dit fibrine vormt een verstevigend netwerk van vezels rond de bloedplaatjesprop die in een eerste fase van de stolling werd gevormd om het lek in de vaatwand te dichten.

Een belangrijke gebeurtenis tijdens het stollingsproces is de vorming van het *protrombinase-complex* op het bloedplaatjesoppervlak. Dit complex bestaat uit *geactiveerd factor X* (FXa) en zijn niet-enzymatische cofactor *geactiveerd factor V* (FVa). Het protrombinase-complex zorgt voor een efficiënte omzetting van protrombine in trombine, een reactie die door FXa wordt uitgevoerd. Het gevormde trombine zorgt op zijn beurt voor de omzetting van fibrinogeen in fibrine dat - zoals hierboven beschreven - voor de versteviging van de bloedplaatjesprop zorgt. Onder optimale omstandigheden (d.w.z. in de aanwezigheid van calcium en een negatief geladen fosfolipidenoppervlak) versnelt FVa de omzetting van protrombine door FXa ongeveer 100 000 keer in vergelijking met wanneer FXa alleen aanwezig is. FVa is dus zeer belangrijk voor de activiteit van het protrombinase-complex en het is daarom niet verwonderlijk dat de trombinevorming door het protrombinase-complex voornamelijk gecontroleerd wordt via de inactivering van FVa door *geactiveerd proteïne C* (APC). Een goede regulatie van de trombinevorming tijdens het stollingsproces is zeer belangrijk om de ongewenste vorming van bloedstolsels te voorkomen.

Het onderzoek dat in dit proefschrift wordt beschreven heeft betrekking op het verband tussen de structuur en functies van factor V (FV), meer in het bijzonder de interacties met trombine, negatief geladen fosfolipiden en APC.

In hoofdstuk I wordt kort samengevat wat er reeds bekend is over de structuur en functie van FV. Hierbij wordt vooral aandacht besteed aan de activering van FV, de interactie van FV en FVa met een negatief geladen fosfolipidenoppervlak en de inactivering van FVa door APC.

In hoofdstuk II worden afwijkingen in het FV-gen beschreven die voor een verhoogd trombose-risico zorgen. Daarbij wordt vooral ingegaan op FV-polymorfismen die leiden tot APC-resistentie en op het moleculaire mechanisme dat daaraan ten grondslag ligt. Bovendien worden ook verworven risicofactoren besproken waarvan bekend is dat ze synergistisch het tromboserisico van genetische FV-afwijkingen versterken.

In hoofdstukken III en IV is de proteolytische omzetting van de inactieve procofactor FV naar de actieve cofactor FVa bestudeerd. De fysiologische activatoren van FV zijn (meizo)trombine en FXa, maar er zijn ook eiwitten van niet-humane oorsprong bekend die FV activeren. Zo bevat het gif van sommige addersoorten (*Viperidae*) een protease dat FV zeer specifiek activeert. In tegenstelling tot (meizo)trombine en FXa, die drie peptidebindingen in het FV B-domein splitsen (namelijk na de aminozuren Arg⁷⁰⁹, Arg¹⁰¹⁸ en Arg¹⁵⁴⁵), activeren deze slangengifeiwitten FV via één enkele 'knip' op positie 1545. Vanwege hun hoge specificiteit en de ongevoeligheid voor protease-remmers die in plasma circuleren, worden FV-activatoren uit slangengif gebruikt in diagnostische tests voor de bepaling van FV-waarden in plasma en voor het vaststellen van afwijkingen in het anticoagulante proteïne C-systeem.

In hoofdstuk III hebben we via homologie-modellering de driedimensionale structuur voorspeld van RVV-V en LVV-V, de FV-activatoren uit het gif van respectievelijk de Russell-adder en de Levantijnse adder. Beide eiwitstructuren vertonen een aantal structurele kenmerken die mogelijk bijdragen tot de opmerkelijk hoge substraatspecificiteit van RVV-V en LVV-V. Zo zijn aan beide zijden van de actieve site twee positief geladen gebieden aanwezig die mogelijk betrokken zijn bij de interactie met FV, net zoals de twee positief geladen exosites van trombine.

Hoewel algemeen aangenomen wordt dat beide exosites van trombine een rol spelen in de activering van FV, was het vooralsnog onduidelijk in welke mate deze twee exosites bijdragen aan elk van de drie knippen die door trombine worden uitgevoerd tijdens de activering van FV. Om dit verder te onderzoeken hebben we in hoofdstuk IV gebruikgemaakt van recombinante FV-varianten die slechts op één van de drie activeringsplaatsen geknipt kunnen worden. De activering van deze FV mutanten door trombine werd gevolgd in de aanwezigheid van DNA-aptameren die specifiek aan één van de twee trombine-exosites binden. Een dergelijke aanpak maakt het mogelijk om de rol van de twee trombine-exosites in elk van de drie afzonderlijke knippen te bestuderen. De resultaten van de experimenten met de recombinante FV-varianten werden bevestigd door gel-electroforetische analyse van de activering van uit plasma gezuiverd FV door trombine in aan- en afwezigheid van de exosite-specifieke DNA-aptameren. Algemeen kan besloten worden dat trombine-exosite I belangrijk is voor de knippen na Arg⁷⁰⁹ en Arg¹⁵⁴⁵,

terwijl exosite II voornamelijk betrokken is bij de knip na Arg¹⁵⁴⁵. Verder werd ook de activering bestudeerd van FV-mutanten waarvan het B-domein helemaal of gedeeltelijk was verwijderd. Uit deze experimenten bleek dat een negatief geladen gebied dat zich net vóór de Arg¹⁵⁴⁵-knipplaats bevindt, noodzakelijk is voor de activering door zowel trombine als RVV-V en LVV-V. Dit doet vermoeden dat RVV-V en LVV-V gebruik maken van exosites voor de herkenning van de Arg¹⁵⁴⁵-knipplaats in FV. Desalniettemin is de interactie tussen de beide slangengifactivatoren en FV waarschijnlijk van een andere aard dan die tussen trombine en FV, aangezien de activering van FV door RVV-V en LVV-V niet wordt beïnvloed door trombine-remmende DNA-aptameren.

De omzetting van protrombine in trombine is een heel belangrijke reactie tijdens de bloedstolling, wat het protrombinase-complex tot een veelbelovend doelwit voor anticoagulante therapie maakt. In hoofdstuk V hebben we gebruikgemaakt van structurele bio-informatica om een grote verzameling chemische stoffen te screenen op moleculen die de interactie van FV of FVa met een negatief geladen fosfolipidenoppervlak verhinderen en zo de vorming van een functioneel protrombinase-complex voorkomen. Moleculen die op basis van hun driedimensionale structuur werden geselecteerd, werden dan op betrekkelijk kleine schaal getest in een gezuiverd systeem waarbij nagegaan werd of ze inderdaad de omzetting van protrombine in trombine door FXa remmen. Actieve moleculen werden vervolgens verder gekarakteriseerd via directbindingsproeven, waarbij gebruikgemaakt werd van oppervlakte-plasmon-resonantie in combinatie met gezuiverd recombinant C2-domein en de gezuiverde lichte keten van FVa. Deze proeven bevestigden dat de geïdentificeerde moleculen de interactie van het FV C2-domein met een negatief geladen fosfolipidenoppervlak voorkomen. Dergelijke moleculen kunnen als leidraad dienen voor de ontwikkeling van nieuwe anticoagulante geneesmiddelen en kunnen gebruikt worden bij onderzoek naar de structuur en functie van FV.

De activiteit van het protrombinase-complex wordt gereguleerd door het proteïne C-systeem via de inactivering van FVa door APC. Onlangs werd door onze groep aangetoond dat de manier waarop APC FVa inactieveert, beïnvloed wordt door heparine, dat de knip na Arg³⁰⁶ stimuleert en deze na Arg⁵⁰⁶ remt. Een structurele analyse van de interactie tussen FVa en APC via computertechnieken heeft verschillende geladen aminozuren aan het oppervlak van FVa geïdentificeerd die mogelijk belangrijk zijn voor de interactie van FVa met APC en heparine. In hoofdstuk VI hebben we de rol van deze geladen aminozuren in de inactivering van FVa door APC onderzocht via mutagenese. Recombinante FV-varianten waarin de lading van deze aminozuren was gewijzigd, werden geïncubeerd met APC en het verlies in FVa-cofactoractiviteit werd gevolgd als functie van de tijd. Uit deze experimenten blijkt dat de interactie tussen FVa en APC een groot oppervlak rond

Samenvatting

de Arg⁵⁰⁶-knipplaats beslaat. Bovendien werd in FV-varianten, waarin de positief geladen aminozuren Lys³²⁰, Arg³²¹ en Arg⁴⁰⁰ waren vervangen, de APC-afhankelijke splitsing na Arg³⁰⁶ niet door heparine gestimuleerd. Deze experimenten ondersteunen de hypothese dat heparine de inactivering van FVa door APC op positie 306 versnelt door de positief geladen exosite van APC en een positief geladen regio in de zware keten van FVa (bestaande uit aminozuren Lys³²⁰, Arg³²¹ en Arg⁴⁰⁰) met elkaar te verbinden.

Ten slotte worden in hoofdstuk VII de belangrijkste bevindingen en conclusies uit dit proefschrift samengevat en besproken.

Dankwoord

“Het leven van een Assistent-In-Opleiding (AIO) is als een sprookje”. Voor een ingeweken Taliban-strijder die wordt bijgestaan door een goede fee gaat deze stelling misschien wel op¹, maar voor een gewone sterveling zoals ik? Ik dacht het niet! En toch ...

Er was namelijk eens, ergens in het verre Rajastan, een ratje. Het was niet zomaar een ratje (gewone ratjes komen immers nooit in sprookjes voor), maar een héél speciaal ratje. Samen met honderden - zo niet duizenden - soortgenootjes leefde het in een tempel die door de plaatselijke bevolking “de Rattentempel” werd genoemd. Waarom die tempel zo heette weet niemand, maar misschien had het wel te maken met het feit dat er zoveel ratjes leefden. Tussen al die ratjes leefde dus dat ene speciale ratje. Iemand die weinig verstand van ratjes heeft zou wel eens kunnen denken dat het ratje zo bijzonder was omdat het het enige witte ratje in de Rattentempel was, maar er was meer aan de hand. Dit ratje had namelijk een heel speciale gave: het kon wensen in vervulling doen gaan! Van heinde en verre kwamen er dan ook mensen naar de Rattentempel afgezakt in de hoop een glimp van het witte ratje op te vangen en zo hun grootste wens in vervulling te zien gaan. De meesten wensten een dure auto, een winnend loterijbiljet of een betere echtgeno(o)t(e), maar niet dat ene echtpaar uit Halle. Zij wensten dat hun jongste zoon als AIO aan de slag kon in Maastricht! Nog steeds begrijpt niemand waarom ze dat deden. Was het in een vlaag van zinsverbijstering, overrompeld door de onverwachte ontmoeting met het witte ratje? Of waren ze gewoon bang dat hun zoontje uiteindelijk straatveger zou moeten worden en de burens daarover kwaad zouden spreken? De echte reden zullen we wellicht nooit kennen, maar één ding is zeker: een week na de wonderbaarlijke ontmoeting met het witte ratje werd hun zoontje als AIO aangenomen in Maastricht!

Het jongetje kon zijn oren niet geloven! Het liefst wou hij zélf naar Rajastan om het ratje persoonlijk te bedanken, maar dat kon natuurlijk niet omdat AIO's nu eenmaal weinig vakantie krijgen. Daarom bracht hij, als offer aan het witte ratje, enkele typische biertjes uit zijn geboortestreek naar zijn nieuwe Nederlandse collega's. Het scheelde geen haar, of het pas begonnen avontuur van onze Halse vriend kwam hier al ten einde! Zijn copromotor Gerry, die nochtans bekend stond als een fijnproever, wist immers niets beter te verzinnen dan pH-indicatorpapier in het goddelijke gerstenat te dompelen! Dergelijke heiligschennis wordt door het witte ratje normaal onmiddellijk afgestraft, maar deze ene keer liet het de barbaarse Nederlander begaan. In de Rattentempel zaten veel Indonesische ratjes die sterke verhalen over hun koloniale overheersers rondstrooiden. Zo hadden ze al verteld over kroketten die uit de muur worden gehaald en over bier zonder kraag dat zelfs slechter smaakt dan een theetje getrokken van het kapsel van koningin Fabiola! Was het door deze verhalen dat het witte ratje de fratsen van Gerry door de

¹ Zie proefschrift en promotiefilmpje Jeroen “Mohammed” Brugge, 2006.

vingers zag? Of wist het slimme ratje dat het de verhalen van zijn Indonesische soortgenootjes met een flinke korrel zout moest nemen en er dus ook Nederlanders met goede smaak bestaan?

Zo'n Nederlander met goede smaak was Guido. Guido was dol op stoofpot van konijn. En dan nog het liefst bereid met pruimen. Omdat het nogal lastig is om op pruimen te jagen, hield Guido het maar bij het jagen op konijnen. Guido wist dat er geen beter vlees bestond dan dat van een Belgisch konijntje en daarom trok hij vaak met zijn jachthonden naar de Ardennen om er wat van deze langorige diertjes te strikken. Af en toe liep er wel eens een hondje verloren en was Guido heel droevig. Dan bracht hij hele dagen in de Ardennen door tot hij zijn geliefde viervoeters had teruggevonden. Omdat je van het wachten op honden best dorst krijgt, was Guido vaak terug te vinden in herberg "Le chien perdu". Waarschijnlijk heeft Guido dáár geleerd dat het ongemanierd is om Belgisch bier met pH-indicatorpapiertjes te bezoedelen. Kortom, Guido was een wijs man. Hij bracht niet alleen het Halse jongetje en diens mede-AIO's veel bij, maar zelfs ervaren wetenschappers kwamen vaak bij hem om raad!

Zo was er Tilman. De man was een kei in het maken van allerhande peptides en wist bovendien ook nog eens alles over proteïne S. Dat is een heel leuk eiwit, zó leuk zelfs dat elke AIO er onderzoek naar zou willen doen. Bovendien had Tilman een hart van goud. Hij zorgde er bijvoorbeeld voor dat er steeds voldoende bier voorradig was zodat de medewerkers van de vakgroep Biochemie nooit te klagen hadden over een droge keel. De goedhartigheid van Tilman ging zo ver dat hij ooit een verwaarloosd straathondje helemaal van Marokko naar Nederland smokkelde om het daar een beter leven te geven. Natuurlijk wist Tilman helemaal niet hoe je een Marokkaanse hond precies moet opvoeden, maar gelukkig was er dan weer goede ouwe Guido om hem met raad en daad bij te staan. Toch was het kwaad dan al lang geschied, want het hondje was zwaar getraumatiseerd geraakt omdat het de lange autorit in Tilmans koelbox onder een kratje Heineken had moeten doorbrengen. Daardoor raakt het arme beestje nu bij elke regenbui vreselijk in paniek, iets wat volgens Guido nooit was gebeurd indien het hondje de hele rit tussen flessen geuze van het Pajottenland had kunnen zitten!

Gerry, Guido en Tilman waren zeker niet de minsten, maar de meest voorname man van de vakgroep was zonder twijfel Jan. Hij stond niet alleen aan het hoofd van de vakgroep Biochemie, maar was bovendien ook de promotor van het Halse jongetje. Jan was gek op pieken en dalen. Dat kwam vooral tot uiting in zijn voorliefde voor trombine-generatiecurves, maar geregeld probeerde hij ook de prestaties van Eddy Merckx te evenaren in het hooggebergte. Net zoals Guido en Tilman was ook Jan een echte dierenvriend, zo was hij bijvoorbeeld verlekkerd op ganzenlevertjes! Ooit heeft Jan zelfs de goudvisjes van Betta van een gewisse dood gered nadat prinses Emma en piraat Mats de visjes iets te ijverig hadden gevoederd. Een zeer nobele daad, al beweren kwatongen dat zijn vrouw Sjoukje die dag een zalmmootje te weinig had gekocht voor bij het avondeten! Vreemd genoeg heeft Betta, de Italiaanse dierenvriendin van de vakgroep, nooit een klacht ingediend wegens goudvismishandeling. Dat heeft waarschijnlijk alles te maken

met het feit dat Betta zelf geen heilig boontje was. Als ze dan al eens legaal in het land verbleef, kwam ze ofwel in aanraking met de milieupolitie of werd ze in het midden van de nacht van haar bed gelicht door de Nederlandse drugbrigade. Desondanks heeft Betta nog steeds een blanco strafblad. Werd haar de hand boven het hoofd gehouden door de Italiaanse maffia? Of was haar geliefkoosd konijn Blackie misschien verre familie van het witte ratje, dat haar daarom beschermde? De waarheid zal wellicht nooit aan het licht komen, maar dat is helemaal niet erg, want Betta is een dierenvriend en die horen helemaal niet in de gevangenis thuis!

Net als Betta hebben Gerry, Jan, Guido en Tilman hun uiterste best gedaan om het Halse kereltje zo goed mogelijk bij te staan, maar zoals jullie zelf al merkten waren ze ook wel eens met andere dingen dan wetenschap bezig. Daarom kreeg het jongetje bijkomende hulp van de Drie Wijzen. Omdat dit een atheïstisch sprookje is, kwamen ze niet uit het Oosten, maar uit Zweden, Frankrijk en de Verenigde Staten. Deze Drie Wijzen, alias Björn, Bruno en Paul, brachten allerlei waardevolle geschenken mee zoals DNA-constructen, gezuiverd factor V in fabelachtig hoge concentratie, een cd van Weezer én de geheime formule voor de bereiding van een elixir met antistollende werking. Maar daarnaast gaven ze ook hun wijsheid met plezier door!

Vanzelfsprekend kon de boog niet altijd gespannen staan. Dan was er Stella, die haar naam zeker niet gestolen had. Stella hield niet alleen van tijd tot tijd van een biertje en deelde die ook graag kwistig uit, maar bakte ook geregeld bitterballen en loempia's voor wie er zin in had. Daarnaast zorgde Stella er ook voor dat op het lab alles organisatorisch vlekkeloos verliep. Maar de vrouw die écht de touwtjes in handen had op de afdeling was Trees. Zonder deze lieve, maar met ijzeren hand regerende secretaresse liep de administratie van heel de vakgroep waarschijnlijk danig in het honderd! Trees zorgde er ook voor dat het koffieapparaat steeds keurig werd bijgevuld en onderhouden. Een zeer belangrijke taak, want de meeste wetenschappers kunnen immers niet zonder hun dagelijkse dosis cafeïne. Daarom alleen al zou Trees op elke Biochemie-publicatie moeten vermeld worden!

Ooit was Trees secretaresse in een gloeilampenfabriek, maar op aandringen van de Indonesische ratjes uit de Rattentempel - Trees kwam namelijk ook uit het Land van Tempo Doeloe - gaf ze die baan met plezier op om als secretaresse aan de Universiteit Maastricht een rol te kunnen spelen in het sprookje van het Halse jongetje. Volgens het originele script zou ze de rol van Assepoester spelen, maar al tijdens de auditie bleek dat dat niet geloofwaardig zou overkomen. Trees had namelijk niet één paar schoenen dat haar paste, maar ze had er zo minstens 40 in haar schoenenkast staan! Het witte ratje besloot dan maar om Trees de rol van Sneeuwwitje te geven. Er was maar één probleem: waar vind je heel snel 7 dwergen? Gelukkig wist Jan hier wel raad mee: hij zocht ze van Membruggen tot Wit-Rusland en vond ze nog ook! Die 7 dwergen (Kristien, Jeroen, Lisbeth, Svetlana, Connie, Olivier en Rudi) maakten heel wat plezier met het Halse jongetje, zowel tijdens als buiten de werkuren. Zo werd er niet alleen vaak stevig geborreld, maar werd er ook al eens getafeltenist, gekwist, een filmpje opgenomen, een

voetbalpronostiek georganiseerd en zelfs gedart. Maar ook met de niet-dwergen van de vakgroep beleefde het jongetje heel wat leuke avonturen!

Toch beleefde het Halse jongetje zijn leukste avontuur met Elke, een meisje dat hij in Maastricht had leren kennen. Elke was een heel lief meisje, alleen gebruikte ze geregeld vreemde woorden zoals "kleinvlees", "tuf", "pitslamp" of "uitleuren". Dat lag niet zozeer aan het meisje zelf, maar veeleer aan haar afkomst. Elke kwam namelijk uit een heel klein dorpje dat diep in de bossen lag verscholen en waar de mensen een nogal bizar taaltje spraken. Ondanks deze kleine taalbarrière konden de twee het toch goed met elkaar vinden. In het begin maakten ze wat fietstochtjes en wandelingen rond haar dorpje, maar al snel ontdekte het meisje dat er leukere dingen bestonden dan madeliefjes plukken en eekhoorntjes tellen. Zo reisden ze al snel de halve wereld rond en leerde het Halse jongetje dat er zowaar nóg vreemdere volkeren en culturen bestonden dan die van zijn vriendinnetje!

Al hoefde hij daar eigenlijk niet eens ver voor te reizen, want de broer van het jongetje had een vriendin uit een dorpje waar het de gewoonte is dat iedereen zijn zakken vult met het geld van iemand anders, pubertjes banken overvallen en het eten met "pwaf" wordt gekruid. Vreemd genoeg was ook dit meisje aardig. "Zou er dan een omgekeerd verband bestaan tussen de aardigheid van een meisje en haar afkomst?", vroeg het Halse jongetje zich af. Volgens Elke was er statistisch gezien geen enkele reden om het tegendeel te beweren. En met deze geruststellende gedachte leefden ze nog lang en gelukkig!

De afgelopen vier jaren waren waarschijnlijk heel anders geweest indien de ontmoeting tussen het witte ratje en het echtpaar uit Halle, mijn ouders, nooit had plaatsgevonden. Hen wil ik hier dan ook nog eens speciaal bedanken, meer nog dan iedereen die in dit sprookje een rol speelde. Al verdient ook Gerry een bijzondere vermelding. Je was misschien niet gek op geuze uit het Pajottenland, maar ondanks deze jeugdzonde had ik me – in alle opzichten – geen betere begeleider kunnen indenken! Ten slotte wil ik de vele vriendjes en vriendinnetjes die de voorbije vier jaar voor de nodige ontspanning en afleiding hebben gezorgd, nog even bedanken. Voor jullie was er jammer genoeg geen eervolle vermelding in het sprookje weggelegd, maar seriemoordenaars, kinderverkrachters, gok-, drugs- en alcoholverslaafden horen nu eenmaal niet in sprookjes thuis!

'Kinnie'

Curriculum vitae

De auteur van dit proefschrift werd op 19 november 1979 geboren in Halle (België), waar hij ook de rest van zijn jeugd doorbracht. Na het verlaten van de plaatselijke basisschool, tegenwoordig het decor van de befaamde politiserie 'Witse', startte hij in het najaar van 1991 zijn middelbare opleiding aan het Onze-Lieve-Vrouwe-College in Halle. In 1997 behaalde hij het diploma Latijn-Wetenschappen, waardoor hem een mooie toekomst als pater of wetenschapper toelachte. Hoewel het vervaardigen en het uitvoerig proeven van trappistenbieren en -kazen hem wel wat leek, werd het vroege opstaan voor het ochtendgebed toch als een struikelblok aangezien. Daarom startte hij in datzelfde jaar met de opleiding bio-ingenieur aan de Vrije Universiteit Brussel (VUB). Die studie rondde hij in 2002 met onderscheiding af, waarbij hij de graad van bio-ingenieur in de cel- en genbiotechnologie verkreeg. In het daaropvolgende jaar verrichtte hij onderzoek naar de structuur en functie van het nucleoside hydrolase van *Mycobacterium tuberculosis* aan het departement Ultrastructuur van de VUB. In februari 2004 begon hij als Assistent In Opleiding bij de vakgroep Biochemie van het Cardiovasculair Onderzoeksinstituut van de Universiteit Maastricht. Onder begeleiding van zijn promotor prof. dr. Jan Rosing en zijn copromotor dr. Gerry Nicolaes verrichtte hij fundamenteel onderzoek naar bloedstollingsfactor V, waarvan de resultaten in dit proefschrift staan beschreven.

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