Lessons from snake venom: new insights into the structural and functional aspects of factor V and factor X

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Chapter 7

General discussion and future directions

Circumventing the paradigm of prothrombinase assembly

The spatiotemporal assembly of the prothrombinase complex is tightly regulated and occurs exclusively on negatively charged membrane surfaces of activated cells or platelets, where activated factor X (FXa) assembles with its cofactor activated factor V (FVa) in the presence of calcium ions [1]. These molecular constraints are paramount to normal hemostasis as they serve to prevent fibrin clot formation at other sites away from the location of vascular injury. The venom of the Australian common brown snake *Pseudonaja textilis* contains a powerful prothrombin activator (Pseutarin C) that is structurally and functionally similar to the mammalian prothrombinase complex [2, 3]. However, both venom-derived FXa and FVa comprise unique molecular adaptations that serve to circumvent the conventional constraints of human FXa-FVa complex assembly [4-6]. For example, the venom-derived homolog of FVa (ptFV) is constitutively active due to the absence of a regulatory B-domain sequence [5] and is able to form a high affinity complex with venom-derived FXa (ptFXa) without the need for anionic phospholipid membranes or calcium ions [7]. In addition, both ptFV and ptFXa display prolonged procoagulant activity in circulation, as both proteins are insensitive to inhibition by their respective plasma regulators activated protein C (APC) [7] and antithrombin [8]. These gain-of-function adaptations enable the 'venom prothrombinase' to escape hemostatic regulation and to initiate indiscriminate clotting throughout the vasculature. The aim of this thesis has been to uncover the unique structural and functional relationships that govern the venom FXa-FVa complex assembly and function, with the intention to harness its potent procoagulant potential.

Part I - Factor V

Alternative splicing of the FV B-domain

In the first part of this thesis, we studied the genomic, structural and functional implications of several molecular modifications found in the *P. textilis* venom FV molecule. FV normally circulates in blood as an inactive procofactor (domain organization A1-A2-B-A3-C1-C2), and is only converted to its active form upon proteolytic removal of its central regulatory B-domain sequence [9, 10]. Previous studies have shown that venom FV is constitutively active due to the absence of a regulatory B-domain in both venom- and liver-derived *P. textilis* FV sequences [5, 7, 11]. In **chapter 2** of this thesis we have investigated whether absence of this B-domain sequence from liver-derived FV is unique to *P. textilis* physiology, or whether this trait is shared among other snakes. In contrast to previous studies [11, 12], we revealed that the liver transcriptome of *P. textilis* and other snake species included both full-length and B-domain deleted transcripts, suggesting unique functional diversification of the FV gene in members of the serpentes suborder. Additional data point to a conserved pre-mRNA exonic splicing enhancer (ESE) motif as potential regulator for alternative splicing of the full-length snake

B-domain transcript [13, 14]. As such, our findings constitute the first report of alternative splicing of the FV B-domain in a non-human species.

Thus far, alternative splicing of the FV B-domain has first been observed in patients that present with a rare bleeding disorder which is caused by supra-physiological plasma levels of Tissue Factor Pathway Inhibitor (TFPI) [15, 16]. In these patients, alternative splicing of the B-domain causes a FV variant known as FV-short to become a carrier protein for TFPI, potentially due to exposure of a high affinity binding site on the acidic region (AR) of the truncated B-domain sequence [17, 18]. Normally, the AR forms an essential part of the B-domain procofactor regulatory region (PRR) that, in conjunction with the B-domain basic region (BR), serves to stabilize the procofactor state of FV [19]. However, the essential BR is absent in FV-short due to activation of an alternative splice donor site and subsequent splicing. In a similar fashion, in **chapter 2** of this thesis we have shown that alternative splicing of the full-length snake FV B-domain also results in complete removal of the BR, leaving only a ~46 amino acid B-domain sequence that is distinctly acidic in nature. Potentially, this alternative acidic region could also function as a neo-epitope for TFPI binding by alternatively spliced snake FV. As TFPI is an essential inhibitor of prothrombinase activity and FXa [17, 20], high TFPI levels in snake plasma could possibly contribute to alternative regulation of prothrombinase activity. Additional research is therefore required to confirm the physiological presence of this alternative snake FV-short molecule in snake plasma, and to evaluate plasma levels of key anti-coagulant proteins such as antithrombin, protein C, and TFPI, as well as to assess the potential role of the truncated B-domain FV variant with regard to plasma TFPI regulation. These studies will undoubtedly provide new insights into the role of alternatively spliced FV molecules in hemostasis and their potential use in novel anti-thrombotic strategies.

Lipid-independent FVa cofactor function

The ability of ptFV to form a high affinity complex with ptFXa without the need for assembly on an anionic membrane surface has enabled Lechtenberg and co-workers to acquire an X-ray structure of the venom prothrombinase complex [21]. In addition to providing original insights into the potential conformation of the human prothrombinase complex, their model also uncovered structural features that were unique to venom ptFV-FXa. In **chapters 3 and 4** of this thesis we examined some of these distinct structural features in more detail and investigated their implications to prothrombinase complex assembly and function. In **chapter 3** we examined whether a non-conserved covalent link between the A2- and A3-domains of venom-derived ptFV induced potential conformational constraints that were essential to its lipid-independent cofactor function [7]. In addition, we investigated whether liver-derived ptFV was able to support lipid-independent prothrombin conversion by venom ptFXa as well. In short, both venom and liver

ptFV were found to support lipid-independent prothrombin conversion, irrespective of the presence or absence of a non-conserved covalent link between the A2- and A3-domain in either ptFV species. In addition, we established that this disulfide bond was not essential to cofactor function upon proteolytic degradation by APC. Moreover, liver ptFV was also functionally resistant to degradation by APC.

In **chapter 4** of this thesis we explored whether the venom C-domain pair was able to induce lipid-independent prothrombinase function by analyzing chimeric variants of FV that comprised either the A-domains of human FV and the C-domains of *P. textilis* venom FV, or vice versa. Functional analysis of these chimeras showed that the venom FV C-domains were of little relevance to its lipid-independent cofactor function, as the chimera comprising the venom A-domain trimer and human C-domain pair was also able to support cofactor function in the absence of phospholipids. In contrast, the chimera that comprised the human A-domain trimer and venom C-domain pair was not able to support lipid-independent cofactor function. These results show that high affinity binding of ptFV to venom ptFXa in solution is exclusively governed by the ptFV A-domain trimer. Additional functional analysis of structural features that are unique to the venom A-domain trimer are required to shed more light on the molecular requirements that govern lipid-independent cofactor function. One such feature includes the C-terminal part of the venom A2-domain which was shown to form an extended interaction interface with ptFXa in the crystallographic model of venom prothrombinase [21].

Functional crosstalk between the A and C-domains of FV

The multi-domain structure of the two chain molecule human FVa is recognized as inherently unstable and prone to spontaneous disassociation upon proteolytic removal of its central B-domain. Previously, we had reported that ptFV is functionally highly stable, as it retains cofactor activity upon extended proteolytic processing by the endogenous enzymatic regulator of FVa activity, APC [7, 22]. In addition, ptFV has been reported to display significantly reduced binding to phosphatidylserine-containing membranes [23]. As shown in **chapter 4**, the C-domains of ptFV confer cofactor stability when linked to the A-domains of human FV while, in turn, the A-domains of human FV improve phospholipid binding by the C-domains of ptFV. These unique findings imply functional crosstalk between the A-domain trimer and C-domain pair. We propose that such crosstalk is facilitated by transient interactions between the A-domain trimer and elongated loops (hFV: Ser1971-Val1987 and Ser2130-Val2146) that cover the crest of the C1- and C2-domains. Other studies have shown that, depending on the relative orientation of the C-domain pair, these elongated loops may potentially associate with each other [24], or alternatively with the A-domain trimer [25]. In addition, a more recent study used atomic force microscopy to show that the C-domains of FV undergo dynamic rearrangements while in solution, suggesting conformational

flexibility [26]. In **chapter 4**, we speculate that such conformational flexibility may be required for high affinity binding to procoagulant membranes. On the other hand, stabilization of inter-domain contacts between the A-domain trimer and C-domain pair may impair membrane association. However, additional engineering of FV is recommended to fully investigate the functional relationship between the elongated loop structures at the apex of the C1- and C2-domain and the A3-C1 domain interaction interface. Also, characterization of FV constructs by CD spectroscopy is suggested to survey the overall folding of the native and chimeric constructs.

The C-domains of ptFV bind phospholipids

It was previously reported that membrane association was abolished in the *P. textilis* venom-FV molecule [27]. However, in **chapter 4** we observed that the venom C-domains were able to bind phospholipid membranes when coupled to the A-domain trimer of hFV, albeit with a lower affinity and reduced phosphatidylserine sensitivity relative to the human C-domain pair. It is generally acknowledged that dedicated phosphatidylserine-binding residues facilitate membrane binding by FV. These residues are located on three separate membrane binding loops which protrude from the base of each C-domain [25, 28-32]. In the **4th chapter**, sequence analysis of these lipid-binding regions in ptFV revealed general sequence conservation of the first and second C1 membrane-binding loops [25]. The essential hydrophobic phospholipid binding pair [31, 32] on the third C1 loop is also partially conserved. However, most of the solvent exposed positively charged residues have been substituted in ptFV. Nonetheless, two essential phosphatidylserine-binding arginine residues remain in ptFV [30]. In addition, two essential aromatic phospholipid-binding residues on the first C2 phospholipid binding loop [29] are conserved as well. In contrast, the ptFV C2-domain displays poor amino acid conservation of the second membrane binding loop and moderate conservation of the third loop. In summary, several essential phospholipid binding features have been preserved in the C-domains of ptFV that could generally enable membrane anchoring, yet marked differences occur within specific phospholipid binding regions that may account for the reduced affinity to phosphatidylserine containing membranes in ptFV. In contrast to previous reports [23], we conclude that the ptFV C-domain pair has retained the ability to bind membrane surfaces, despite marked differences within specific phospholipid binding regions.

The venom A-domain trimer impedes phospholipid binding

In **chapter 4**, we show that the C-domains of ptFV can bind anionic phospholipids when coupled to the human A-domain trimer. Correspondingly, the chimeric variant that comprised the venom A-domain trimer and human C-domain pair displayed reduced phospholipid binding relative to hFV. This observation indicated that the venom A-domain trimer was less conducive to phospholipid binding. We

speculate that the unique disulfide bond between the A2- and A3-domain in ptFV may potentially constrain cofactor movement, impeding structural rearrangements required for phospholipid binding [23]. As removal of the disulfide bond from ptFV was shown to decrease overall thermal stability in **chapter 4**, we suspect that phospholipid binding might potentially be augmented in the variant lacking the covalent link between the A2- and A3-domain. Assessment of phospholipid binding by this variant is therefore recommended to investigate the role of this covalent link in ptFV with regard to phospholipid binding [22]. Efforts to introduce this covalent link in human FV have not been successful thus far, limiting our ability to study its effects on phospholipid binding and cofactor stability in the setting of human FV [33]. Implementation of dedicated protein modeling software, such as Molecular Operating Environment (MOE) [34], is recommended to improve disulfide bridging *in silico* before additional engineering strategies are attempted. Specifically, mutation of amino acids that surround a potential Cys-Cys pair may improve formation and stability of a disulfide bond [35, 36].

Part II - Factor X

Modulating inhibitor sensitivity in FXa

The second part of this thesis is focused on the structural and functional aspects of venom FX. In **chapter 5** of this thesis we discovered that venom derived FXa was highly insensitive to direct FXa inhibitors, unlike any FXa species known to date. This essential finding formed the basis of a new line of research that focused on developing a FX-based antidote against the direct FXa inhibitors. Inhibition of FXa by direct FXa inhibitors such as apixaban, rivaroxaban, or edoxaban is steadily becoming the mainstay of oral anticoagulant therapy due to their ease of dosing and relatively short half-life [37-41]. However, a major drawback to the use of direct FXa inhibitors is the absence of a safe and effective reversal strategy in the event of (trauma induced) bleeding [42-45]. Using a combined computational and biochemical approach we revealed that insertion of a heterologous segment into the 99-loop of venom FXa resulted in direct FXa inhibitor insensitivity in venom FXa. Introduction of similar structural features into the 99-loop of human FXa led to the engineering of FXa variants that were able to support coagulation in human plasma spiked with (supra-)pharmacologic concentrations of direct FXa inhibitors. As such, these FXa variants have the potential to be employed to bypass the direct FXa inhibitor-mediated anticoagulation in patients that require restoration of blood coagulation. The inhibitor-insensitive FXa variants have been patented [46], and are currently in (pre)clinical development as direct FXa inhibitor reversal agent. In addition to this work, we also applied insertional mutation of the 99-loop to successfully engineer inhibitor-insensitivity in thrombin. This work was also filed in a second patent on inhibitor-insensitive serine proteases [47]. Taken together, our findings highlight the general significance of 99-loop architecture in

modulating inhibitor insensitively in trypsin-like serine proteases and demonstrate the potential of studying natural homologs as a foundation for knowledge-based protein engineering.

Despite these evident successes, additional scientific work is still required to address a number of fundamental matters concerning chimeric FXa and thrombin variants. Essentially, we have not been able to clearly demonstrate how the length and amino acid composition of the 99-loop precisely modulates enzymatic activity and inhibitor insensitivity. For example, it is still unclear whether the inhibitor insensitivity can also result from steric hindrance by the 99-loop (potentially due to extension over the active site) as observed in kallikreins [48], in addition to an effect on overall protein motion and S4 subsite architecture [49, 50]. Furthermore, it is uncertain whether the 99-loop maintains a similar fold in chimeric FXa as that observed for the secondary structure of the 99-loop in venom derived FXa [21]. Structural and functional characterization of additional FX variants with 99-loop insertions varying in length and amino acid composition is therefore required to shed more light on these issues. Interestingly, a decreased sensitivity towards direct FXa inhibitors was also observed in FXa variants comprising substitutions at position Phe174 [51]. This amino acid forms part of the S4 subsite that is engaged by the direct FXa inhibitors [37, 39, 41]. Therefore, additional substitutions targeting the 174 position, as well as the amino acids immediately surrounding this position, should be investigated in more detail for their potential to modulate the sensitivity to the direct FXa inhibitors.

Differential inhibition of FXa in non-human plasmas

The discovery of inhibitor insensitive FXa variants had prompted us to categorically evaluate global clotting parameters in non-human plasma in order to facilitate the use and interpretation of animal models for the preclinical assessment of FX(a)-based bypassing agents. As outlined in **chapter 6** of this thesis, we assayed mouse, rat, rabbit, porcine, goat, and human pooled plasma using relevant coagulation assays such as the prothrombin time (PT), activated partial thromboplastin time (APTT), and calibrated automated thrombography (CAT). In short, we established that rabbit, rat, and mouse plasmas exhibited robust clotting pathways that differ distinctly from human, goat and porcine plasma when evaluated in clinical coagulation assays. In addition, we discovered fundamentally higher rates of FXa inhibition in rabbit, rat and mouse plasma, pointing to an overall higher anticoagulant threshold in these plasmas relative to human, goat, and porcine plasma. Overall, rat plasma was established as relevant small animal model for the preclinical assessment of human FX(a) variants. Apart from general implications on the selection preclinical models, it is thought-provoking to point out that higher FXa-inhibitory rates were observed in smaller mammals and vice versa, suggesting a potential link between species physiology, heart rate, and anticoagulation. On

another note, an association between physiology and coagulation has been found in human subjects also. For example, several studies have reported an increased thrombin generation potential in humans with a higher body mass index (BMI) [52-54]. On the other hand, a higher BMI may also be linked to induced coagulation through inflammatory pathways [55, 56]. In general, we advocate studying non-human coagulation systems in order to generate novel insights into the relationship between physiology and coagulation. With respect to the regulation of FXa, efforts should be made to assess the levels of FXa inhibitors such as TFPI, AT, protein Z-dependent protease inhibitor (ZPI), and alpha2-macroglobulin (a2M) in plasmas from larger and smaller mammals. Insights obtained from these types of studies are especially important, as the hemostatic system not only protects against thrombosis and bleeding, but is also intricately linked to systemic processes such as angiogenesis [57], inflammation [58], and innate immunity [59].

Conclusions

In this thesis, we have studied the functional and structural properties of the powerful prothrombin activator complex (ptFV-FX) from the venom of the Australian snake *P. textilis*. With respect to the non-catalytic subunit of this complex, we have found that 1) the absence of a regulatory B-domain in ptFV is most likely the result of a previously unidentified alternative splicing event. In addition, we have uncovered that 2) the unique disulfide bond in ptFV is not required for lipid-independent cofactor function. Furthermore, we have 3) established the presence of functional crosstalk between the A-domains and C-domains of FV by characterizing chimeras of human and ptFV. Therefore, by studying the venom-derived FV molecule we were able to shed new light on structural and functional relationships that govern FV function and prothrombinase complex assembly. With respect to the venom catalytic subunit ptFXa we have shown that 4) the elongated 99-loop mediates resistance to plasma-based FXa inhibitors and synthetic FXa inhibitors and that 5) introduction of this loop into human FXa enables protease function in plasma spiked with supra-physiological concentrations of direct oral FXa-inhibitors. As such, the ptFXa molecule proved to be an exceptional blueprint for the rational design of a unique gain-of-function adaptation to human FXa. In conclusion, our studies highlight the significance of studying the structural and functional aspects of venom-derived proteins for prospective protein engineering strategies.

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