Two parallel prothrombin activator systems in Australian rough-scaled snake, *Tropidechis carinatus*

Structural comparison of venom prothrombin activator with blood coagulation factor X

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Summary

It is uncommon for similar pathways/systems to be involved in highly divergent functions within single organisms. Earlier, we have shown that trocarin D, a venom prothrombin activator, from the Australian rough-scaled snake *Tropidechis carinatus*, is structurally and functionally similar to the blood coagulation factor Xa (FXa). The presence of a haemostatic system in these snakes implies that they have two parallel prothrombin activating systems: one in the plasma, that participates in the life saving process of blood clotting and the other in their venom, where it acts as a toxin. Here, we report the complete cDNA sequence encoding the blood coagulation factor X (FX) from the liver of *T. carinatus*. Deduced *T. carinatus* FX sequence shows ~80% identity with trocarin D but ~50% identity with the mammalian FX. Our present study confirms the presence of two separate genes – one each for FX and trocarin D, that code for similar proteins in *T. carinatus* snake. These two genes have different expression sites and divergent uses suggesting that snake venom prothrombin activators have probably evolved by the duplication of the liver FX gene and subsequently marked for tissue-specific expression in the venom gland.

Keywords

Prothrombin activation, factor X, haemostasis, snake venom

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Introduction

Blood coagulation cascade involves a series of proteolytic activation reactions of several coagulation factors culminating in the formation of a fibrin clot. The activation of the zymogen prothrombin to thrombin is the key reaction in this cascade. This activation is catalyzed by prothrombinase, which is a complex of the vitamin K-dependent serine proteinase FXa and its cofactor factor Va (FVa). The complex is formed in the presence of calcium on the surface of negatively charged phospholipid membranes (1). During the activation of prothrombin, two peptide bonds, Arg_{273}-Thr_{274} and Arg_{322}-Ile_{323}, are cleaved resulting in the formation of mature thrombin (2). FXa alone can activate prothrombin, but at a very slow rate. This activation rate is enhanced by about five orders of magnitude by the formation of the prothrombinase complex (3–5). Therefore, for the activation of prothrombin to thrombin at a physiologically relevant rate, formation of the prothrombinase complex is a crucial step in blood coagulation.

Apart from the *in vivo* physiological activator FXa, prothrombin is additionally activated by several exogenous factors isolated from various sources, including snake venoms (6, 7). Depending on the cofactor requirements for their optimal activity, exogenous prothrombin activators are classified into four groups, namely groups A, B, C and D (8). Group D prothrombin activators are serine proteinases that cleave the same two peptide bonds in prothrombin (Arg_{273}-Thr_{274} and Arg_{322}-Ile_{323}) and generate mature thrombin (9–11). The prothrombin converting activities of group D members are stimulated by the addition of FVa, Ca^{2+} ions and phospholipids (cofactors of the prothrombinase complex) (9, 12), to the same extent as that of FXa. They form a tight 1:1 complex with FVa in the presence of Ca^{2+} ions and phospholipids (13).

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Recently, we purified and characterized several group D prothrombin activators such as trocarin D, hopsarin D, notecarin D, and notanarins D1 and D2 from a number of Australian elapid venoms and determined the amino acid sequences of trocarin D and hopsarin D (9, 11). These studies indicate that group D prothrombin activators from the Australian elapid snakes are true structural and functional homologues of mammalian FXa (9–11). Structurally, they have the same domain architecture as mammalian FXa. The light and heavy chains in these proteins are linked by a single disulfide bond. The light chain contains an N-terminal Gla domain followed by two epidermal growth factor (EGF)-like domains and the heavy chain contains a serine protease domain. The characteristic catalytic triad His_{236}, Asp_{282} and Ser_{379}, typical for a serine proteinases, is also found conserved in the heavy chain (9–11).

Snakes have their own haemostatic systems, as found in the case of other vertebrates (14). This necessitates the presence of a coagulation factor, factor X (FX) or equivalent protein in its plasma. Thus, there are two prothrombin activating systems in these snakes; a toxin Trocarin D (9) produced in their venom glands, and the other is a haemostatic factor produced in the liver. The difference in their functional roles, therefore, mandates a highly tissue-specific expression of these closely related proteins. Further, their levels of expression are also different: Trocarin D is expressed in the venom gland in high quantities (~1,150 times higher) compared to FX expression in liver (9). Moreover, the regulation of expression of these two proteins is also distinct. In the liver, FX is expressed constitutively (15, 16) and hence produced at a steady state level. In contrast, the expression of venom prothrombin activators is inducible (17, 18); when the venom gland is empty, its expression reaches the maximum, and when the gland is full its expression slows down or stops. Therefore, it is interesting to study the gene structure and regulation of expression of these two closely related proteins with divergent functional roles in the same snake species. As a first step, we have cloned and sequenced a cDNA encoding FX from the liver of *T. carinatus* snake. The deduced amino acid sequence shows that FX produced in the liver is structurally similar to mammalian FX as well as to its own venom prothrombin activator trocarin D. This is the first amino acid sequence of a reptilian coagulation factor. This study will contribute significantly towards the understanding of the structure-function relationships and the mechanism of FXa-mediated prothrombin activation. It will also aid in improving the expression of these proteins, which have a therapeutic potential for the treatment of FX-deficient as well as FVIII and FIX inhibitor patients. Furthermore, it will help us to establish an evolutionary relationship between the physiological prothrombin activator FX in the blood, its venom prothrombin activator and the other FX proteins.

Materials and methods

Liver of *Tropidechis carinatus* was purchased from the Venom Supplies Pty Ltd, Australia. The RNeasy RNA isolation kit, One step RT-PCR kit, Qiaquick gel extraction and PCR purification kit, pDrive cloning kit, QiaPrep mini prep kit were purchased from Qiagen (Valencia, CA, USA), SMART RACE cDNA amplification kit and BD Advantage 2 PCR Enzyme system were bought from Clontech Laboratories inc (Palo Alto, CA, USA). The ABI PRISM® BigDye® terminator cycle sequencing ready reaction kit was purchased from Perkin Elmer (Foster City, CA, USA). Oligonucleotides were custom synthesized from 1st BASE (Singapore). All other chemicals and reagents were of the purest grade available.

RNA isolation

Total RNA was isolated from the liver tissues of *T. carinatus* using a rotor homogenizer and the RNeasy mini kit. RNA purity and quality was checked by agarose gel (0.8%) electrophoresis. Amount of RNA was quantified spectrophotometrically.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

One step RT-PCR was carried out in a final volume of 25 µl using 100 ng of total RNA. FX sequence of mammalian and non-mammalian sources were aligned with that of trocarin D and sense (FXF: 5'-ATTGAAAGGGAATGC-3') and antisense (FXR: 5'-TGCACATCCTTCCCC-3') primers were designed from the most conserved sequence.

The RT-PCR conditions were: reverse transcription at 50°C for 30 min followed by inactivation of reverse transcriptase and activation of *Taq* polymerase at 95°C for 15 min. PCR was performed as follows: 35 cycles of one step each at 94°C for 1 min, 54°C for 45 sec, 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products were purified using Qiagen PCR purification kit according to the manufacturer's instruction.

5'- and 3'- Rapid Amplification of cDNA ends (RACE)

5'- and 3'- RACE libraries were created and amplifications were carried out with Clontech's SMART RACE cDNA amplification kit. Universal primer mix (UPM: 5'-CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT-3'; 5'-CTAATACGACTCACTATAGGGC-3') from the SMART RACE kit and gene-specific oligonucleotides were used for amplification. The gene-specific oligonucleotides (GSP1: 5'-TCAGCAGTGGGAAGGCAGGCAGGAACC-3'; GSP2: 5'-GGTTCCTGCCTGCCTTCCCACTGCTGA-3') used for RACE were designed based on the sequence data obtained from RT-PCR product. In each case of 5'- and 3'- RACE, the primers were designed a few hundred bases downstream and upstream of the obtained sequence, respectively, to get an overlap sequence to confirm the RACE sequence with previously obtained sequence. Primers were designed based on the requirements for touchdown PCR (Clontech manual), which requires a Tm ~ 70°C, GC content close to 50–70% and a primer length of 23–30 nucleotides.

Following the initial RACE reactions, 5 µl of the PCR product from each reaction was analyzed in 1% agarose gel. A prominent band with a number of background bands was observed in both the 5'- and 3'- RACE reactions. The remaining PCR products from both the RACE reactions were purified and used as templates for PCR re-amplification reaction. A 2nd set of nested 5'- and 3'- gene specific primers were designed few bases upstream and downstream, respectively, from the 5'GSP1 (5'GSP2: 5'-TGCAGAAGTGCCAACAGTTACCATTG-3') and 3'GSP1

(3'GSP2: 5'-CCTTCCCACTGCTGATTTTGCCAACC-3' oligonucleotide, respectively. These nested gene- specific RACE primers and nested universal primer (Nested UPM: 5'- AAGCAGTGGTATCAACGCAGAGT-3') from the Clontech's kit were used for nested PCR amplification.

Amplification and cloning of full-length FX cDNA

Based on sequencing of the 5'- and 3'- RACE products, a new set of gene-specific primers were designed to amplify the full length FX cDNA. Amplified PCR product was purified, cloned into pDrive cloning vector and subsequently transformed into DH5α strain of *E. coli*. Ampicillin (100 mg/ml) and kanamycin (30 mg/ml) were used for selection and 80 mg/ml X-gal and 0.5 ml/l of 100 mM IPTG, respectively, were used for blue and white colony selection.

DNA sequencing was carried out using an ABI PRISM® 3100 automated DNA sequencer. All sequencing reactions were carried out using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit according to manufacturer's instructions.

Sequence analysis

Sequence analysis was carried out using BLASTX program at the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). Sequence alignments were carried out using the GeneDoc/DNAMAN program. Prediction of signal peptide and N-glycosylation site was carried out using PSORT and NetGlyc prediction site, respectively, at Expasy web site (http://tw.expasy.org/).

Phylogenetic tree

A neighbour-joining phylogenetic tree for FX sequences along with the prothrombin activators – trocarin D, hopsarin D and pseutarin C (catalytic subunit) was plotted using the software DNAMAN. *Tropidechis* FX was sequenced and all other FX and trocarin D, hopsarin D and pseutarin C (catalytic subunit) sequences were obtained from GenBank. The neighbour-joining tree represents boot strap value after 1000 replicates.

Results

cDNA sequence of FX of *Tropidechis carinatus* liver

The precursor of coagulation factor FX is synthesized in the liver (16). The full length cDNA sequence of FX of *Tropidechis carinatus* liver was obtained by RT-PCR followed by 5'- and 3'-RACE experiments. The sense-primer for RT-PCR was designed based on the sequence of 13 –17th amino acid residues of the Gla domain (FXF: LEREC) and the antisense-primer was designed based on the C terminal region (FXR: GEGCA) of the serine protease domain. RT-PCR was carried out using these primers (FXF and FXR), which yielded a PCR product of 1.2 kb size. The cloned PCR product was sequenced and the deduced amino acid sequence showed high similarity with blood coagulation FX.

The 5'- and 3'-RACE libraries were created and amplification of cDNA ends was performed using gene-specific RACE primers (see Methods for detailed primer design). 5'-RACE using antisense oligonucleotide 5'GSP1 and UPM gave a faint PCR band of ~600 bp along with a number of background bands. This PCR product was reamplified using the second set of nested antisense gene-specific primers 5'GSP2 and NUPM (nested UPM) and a clear band of ~500 bp was obtained. In the 3'-RACE reaction, the primers 3'GSP1 and UPM were used in touchdown PCR. The PCR product was reamplified using the nested 3'GSP2 and NUPM primers to reduce the background. A clear band ~900 bp was obtained upon nested amplification. The 5'- and 3'-RACE products were cloned and sequenced. The cDNA and the deduced amino acid sequences, as expected, showed 100% identity in the overlapping regions obtained from the sequence of the RT-PCR product.

Using the sequence data generated by RACE reactions, new sets of gene-specific primers were designed to generate the full length FX cDNA of *T. carinatus* and to confirm the sequence. Seven positive clones were screened and sequenced from both the directions at least three times. The full length FX cDNA sequence was 1452 bp long and was predicted to encode the FX protein of 483 amino acid residues (Fig. 1). The sequence has been submitted to GenBank (AY651849).

Sequence identity with venom prothrombin activators and other FX

As expected, *T. carinatus* liver FX protein sequence shares high percentage of identity with mammalian and piscine FX sequences as well as venom prothrombin activators. We aligned its sequence with trocarin D, hopsarin D, pseutarin C (catalytic subunit) and other FX sequences (Fig. 2). *T. carinatus* liver FX has similar domain architecture. The light chain has Gla, EGF-I and EGF-II domains and the heavy chain has a serine protease domain. The deduced FXa sequence of *T. carinatus* shows ~ 80% identity to trocarin D. This data clearly indicates that there are two parallel prothrombin activator systems in *T. carinatus*. The one expressed in the liver is involved in blood clotting process, and the other expressed in the venom glands acts as a toxin. *T. carinatus* liver FX protein sequence (including activation peptide) shares 43–54% identity with FX from all other known sources.

Proteolytic maturation

The precursor of FX includes a signal peptide and a propeptide region. Signal peptides promote translocation and secretion. Upon translocation, signal peptides are cleaved off at signal processing sites. Signal peptides of human as well as other FX are processed at –17 position. According to –3,-1 rule (19) the residue at –3 position of the signal peptide must not be aromatic, charged, large or polar. Since *T. carinatus* FX contains a charged residue (Glu) at –20 position it is most likely that *T. carinatus* FX is processed at –18 position instead of –17. However, the signal peptide prediction algorithm (SignalP 3.0; http://www.cbs.dtu.dk/services/SignalP/) predicted the possible processing site between the residue –20 and –21. The propeptide region plays a critical role in the γ-carboxylation of Glu residues in the Gla domain (20) (for details, see below). During maturation, the propeptide region is removed by cleavage at a dibasic site (21, 22). With the exception of human FX (23) all FX including *T. carinatus* FX have a dibasic sequence at the –1 and –2 positions. Subsequently, the single chain precursor is converted into a two-chain mature zymogen FX by two internal cleavages around Arg-

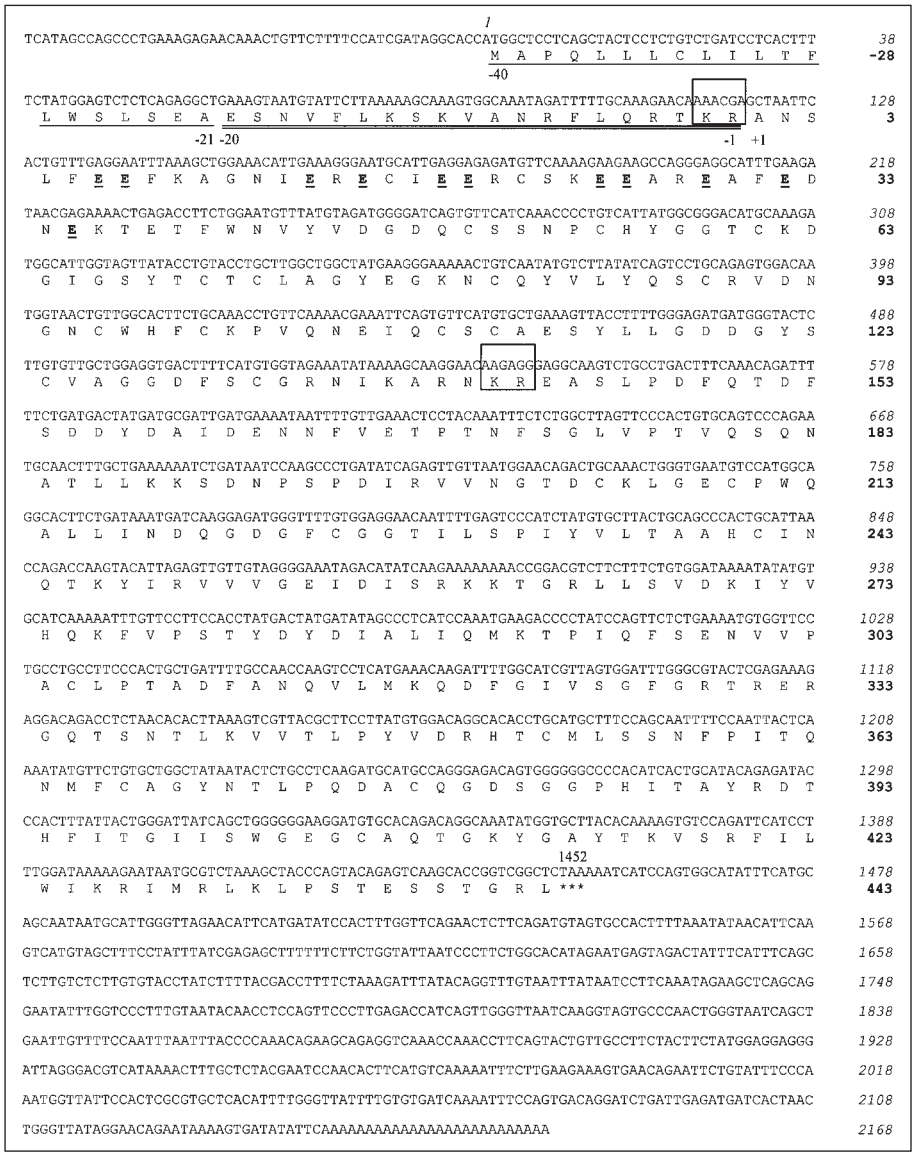

Figure 1: Complete nucleotide sequence of cDNA encoding blood coagulation factor FX of *Tropidechis carinatus*. The amino acid sequence is shown in single letter code beneath the nucleotide sequence. Nucleotide (in italics) and amino acid numbers are shown in the column on the right. Numbering starts with Ala1 at the amino terminus of the light chain of the mature zymogen. The putative signal peptide is underlined (from –40 to –21) and the propeptide is doubly underlined (from –20 to –1). The sequences of the propeptide and internal cleavage sites are boxed. Putative γ-carboxylated Glu residues are in boldface and underlined.

Xaa-Lys/Arg-Arg and release of a short peptide (21, 22). In venom homologues, trocarin D and hopsarin D, the cleavage appears to be after the Arg-Asn-Lys as their light chains end with KARNK (9, 11). Since a similar sequence is also found in *T. carinatus* FX, it may be processed similarly.

Proteolytic activation

The zymogen FX has negligible procoagulant activity (3–5). When required, it is activated to FXa by TF-VIIa (24, 25) or IXa-VIIIa (24, 26) complexes through a proteolytic cleavage at Arg_{194}-Ile_{195} (human FX numbering) and removal of the activation peptide. The proteolytic activation site is conserved in *Tropidechis* FX. The size of activation peptide segment varies considerably among different FX proteins. Its size appears to reduce during evolution; fish FX have the longest activation peptides (54–66 residues), whereas mammals have the shortest (49–52 residues). However, reptilian *Tropidechis* FX has intermediate size of 57 residues. Interestingly, the enzymatic subunit (which is structurally similar to group D prothrombin activators and FX) of pseutarin C, a group C prothrombin activator from snake venom, has the shortest activation peptide of 27 residues (27).

Post translational modifications

Disulfide bonds

Disulfide bonds are important in determining the protein folding and overall three dimensional structure and stability of the protein. All the 24 cysteine residues are conserved in *Tropidechis* FX which form 12 disulfide bonds; one in the Gla domain (Cys_{17}, Cys_{22}), three in EGF-I domain (Cys_{50}-Cys_{62}, Cys_{56}-Cys_{71}, Cys_{73}-Cys_{82}), three in EGF-II domain (Cys_{90}-Cys_{101}, Cys_{97}-Cys_{110}, Cys_{112}-Cys_{125}), four in the catalytic domain (Cys_{215}-Cys_{220}, Cys_{236}-Cys_{252}, Cys_{378}-Cys_{392} and Cys_{403}-Cys_{431}) and one inter-chain disulfide bond between Cys_{133}-Cys_{330} (Fig. 3). In hopsarin D, the first cysteine residue (11) (Cys_{215}) and in Zebrafish the last cysteine residue (28) (Cys_{431}) on the heavy chain is found to be replaced by serine and valine respectively. Hopsarin D has 10-fold lower activity compared to trocarin D

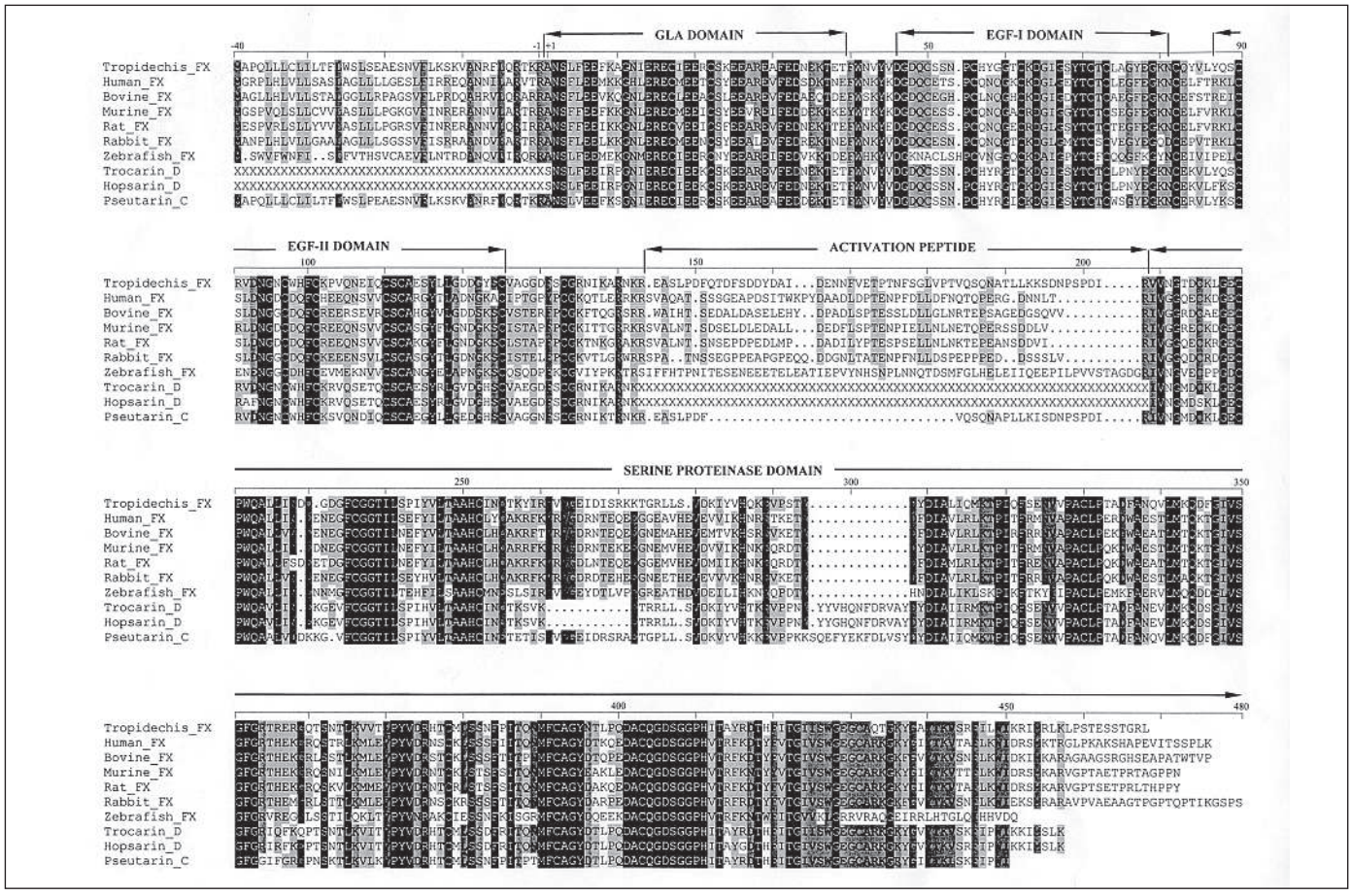

Figure 2: Alignment of deduced amino acid sequence of *Tropidechis carinatus* FX with sequences of mammalian and non-mammalian FX and groups C and D prothrombin activators. Alignment was carried out using the software DNAMAN; gaps (-) are introduced for optimal alignment. "X" indicates sequence not known. Conserved residues (100%) are shaded in black, 80% conserved ones in dark grey and 60% conserved ones in light grey. Pseutarin C catalytic subunit is shown as "Pseutarin_C". The sequences have been taken from the following sources: Tropidechis FX (this paper): AY651849; Human FX: P00742; Bovine FX: P00743; Murine FX: AAH03877; Rat FX: NP 058839; Rabbit FX: AF003200; Zebrafish FX: AAM88343; trocarin D from *Tropidechis carinatus*: P81428, hopsarin D from *Hoplocephalus stephensi*: P83370; Pseutarin C catalytic subunit: AY260939.

and FXa (11). It is not clear whether the absence of the 11th disulfide linkage results in the lower catalytic activity.

γ-Carboxylation

γ-Carboxylation is one of the distinct characteristics of vitamin K- dependent serine proteinases. Gla residues confer calcium binding properties on proteins (29, 30). In the presence of Ca^{2+} ions, these proteins undergo conformational changes and bind to membranes via a Ca^{2+} bridge to express procoagulant activity (31–35). γ-Carboxylation is catalyzed by a specific carboxylase. The recognition site for γ-carboxylase is located in the propeptide region. Two critical residues shown to be important for the recognition of carboxylase, Phe_{-16} and Ala_{-10} (36), are conserved in *T. carinatus* FX. There are 12 Glu residues in the Gla domain of *T. carinatus* FX; all of them are at identical positions compared to trocarin D and hopsarin D. We have shown that all except Glu_{38} residues are carboxylated in trocarin D (9). Based on the homology (Fig. 4), we propose that only the first 11 Glu residues are carboxylated in *T. carinatus* FX. First ten of Gla residues are conserved in all the FX sequences. The 11th Gla is not conserved in human and two piscine FX, whereas the 12th Gla is not conserved in reptilian FX. Either the location of 11th Gla or the presence of 12th Gla does not appear to severely affect the activity.

β-hydroxylation

The invariant Asp_{64} of the mature FX protein (homology numbering, Fig. 2) in human is posttranslationally hydroxylated to β-hydroxyaspartic acid (Hya) in EGF-I domain (37). This Asp_{64} is conserved in *T. carinatus* FX. Even the conserved consensus sequence Cys-Xaa-Hya-Xaa-Xaa-Xaa-Xaa-Tyr/Phe-Xaa-Cys-Xaa (38, 39) surrounding the Hya residue is also conserved in *Tropidechis* FX. But in spite of having this consensus sequence, trocarin D is not hydroxylated at this position (11). It is not known whether Asp_{64} in *T. carinatus* FX is β-hydroxylated and if so, then what is its biological significance (11).

Glycosylation

Glycosylation is known to play an important role in the stability as well as folding (40). In general, glycosylation has been

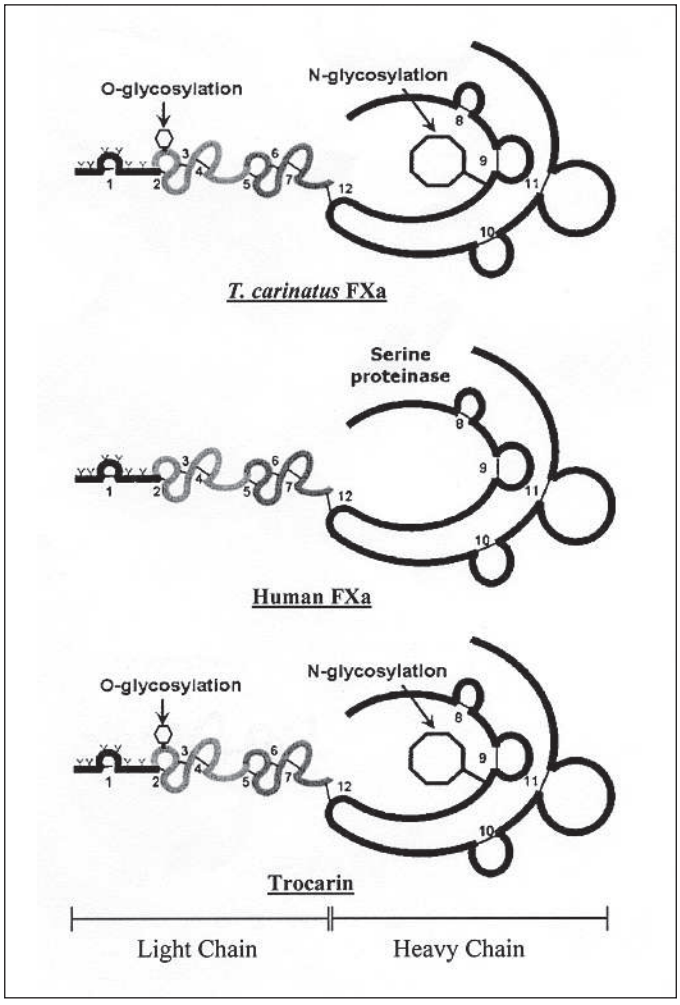

Figure 3: Schematic representation of the domain architecture of *T. carinatus FXa*. Similar to the human FXa and trocarin D, *T. carinatus* FXa contains a light chain and a heavy chain linked by a disulfide bond. Unlike Human FXa, *T. carinatus* FX and trocarin D are O- and N-glycosylated both in the light and heavy chains, respectively.

thought to play a role in the stabilization of protein conformation and protection from proteolysis (41, 42) as well as cell-surface recognition phenomenon in multicellular organisms (43). Wang et al. (44) have also shown that glycosylation enhances the thermal stability of proteins. Although the zymogen FX is a glycoprotein, all carbohydrate moieties are found on the peptide segment that is removed during activation (40, 45). These carbohydrate moieties are important for the activation of zymogen FX by both intrinsic and extrinsic tenase complexes (45, 46). Besides the two potential *N*-glycosylation sites on the activation peptide, we found one potential *O*-glycosylation site at Ser_{52} position of light chain and *N*-glycosylation site on the Asn_{254} residue of the heavy chain of *Tropidechis* FX (Fig. 3). Interestingly, the group D prothrombin activator trocarin D from the same snake and hopsarin D from *Hoplocephalus stephensi* have similar *O*- and *N*-glycosylation sites (9, 11). We have determined the structure of carbohydrate moieties linked at these sites in trocarin D (47). All the non-mammalian FX have a similar potential *N*-glycosylation site at this position in the heavy chain. However, in case of mammals, glycosylation is restricted to the activation peptide only such that active FXa is not glycosylated. This finding suggests that the *N*-glycosylation site exists in FXa of the lower vertebrates but not in mammalian FXa. This finding provides a new insight in terms of evolution of the coagulation factors.

Discussion

So far, a number of procoagulant proteins from snake venoms have been purified and characterized (12, 48–51). Rosing and his co-workers first isolated and purified prothrombin activators from *Notechsis scutatus scutatus* and *Oxyuranus scutellatus scutellatus* venoms (14, 48). Their studies showed that the prothrombin activation by the *Notechis* activator was stimulated by the presence of FVa, phospholipids and Ca^{2+}. Thus, the protein described by them functionally resembles FXa with respect to catalytic properties. Recently, we isolated and characterized trocarin D from the venom of *T. carinatus* (Australian rough-scaled snake) (9). As mentioned in the Introduction, trocarin D as well as other group D prothrombin activators are structurally and functionally similar to blood coagulation FXa (9–11).

The initial blood coagulation cascade is believed to have evolved over 450 million years ago (52) before the divergence of tetrapods and teleosts and the complete/total cascade was formed by gene duplication and diversification (53, 54). Coagulation cascades similar to that of mammals exist even in non-mammalian vertebrates, such as amphibians, birds, fishes and reptiles (14, 28, 55–58). We have shown that *Tropidechis* snake plasma treated with FX-activating proteinase from Russell's viper venom (RVV-X) leads to the activation of a protease which cleaves FXa specific chromogenic substrate (58). This observation clearly indicates the presence of a zymogen similar to FX in snake plasma, which plays a crucial role in hemostasis.

In this paper, we determined the full length cDNA sequence of plasma prothrombin activator- liver FX from *T. carinatus*. Structurally, *T. carinatus* FX shows all the characteristic features

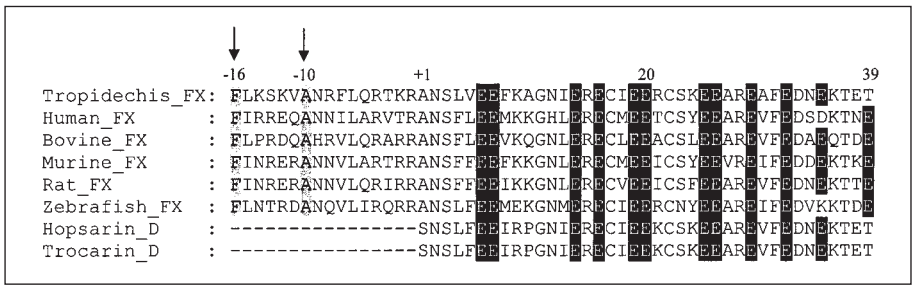

Figure 4: γ-Carboxylation of Glu residues in the Gla domain. The residues that are carboxylated are shaded in black. Phe-16 and Ala-10, shaded in grey, are known to play critical role in the enzyme recognition site for γ-carboxylation.

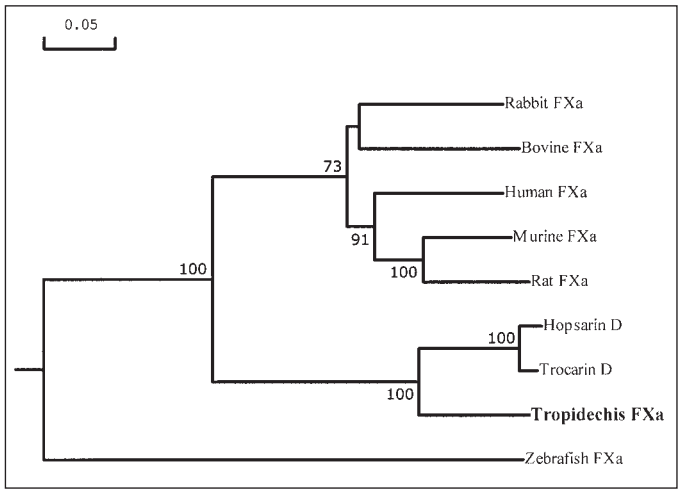

Figure 5: Phylogenetic relationships among FX proteins. Sources of the sequences are the same as in Figure 2. The neighbour-joining tree represents bootstrap values after 1000 replicates. Nodes with confidence value greater than 70% are indicated. Tree was plotted using the software DNAMAN.

including the domain architecture and 24 conserved cysteines found in other FX sequences as well as its venom prothrombin activator trocarin D. It shares >50% identity with other FXs, but much higher (~80%) identity with its venom protein trocarin D. As observed in other known FX proteins, it contains a 40-residue long pre-pro leader sequence, which is removed during maturation (16, 59). The sites for signal peptide processing, proteolytic maturation and activation are conserved in *T. carinatus* FX. In terms of posttranslational modifications such as γ-carboxylation, *N*- and *O*-glycosylation, it is closer to its venom prothrombin activator, trocarin D rather than to other FX proteins. All FX proteins are glycosylated. Interestingly, activated FXa proteins in lower vertebrates (fishes and reptiles) retain the carbohydrate moieties as they are located on the light and heavy chains, whereas in the case of mammalian FX proteins the glycosylated segment is removed during activation. Our preliminary studies on the de-glycosylation of trocarin D indicated that glycosylation plays a critical role in its stability and de-glycosylation results in autolytic destruction. Thus glycosylation in activated FXa in mammals might have been eliminated during the course of evolution probably to make FXa short-lived after activation.

The phylogenetic tree generated by neighbor joining method (Fig. 5) shows that all the vertebrate FX proteins have evolved from a common ancestor. The venom prothrombin activators, such as trocarin D and hopsarin D, seem to have branched recently. We hypothesize that group D prothrombin activators have evolved by duplication of liver FX gene and subsequently marked for tissue-specific expression in the venom gland. Thus liver FX is 'modified' and recruited to be used as an offensive weapon in capturing the prey.

In this paper we have shown that FX is synthesized in *T. carinatus* liver and is probably released into its plasma. As shown earlier (58), RVV-X activates a plasma protein resulting in the formation of FXa-like activity. Taken together, we believe that this protein plays a crucial role in the hemostasis of *T. carinatus*. In a separate study, we have shown that the prothrombin activator, trocarin D is structurally and functionally similar to mammalian FXa (9). This venom protein acts as toxin and induces cyanosis and death in mice. Therefore, *T. carinatus* has two similar but diverse prothrombin activating enzymes that are encoded by two different genes. Despite similar structural and catalytic properties, these two proteins are involved in distinctly divergent functions within the same organism. This is a very rare phenomenon. These two closely related proteins are encoded by two separate genes with different outcomes of regulation, possibly resulting from different regulatory controls. Further studies to understand tissue-specific expression and transcriptional control of these two proteins will be of great interest. Such studies may help us to express and produce large amounts of recombinant FX and related proteins that have therapeutic potential. We are currently attempting to decipher the subtle differences between these two closely related proteins to understand the functional dichotomy. Finally, these studies may also aid in clarifying the molecular details of the prothrombinase complex formation.

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