

Prothrombinase complexes with different physiological roles

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It is a curious irony that a protein complex that plays a vital role in protecting one organism from bleeding to death is also utilised by another to immobilise its prey, commence digestion by its trypsin-like activity and ensure its own survival. In addition to an armamentarium that includes phospholipases and neurotoxins, venom from the Australian elapid snakes is a rich source of prothrombinase (1, 2). This complex is composed of factor FXa- and FVa- like proteins, both of which have a significant sequence identity with their human counterparts (3, 4). Two recent reports, one of which is featured in this issue of *Thrombosis and Haemostasis* (5), provide important insight into the characteristics of the FX- and FV-like proteins in the venom of Australian elapid snakes, and how they differ from the protein prothrombinase proteins involved in haemostasis (6). What is evident for these snake venoms is that they possess different potent prothrombotic activities that function in the circulation of the prey to induce haemostasis and immobilisation in the prey.

The prothrombinase activators from Australian snake venoms are distinguished by their cofactor requirement for blood coagulation and by their capacity to cause coagulation of anticoagulated blood (e.g. in patients on heparin or warfarin). The activity of the Group C prothrombin activators requires Ca^{+2} only for activation, whereas the Group D activators require Ca^{2+} , phospholipid and FVa (7, 8). The Group C prothrombin activator from the Australian elapid snake *Pseudonaja textilis* is a large protein complex made up of catalytic (pt-FXa) and non-catalytic (pt-FVa) subunits that bear functional resemblance to their mammalian counterparts but have unique characteristics (5, 6, 9, 10). The *P. textilis* prothrombinase represents approximately 40% of the dry weight of the venom, while two closely related species, *Oxyuranus scutellatus* (Coastal taipan) and *Oxyuranus microlepidotus* (Inland taipan), have 10–20% prothrombinase complex in the venom (11). However, during envenomation, both taipan species deliver approximately the same amount of functional prothrombin activator as *P. textilis*. What is notable about these prothrombinases is that their potency is several orders of magnitude greater than the mammalian prothrombinases in the

rate of conversion of prothrombin to thrombin. This can be explained by unique structural features of both components that include Ca^{+2} and phospholipid independence, resistance to cleavage by activated protein C and loss of the thrombin cleavage site on the FV-like protein.

Moreover, Australian elapid snakes are unique in that they possess two closely related but distinct prothrombinases, one in the venom and the other produced by the liver and present in plasma (12). The complex produced by the liver to provide blood circulating components fulfils the same function as its mammalian counterpart for haemostasis; while we have seen that the complex in the venom acts as a toxin, it has been evolutionary focussed to clot the blood of the prey. The presence of the same catalytic activity in proteins with different physiological roles is unusual and is suggestive of altered evolutionary pathways. It seems likely that the evolutionary process involves duplication of genes that produce normal blood circulating proteins and subsequent selective expression of these in the venom gland (13). Considerable insight into the evolutionary process has been provided by Reza et al (9) who identified two isoforms of FXa in the liver of *P. textilis*. One of these, PFX1, is more closely related to an FX-like cDNA of a related snake *Tropidechis carinatus* than its own plasma counterpart in sequence identity and size of the activation peptide. On the other hand, isoform PFX2 is more similar to the corresponding *P. textilis* venom cDNA (94% identity) and has 79% identity to the venom gland cDNA from *T. carinatus*. These authors proposed a model involving duplication of the ancestral gene followed by deletion in the activation peptide and insertions in the heavy chain.

A second gene duplication event followed giving rise to FX isoforms expressed either in the liver or the venom gland. Quantitative polymerase chain reaction (PCR) revealed that PFX2 in the liver is expressed approximately 5.6×10^4 lower than the functionally important PFX1. If this is indicative of protein expression, PFX2 would not be present in sufficient amount to impact on haemostasis and thus it may represent an inactive intermediate in the evolutionary pathway. Thus, PFX2 may be a

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precursor to a form that was recruited to the venom gland and expressed there at high levels as a toxin. It is of interest to point out that in an animal model, heparin-antithrombin inhibitor complex was shown to protect animals from a fatal outcome by intravenously injected *P. textilis* prothrombin activator, further supporting the similarity of *P. textilis* venom prothrombin activator to mammalian blood prothrombinase complex (14).

Given the tissue-specific expression of the venom genes it seems likely that they may have acquired or modified control elements during the process of recruitment. Support for this was provided by Reza et al (9, 10). They showed that Trocarin D (venom protein) and TrFX (the corresponding liver protein) had identical gene structure and a well conserved DNA sequence, with the exception of intron 1 where Trocarin D had three insertions and two deletions of nucleotides compared to TrFX. DNA sequencing of the putative promoter regions of both genes revealed identity except for a 264bp insert upstream of the start codon (-33bp to -297bp) in the Trocarin D promoter (10). This insert in the promoter has three putative TATA boxes, a Y box and a GATA-4 binding site. This segment is referred to as the Venom Recruitment/ Switch Element (VERSE). A similar VERSE is found in the *P. textilis* pt-FX gene, 271bp in length, containing the same control elements and showing 95% identity (10). In that case the segment was inserted 11bp upstream compared to Trocarin D, most likely representing an independent insertional event during evolution. The authors speculated that this insertion might be responsible for switching from constitutive expression of the liver FX gene to inducible high level expression of the gene in the venom gland. Using a Luciferase reporter assay they showed that VERSE enhanced activity to the same extent as the Trocarin D promoter in which it is contained, indicating that this segment contains the functional cis-elements.

The VERSE promoter was equally effective in stimulating activity in venom gland cells. The promoter activity was 20–50 times higher than that observed with the liver TrFX promoter, co-

inciding with previous data showing that Trocarin D is expressed approximately 30 times higher in the venom gland than TrFX in the liver (10). More detailed functional analysis of VERSE revealed that TLB2 (putative TATA box) is the primary transcription initiator and GATA-4 and Y box regions have up-regulatory effects on expression. Three other cis-elements were also identified with either up-regulatory or suppressor activities. Comparable levels of expression of the VERSE driven construct in mammalian cells and in venom gland cells suggest that VERSE is not responsible for the inducibility of FX in the venom gland. The presence of scaffold matrix attachment regions in the insertions/deletions in intron 1 of Trocarin D might provide the solution to the inducibility, since such regions are implicated in gene regulation elsewhere (15, 16).

The second member of the prothrombinase complex, venom-derived FV (pt-FV), shows approximately 44% homology with mammalian FV and possesses a similar domain structure (4, 7). However, it is evident that the B-domain is markedly shorter than that present in mammalian FV, 46 amino acids compared to approximately 800 amino acids. This is of considerable interest since the accepted role for the B-domain in mammalian FV is to maintain the molecule in an inactive form (17). This domain is removed by thrombin cleavage at three sites during the process of activation of FV to FVa. This is further substantiated in a modified form of FV in which a large segment of the B-domain is deleted (FVdes⁸¹¹⁻¹⁴⁹¹, FV-810), rendering it constitutively active (18). It seems likely that FV is maintained in its inactive cofactor state at least in part by a cluster of basic amino acids (18 out of 46 residues) (19). Deletion of these amino acids bypasses the requirement for proteolysis of FV to generate an active molecule. These residues appear to provide only part of the conformational restraints to maintain FV in an inactive form. Unlike the remainder of the B-domain this short basic region is highly conserved amongst mammals and other species.

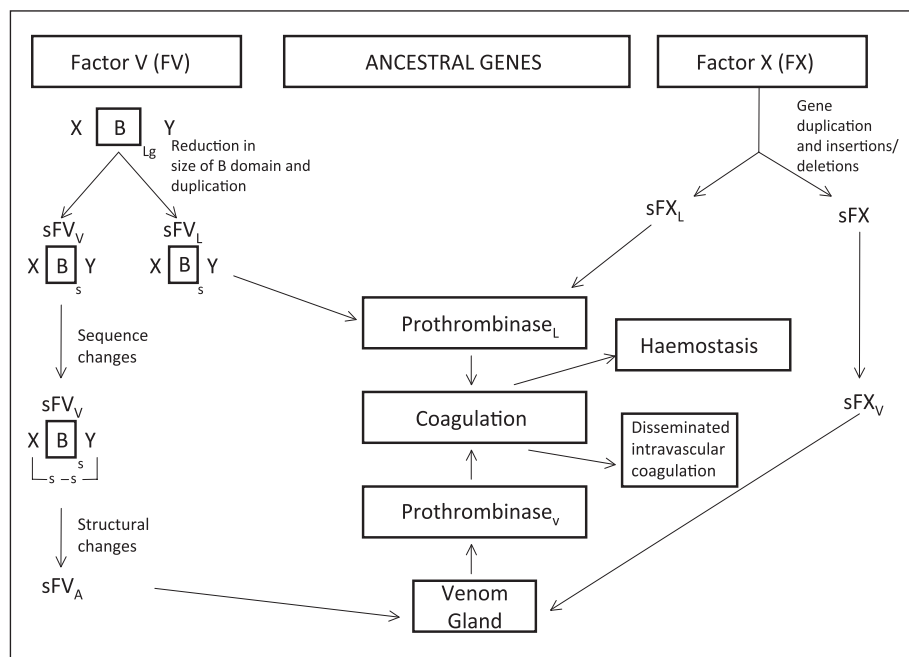


Figure 1: Evolutionary development of elapid snake prothrombin activators. The two major components of the elapid snake venom prothrombin activators, FV and FX, evolved from ancestral genes by different pathways. In the case of FV, this involved a marked decrease in the size of the B domain, capacity to function in the absence of anionic membranes, and the presence of a disulfide bond that holds the heavy and light chains together. These changes resulted in structural modification to the protein that led to constitutive activation and resistance to cleavage. During this process duplication led to venom and liver forms of the gene. It seems likely that the liver form is not constitutively active. For FX this involved duplications, insertions and deletions that altered the protein and its expression. These changes have enabled FV and FX from the venom to form a very potent prothrombinase complex. B_{lg} refers to large B domain; B_s short B domain sFV; snake FV; sFX; snake FX; X is A1 and A2 and Y refers to A3, C1 and C2 domains for FV.

More recently Bos et al (6) employed recombinant (pt-rFV) and native (pt-FVa) forms of venom-derived FV from *P. textilis* to investigate in more detail biological properties of this protein and how it so efficiently leads to blood coagulation. Pt-rFVa was assembled with a native form of *P. textilis* (pt-FXa) – membranes to rapidly convert prothrombin to thrombin revealing that pt-rFVa was constitutively active. Under the same conditions human FV failed to convert prothrombin to thrombin. Another unique feature of the pt-FXa-pt-rFV complex, whether in solution or in the presence of anionic membranes, is its capacity to convert prothrombin to thrombin, a property not seen with FXa-FVa (6).

The protein C anticoagulant pathway is the major mechanism for controlling thrombosis and requires four essential components: endothelial cell protein C receptor, thrombin, thrombomodulin and proteins C and S (20). On dissociation from its receptor, activated protein C binds protein S on appropriate surfaces, which enables it to inactivate FVa by cleaving it at three sites (Arg³⁰⁶, Arg⁵⁰⁶, Arg⁶⁷⁹). The absence of one of these sites and modification to the other two in pt-FV makes it resistant to cleavage by activated protein C, and thus resistant to inactivation under conditions where mammalian FVa is completely inactivated (6). However, while pt-FVa was cleaved at higher concen-

trations of activated protein C, it retained full procoagulant activity. An explanation for this behaviour was provided by the observation that a large fraction of pt-FV was held together by one or more disulfide bonds. This molecule was also resistant to inactivation by thrombin.

In conclusion, the work reported by Kwong et al (5) and Bos et al (6) provide important mechanistic data that assist in explaining why such proteins with similar catalytic activity have evolved to perform quite different physiological functions. The focus here is the prothrombinase, the FXa-FVa complex, which functions in the blood to respond to injury and minimise bleeding. This functional role is highly conserved in a variety of species including snakes. In snakes a closely related prothrombinase complex has been recruited into the venom gland as a toxin to assist in immobilising prey by inducing a massive thrombotic event such as a disseminated intravascular coagulation (11, 21). This evolution has been achieved by distinct processes for FXa- and FVa-like proteins (Fig. 1).

The capacity of both venom factors to associate with members of the mammalian prothrombin activator provides the potential for the development of novel therapeutics capable of preventing blood loss both topically and when administered intravenously.

References

- Masci PP, Whitaker AN, de Jersey J. Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. *Biochem* 1988; 17:825–835.
- Kini RM. The intriguing world of prothrombin activators from snake venom. *Toxicon* 2005; 45:1133–1145.
- Joseph JS, Chung MC, Jyeaseelan K, Kini RM. Amino acid sequence of trocarnin, a prothrombin activator from *Tropidochis carinatus* venom: its structural similarity to coagulation factor Xa. *Blood* 1999; 94:621–631.
- St. Pierre L, Masci PP, Filippovich I et al. Comparative analysis of prothrombin activators from the venom of Australian elapids. *Mol Biol Evol* 2005; 22:1853–1864.
- Kwong S, Woods AE, Mirtschin PJ, et al. The Recruitment of Blood Coagulation Factor X into Snake Venom Gland as a Toxin: The Role of Promoter Cis-elements in Its Expression. *Thromb Haemost* 2009; 469–478.
- Bos MH, Boltz M, St Pierre L, et al. Venom factor V from the common brown snake escapes hemostatic regulation through procoagulant adaptations. *Blood* 2009; 114:686–692.
- Rao VS, Swarup S, Kini RM. The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V. *Blood* 2003; 102:1347–1354.
- Rao VS, Swarup S, Kini RM. The catalytic subunit of pseutarin C, a group C prothrombin activator from the venom of *Pseudonaja textilis*, is structurally similar to mammalian blood coagulation factor Xa. *J Thromb Haemost* 2004; 92:509–521.
- Reza MA, Minh LT, Swarup S, Kini RM. Molecular evolution caught in action: gene duplication and evolution of molecular isoforms of prothrombin activators in *Pseudonaja textilis* (brown snake) *J Thromb Haemost* 2006; 4:1346–1353.
- Reza MA, Swarup S, Kini RM. Structure of two genes encoding parallel prothrombin activators in *Tropidochis carinatus* snake: gene duplication and recruitment of factor X gene to the venom gland. *J Thromb Haemost* 2007; 5:117–126.
- Masci PP. PhD Thesis. Studies of coagulation and fibrinolysis using Australian snake venoms: From molecular toxicology to novel therapeutic agents. 2000.
- Masci PP, Tibbals J. Genus *Pseudonaja* from snakes. In *The Biology of Australian Snakes*, Sutherland SK and Tibbals J. Ed Oxford University Press 2001; 99–129.
- Fry BG, Scheib H, van der WL, et al. Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (*Caenophidia*). *Mol Cell Proteomics* 2008; 7:215–246.
- Tibbals J, Sutherland SK, Rivera R, et al. The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. *Anaesth Intensive Care* 1992; 20:28–32.
- Bode J, Kohwi Y, Dickinson L, et al. Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* 1992; 25:257–290.
- van Drunen CM, Oosterling RW, Keultjes GM, et al. Analysis of the chromatin domain organization around the plastocyanin gene reveals an MAR-specific sequence element in *Arabidopsis thaliana*. *Nucleic Acids Res* 1997; 25:3904–3911.
- Mann KG, Kalafatis M. Factor V: A combination of Dr Jekyll and Mr Hyde. *Blood* 2003; 101:20–30.
- Tozo R, Camire RM. Removal of B-domain sequences from factor V rather than specific proteolysis underlies the mechanism by which cofactor function is realized. *J Biol Chem* 2004; 279:21643–21650.
- Zhu H, Toso R, Camire RM. Inhibitory sequences within the B-domain stabilize circulating factor V in an inactive state. *J Biol Chem* 2007; 282:15033–15039.
- Esmon CT. The protein C pathway. *Chest* 2003; 124 (3 Suppl): 26S–32S.
- Masci PP, Rowe EA, Whitaker AN, et al. Fibrinolysis as a feature of disseminated intravascular coagulation (DIC) after *Pseudonaja textilis textilis* envenomation. *Thromb Res* 1990; 59:859–870.