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The unique procoagulant adaptations of pseudonaja textilis venom factor V and factor X

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Chapter 1

General Introduction and Outline of the Thesis





Hemostasis

The cardiovascular system is responsible for the continuous flow of blood that delivers essential nutrients and oxygen to cells throughout the body and transports metabolic degradation products away from distal tissues. Upon vascular damage, a process known as blood coagulation is initiated, which acts to form a hemostatic plug at the site of injury to arrest bleeding and maintain hemostasis. This process consists of two main components known as primary and secondary hemostasis. In primary hemostasis, the loss of blood is initially limited by the contraction of smooth muscle cells underlining the vascular endothelium, resulting in a reduction of blood flow. Subsequently, platelets are activated and aggregate at the site of injury, forming a physical barrier known as the primary platelet plug¹. Secondary hemostasis serves to stabilize and strengthen the blood clot by forming an insoluble mesh of fibrin in and around the platelet plug². The deposition of fibrin is the result of a series of enzymatic reactions that take place on the surface of activated platelets and endothelial cells via a 'cascade' principle (**Figure 1**). This so-called coagulation cascade traditionally consists of two different initiation pathways that both culminate into the common pathway with the activation of factor X (FX), the generation of thrombin, and ultimately the formation of fibrin.

Coagulation Cascade

In the coagulation cascade, the intrinsic pathway is triggered by negatively charged surfaces, such as kaolin or glass³. This results in the activation of factor XII (FXII), prekallikrein, and high molecular weight kininogen. Upon the activation of FXII, the coagulation proteins factor XI (FXI), factor IX (FIX), and FX are converted to their activated state via a series of concerted proteolytic reactions.

The extrinsic pathway starts upon the exposure of subendothelial tissue factor (TF) to blood and the subsequent binding of circulating factor VII(a) (FVII(a)) to TF. The resulting FVIIa-TF complex immediately initiates the common pathway by converting factor X to factor Xa (FXa). In addition, the TF-FVIIa complex activates part of the intrinsic pathway by proteolytic conversion of FIX to factor IXa (FIXa). The extrinsic pathway is activated by external trauma and is therefore considered as the most common trigger of the coagulation cascade.

Both the intrinsic and extrinsic pathways converge into the common pathway with the generation of FXa. FXa assembles into the prothrombinase complex by binding to its cofactor factor Va (FVa). The spatiotemporal assembly of the prothrombinase complex occurs exclusively on the surface of anionic membranes in the presence of calcium

ions. This complex rapidly converts prothrombin into the active protease thrombin, a key step in the formation of a blood clot. Subsequently, thrombin converts soluble fibrinogen into the insoluble fibrin which spontaneously polymerizes into long fibrin strands. In addition, thrombin initiates several positive feedback loops by activating FXI and the cofactors FVIII and FV in order to generate sufficient amounts of fibrin. The fibrin strands are cross-linked by factor XIIIa (FXIIIa) to create a strong and dense fibrin network resulting in the formation of a stable blood clot.

Coagulation Factor V

Coagulation FV circulates in plasma as a large single-chain procofactor of ~330 kDa, comprising an A1-A2-B-A3-C1-C2 domain structure (**Figure 2A**). The B domain contains a basic and a complementary acidic region that interact with each other, thereby creating an autoinhibitory conformation that is assumed to prevent FXa binding^{4,5}. Factor V is mainly synthesized by the liver and excreted in the plasma. The majority of the total FV pool circulates in plasma, whereas ~20% is stored in platelet α -granules and is secreted upon platelet activation⁶.

The activation of factor V is facilitated by FXa and meizothrombin in the initiation phase and subsequently by thrombin in the propagation phase of coagulation⁷⁻¹⁰. Sequential cleavage at B domain residues Arg709, Arg1018, and Arg1545 results in the dissociation of the B domain and the generation of a heavy (A1-A2) and light (A3-C1-C2) chain that are non-covalently linked by divalent metal ions (**Figure 2A**)^{11,12}. Once partially or fully activated, FVa is able to serve as a cofactor for FXa through assembly into the prothrombinase complex. This complex increases the catalytic rate of prothrombin activation by five-orders of magnitude relative to membrane-bound FXa¹³, illustrating the physiological relevance of the prothrombinase complex in prothrombin activation.

Apart from its procoagulant function, thrombin fulfills an anticoagulant role by converting protein C to the protease activated protein C (APC) in the presence of thrombomodulin and the endothelial protein C receptor¹⁴. Activated protein C plays a crucial role in the downregulation of the procoagulant response by selective proteolysis and subsequent inactivation of FVa. It exerts this function by cleaving FVa at the A2 domain residues Arg306, Arg506, and Arg679, resulting in the dissociation of the A2 domain^{15,16}. The kinetically rapid cleavage at Arg506 results in a partially active FVa intermediate, which is fully inactivated by the relatively slow proteolysis at Arg306¹⁷. The functional relevance of the Arg679 cleavage is currently not fully understood. The APC-induced inactivation of FVa is further stimulated by the nonenzymatic cofactor protein S, which specifically accelerates the cleavage at Arg306¹⁸.

Coagulation Factor X

Coagulation FX is a vitamin-K dependent serine protease and one of the central components of the coagulation cascade (**Figure 2B**). Factor X is composed of a heavy and light chain that are covalently linked via a disulfide bond. The light chain comprises a γ -carboxyglutamic acid-containing domain that is responsible for anionic phospholipid binding and two epidermal growth factor-like domains that have been implicated in macromolecular interactions^{2,19-22}. The heavy chain consists of an activation peptide that prevents unwanted catalytic activity and the serine protease domain that comprises the active site which is hallmarked by the catalytic triad residues His⁹⁵, Asp¹⁰², and Ser¹⁹⁵ (chymotrypsinogen numbering).

Factor X circulates in plasma in a zymogen form and is converted into FXa (**Figure 2B**) by the intrinsic (factor VIIIa (FVIIIa) and FIXa) or extrinsic (TF and FVIIa) tenase complexes via proteolytic removal of the activation peptide upon cleavage of the highly conserved Arg¹⁵-Ile¹⁶ scissile bond. The transition to a fully active protease requires conformational changes in the serine protease domain that realign the active site and exosite regions^{23,24}. These conformational changes are induced when the newly formed heavy chain N-terminus (Ile¹⁶) engages with the active site residue Asp¹⁹⁴ to form a Ile¹⁶-Asp¹⁹⁴ salt bridge^{23,24}. The matured FXa comprises the essential S1 and S4 subpockets which determine the substrate specificity^{24,25}.

The central negative regulators of FXa are the active site inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin. TFPI is an anticoagulant protein that downregulates the early phases of the procoagulant response. Several alternatively spliced isoforms of TFPI are expressed; however, each splice variants comprises the Kunitz 1 and 2 domains. The Kunitz-1 domain of TFPI inhibits FVIIa that is complexed with TF, and the Kunitz-2 domain is responsible for FXa inhibition^{26,27}. Since TFPI is a weak inhibitor of TF-FVIIa in the absence of FXa, TFPI is often described as a FXa-dependent inhibitor of TF-FVIIa. As a consequence, TFPI appears to simultaneously inhibit FXa and TF-FVIIa immediately after the activation of FX by the TF-FVIIa complex²⁸. The other physiological relevant inhibitor of FXa is the serine protease inhibitor antithrombin. The inhibitory effect of antithrombin is relatively low but can be stimulated up to four-orders of magnitude in the presence of heparin. Antithrombin inhibits FXa via a substrate recognition sequence within its exposed reactive center loop^{29,30}. This sequence acts as a bait region that is cleaved by FXa resulting in a covalent antithrombin-FXa complex^{29,30}. Subsequently, this complex undergoes a major conformational change that stably traps and fully inactivates FXa^{30,31}.

Inhibition of Factor Xa and Thrombosis

A disruption of the hemostatic system can result in life-threatening bleeding disorders, such as hemophilia or pathological thrombosis. Because of its central role in the coagulation cascade, FXa has been a major drug target for antithrombotic therapy. Traditionally, oral vitamin K antagonists were used for the prevention and treatment of thrombosis, which effectively inhibit the hemostatic vitamin K-dependent proteases. However, their narrow therapeutic range and variable response to dosing provide several disadvantages that significantly affect their efficacy. In the past decades, the direct oral anticoagulants (DOACs) have been developed as an alternative anticoagulant treatment option for thrombosis-related disorders. The DOACs are small molecules that reversibly engage the active site of the key proteases FXa (apixaban, rivaroxaban, edoxaban, and betrixaban)³²⁻³⁵ or thrombin (dabigatran)³⁶. These drugs are characterized by a superior pharmacodynamic profile relative to the vitamin K antagonists, and as such these drugs are considered as the preferred anticoagulant therapy³⁷. Currently, the DOACs have been clinically approved for the prevention of stroke in atrial fibrillation patients, the treatment of thromboembolism, and thromboprophylaxis following orthopedic surgery³⁸⁻⁴².

Pseudonaja textilis Venom Factor V and Factor X

The procoagulant venom of the Australian Elapid snakes comprises a powerful prothrombin-activating enzyme complex consisting of FV- and FX-like proteins that are specifically expressed in the venom gland⁴³⁻⁴⁵. These coagulation proteins have evolved into potent toxins due to some remarkable gain-of-function adaptations that enable this prothrombinase-like complex to initiate coagulation in an uncontrolled manner⁴⁶⁻⁴⁸. In contrast to human FV, the venom FV homolog from *Pseudonaja textilis* (v-ptFV) was found to be a constitutively active cofactor for the venom-derived FXa (v-ptFXa)⁴⁷. Furthermore, both v-ptFV and v-ptFXa bypass the normal requirement of membrane binding by forming a high affinity complex in the absence of anionic phospholipids^{47,49}. In addition, v-ptFV and v-ptFXa are functionally resistant or display a reduced sensitivity towards their natural inhibitors APC or antithrombin, respectively^{45-47,49}.

Outline of this Thesis

In this thesis, we focus on the unique evolutionary adaptations of the *P. textilis* venom-derived proteins v-ptFV and v-ptFX. By using biochemical approaches, we assess the structure-function relationships of several uniquely modified structural elements.

Chapter 1

Secondly, we address current and newly emerging reversal agents for FXa-inhibiting DOACs as therapeutic option for life-threatening bleeding complications.

Identification of the binding sites responsible for protein-protein interactions provides fundamental information on functional important regions and allows for a better understanding of the biology of the specific proteins involved. In **chapter 2**, we review the current literature on the interactive residues and regions of FVa that are essential for prothrombinase assembly and prothrombin binding, forming the basis for further studies on functionally important regions in FVa.

The functional APC resistance of v-ptFV was studied in **chapter 3**. Since v-ptFV does not comprise an Arg306-like cleavage site that is essential for mammalian FV inactivation, we hypothesized that introduction of the human Arg306 region would inactivate v-ptFV upon APC-mediated proteolysis.

Sequence analysis has shown that ptFV comprises a significantly extended A2 domain C-terminus (A2T) compared to other FV species. In human FV, this structural element has been implicated in the formation of the prothrombinase complex and the engagement of prothrombin. In **chapter 4** we examined the functional role of the extended A2T in ptFVa.

In **chapter 5**, we studied the functional relevance of a particular modification in v-ptFX. The activation peptide of v-ptFX is significantly shortened compared to the FX activation peptide of other known organisms. We hypothesize that this venom-derived activation peptide plays an important role in the unique procoagulant characteristics of v-ptFX.

The additional focus of this thesis is on the reversal agents for the direct FXa inhibitors. Bleeding complications associated with DOAC-use remain the main concern in clinical practice. Patients presenting with major bleeds may require rapid reversal of the anticoagulant DOAC activity. **Chapter 6** summarizes the antidotes and bypassing agents that are currently approved or in (pre)clinical development.

In **chapter 7** we characterized a novel FX-based bypassing agent for the direct FXa inhibitors. In this study, several FX variants were generated by substituting a single residue in the FX S4 subsite. The FX variants were characterized and tested for their ability to restore thrombin generation in the presence of the direct FXa inhibitors.

Finally, **chapter 8** provides a general summary and discussion of this thesis.

Figures

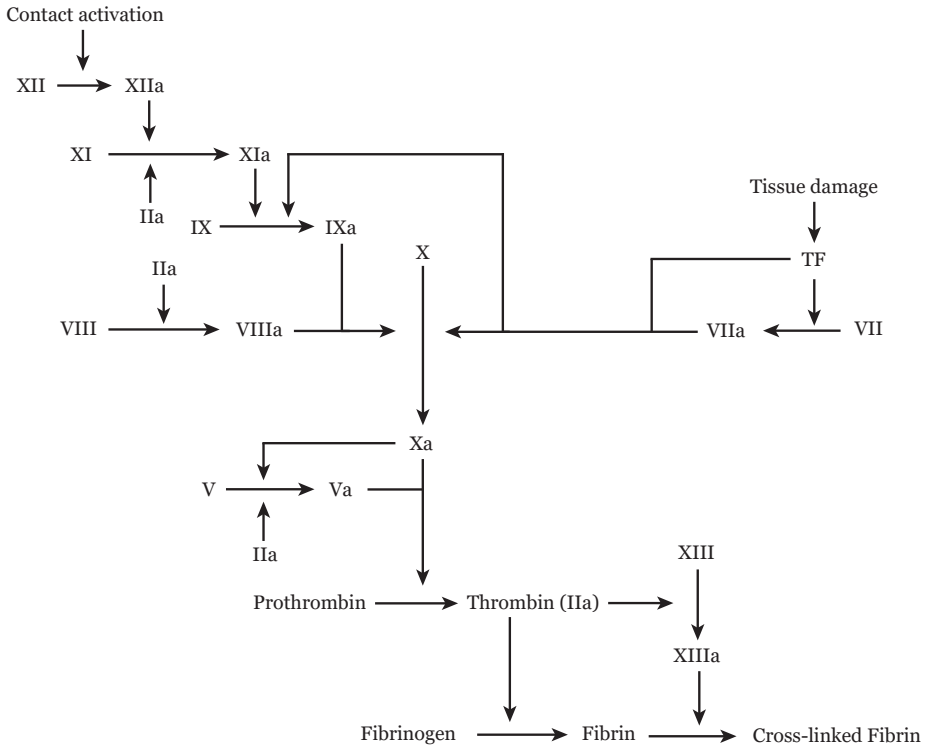


Figure 1. Schematic representation of the procoagulant reactions in the coagulation cascade. Coagulation is triggered by contact activation or tissue injury and activates the intrinsic or extrinsic pathway, respectively. The activation of factor Xa initiates the common pathway and ultimately results in the formation of a fibrin network. TF indicates tissue factor, and the blood coagulation factors are indicated by their Roman numerals.

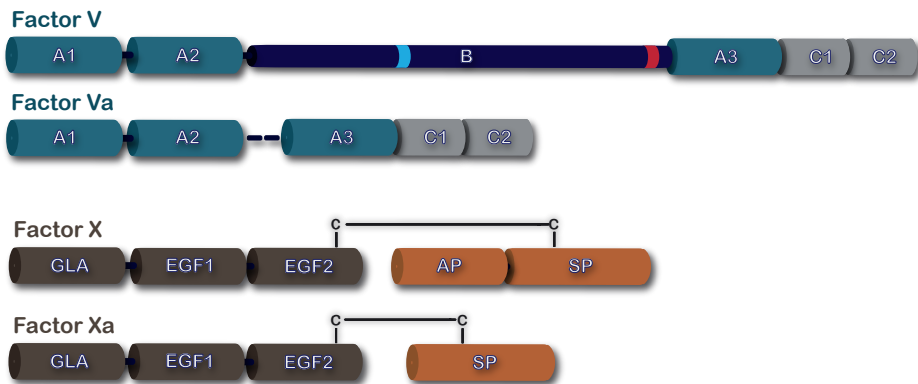


Figure 2. Schematic representation of precursor and activated forms of human coagulation factor V and factor X. (A) Factor V is composed of an A1-A2-B-A3-C1-C2 domain structure in which the A domains are involved in macromolecular interactions and the C domains interact with the lipid membrane layer. The B domain comprises the basic (blue) and acidic (red) regions that keep factor V in a precursor state. Upon activation of factor V, the B domain is proteolytically removed thereby generating factor Va. **(B)** Factor X comprises a lipid-binding γ -carboxyglutamic acid (GLA) domain, two epidermal growth factor (EGF) domains, an activation peptide (AP), and the serine protease (SP) domain. Factor X is converted to factor Xa upon cleavage and dissociation of the activation peptide.

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