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The carboxyl-terminal region of coagulation factors: role in biosynthesis and function of FVII and FX

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INTRODUCTION

1.1 Haemostasis and blood coagulation

1.1.1 Haemostasis

Blood coagulation is a host defence system that assists in maintaining the integrity of the closed, high-pressure mammalian circulatory system after vascular damage.

At the place of injury, blood is converted from a fluid phase into a solid phase, to prevent further blood loss. The physiological mechanisms whereby blood is maintained fluid under normal conditions, but is allowed to clot in case of trauma, are designed ”haemostasis”, and are finely regulated by various cellular and molecular components, primarily the vascular endothelium, platelets and a number of circulating and transmembrane proteins [1] . The haemostatic balance is the result of the activity of two pathways: the procoagulant pathway, which promotes coagulation, and the anticoagulant pathway, which keeps the blood fluid. The haemostatic balance under normal conditions shifts towards the anticoagulant pathway, to allow the blood flow, while in the case of vascular damage blood coagulation is activated, and the balance shifts towards the procoagulant pathway.

The events that take place following vascular damage can be schematically divided in three phases:

1) Activation of endothelial cells and platelets: endothelial cells tile the walls of the blood vessels. In the resting state they inhibit the platelet adherence and thus the activation of blood coagulation. Moreover, the synthesis of prostacyclin and heparin-like substances, and the presence of protein complexes (thrombin-thrombomodulin), leading to generation of anticoagulant proteins (activated protein C), prevent clot formation in normal blood vessels [2]. After vessel injury, damaged endothelial cells expose negatively charged phospholipids and release procoagulant proteins [3]. These events cause platelets to adhere to the damaged vessel wall. This interaction requires von Willebrand factor (vWF), a large multimeric plasma protein that binds to a specific receptor (glicoprotein Ib) on the platelet membrane, and acts as a bridge between sub-endothelial collagen and specific receptors (GPIb) on the platelet membrane [2]. The phospholipids composition of the platelet membrane changes, resulting in the exposure of negatively charged phopshatidylserine on the outer leaflet of platelet membrane [4]. The activation of platelets by thrombin, ADP, thromboxane A2 or epinephrine triggers characteristic morphological and biochemical alterations in the platelet. Activated platelets secrete α-granules, containing fibrinogen, Factor V (FV), Factor VIII (FVIII), vWF and other proteins involved in haemostasis, and δ-granules, containing calcium ions and ADP, and aggregate at the site of injury, forming a

sort of plug that provisionally blocks blood loss. The expression on the platelet surface of a receptor (glycoprotein IIb-IIIa) for plasma proteins (fibrinogen) mediates platelet aggregation [2].

2) Blood coagulation: the exposure of blood to tissue factor (TF) present on the membrane of sub-endothelial cells triggers the activation of the coagulation cascade, culminating in thrombin generation. Newly-formed thrombin converts soluble fibrinogen into insoluble fibrin that precipitates at the site of injury, stabilizing the platelet plug. In addition, thrombin further stimulates platelet activation.

3) Inflammation and wound healing: vascular injury is always accompanied by inflammation and repair reactions. Thrombin plays a key role in these processes by chemotactically drawing leukocytes to the site of injury and by stimulating tissue remodelling and mitogenesis. P-selectin expressed on the platelet membrane in the haemostatic plug acts as a receptor for monocytes and neutrophils which, in addition to providing ideal membrane surfaces for blood coagulation, sustain the inflammatory response. During wound healing, the fibrin clot is degraded by the serine protease plasmin, a process known as fibrinolysis [6].

1.1.2 The cell-based model of blood coagulation

In the classical view coagulation is represented as a “cascade” or “waterfall” model divided into two pathways: an “intrinsic pathway”, so named because all the components are present in blood and an “extrinsic pathway”, in which the subendothelial cell membrane protein tissue factor (TF) is required in addition to circulating components. The initiation of both pathways resulted in activation of Factor X (FX) and the eventual generation of a fibrin clot through a common pathway [7]. Although these concepts represented a significant advance in the understanding of coagulation and served for many years as a useful model, more recent clinical and experimental observations [8] explain how the cascade/waterfall hypothesis does not fully and completely reflect the events of hemostasis in vivo [9].

A cell-based model of coagulation explain, in a more physiological way, how coagulation cascade evolves in consequence of a vascular injury, underlying the roles of cellular elements. The cascade model included the recognition of negative charged phospholipids, principally phosphatidylserine, as a requirement for the assembly and the full function of

coagulation complexes, but the role of cells, especially platelets, was thought to be primarily to provide anionic phospholipids and not to be actively involved in the process.

Several cells play different roles in the coagulation process, due to their procoagulant and anticoagulant properties. Blood platelets and TF-bearing microparticles (MPs) play a major role in supporting procoagulant reactions, supplying negatively charged phospholipids essential for the correct assembly of molecular complexes. Microparticles are vesicles that carry a cytoskeleton surrounded by a membrane consisting of a phospholipid bilayer which shows a high density of negative charged phospholipids, particularly phosphatidylserine, on its outer membrane layer [10]. Among the various hypothesis functions of MPs and one of the most studied is their possible role in hemostasis and thrombosis, and the capacity by monocytes of shedding microparticles selectively enriched in TF has been observed [11]. Vascular endothelial cells play a key role in maintaining the anticoagulant properties of the vasculature. Thus, the process of coagulation is prevented, at least in part, by keeping the two cell types (platelets and endothelial cells) apart until an injury makes activation of the coagulation system indispensable.

Formation of an impermeable platelet and fibrin plug at the site of vessel injury is essential, but it is also required that procoagulant substances activated in this process remain localized to the site of injury. This localization is mediated by the different distribution of procoagulant reactions on specific cell surfaces.

According to this model, coagulation pathway proceeds as a sequence of events localized on the site of vessel injury. In this view, coagulation occurs in distinct overlapping steps: initiation, amplification and propagation.

Initiation phase

TF is the primary physiologic initiator of blood coagulation, thus this process starts by the exposure of TF-expressing cells to flowing blood (Fig.1.1.2.1). It is structurally unrelated to the rest of the coagulation proteins and is an integral membrane protein [12]. TF is expressed constitutively on cells such as smooth muscle cells and fibroblasts but not on resting endothelium; it is also expressed in several other districts and constitutes an hemostatic envelope normally not in contact with blood [13].

Disruption of the endothelium or activation of endothelial cells or monocytes results in the exposure of TF on blood flow [14]. Stronger evidence suggests that TF circulates in blood exposed on the surface of MPs that derive from various cell types: white blood cells,

endothelial cells, and platelets, and might play important roles in development of pathological hemostasis (thrombosis) opposing to normal clotting [15].

Upon an injury in the vessel wall plasma comes into contact with TF-bearing cells and FVII binds tightly with TF being rapidly converted to FVIIa. The new-formed complex TF/FVIIa activates small amounts of FX and Factor IX (FIX). Activated FX (FXa) associates with its cofactor, activated Factor V (FVa), and forms the prothrombinase complex on the surface of the TF-bearing cells [16], leading to the conversion of small amounts of circulating Prothrombin (II) to Thrombin (IIa). The active form of FV derives from one of several sources. The adhesion process to components as collagen partially activates platelets and promotes secretion of partially activated FV from their α-granules [17]. Zymogen FV can also be converted to FVa by FXa [18] or by noncoagulant pro- teases [19].

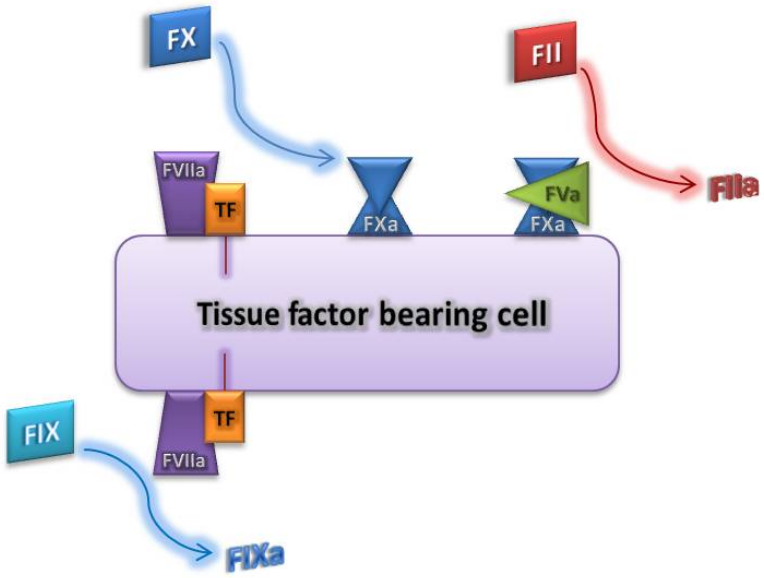

Figure 1.1.2.1. Factor VIIa bound to TF activates both factor X and factor IX. Factor Xa formed by factor VIIa/TF binds to factor Va and converts a small amount of prothrombin to thrombin.

The localization to the cell surface make FXa relatively protected from inactivation mediated by protease inhibitors. However, FXa molecules that dissociate from TF-bearing cells are rapidly inhibited in the fluid phase by Tissue Factor Pathway Inhibitor (TFPI) and Antithrombin (AT). Thus, the presence of inhibitors localizes FXa activity to the surface on which it was converted to the active enzyme form. Contrarily, FIXa can move from TF-bearing cells to the platelet surface since it is not inhibited by TFPI and more slowly inhibited by AT than FXa.

Low level activity of the TF pathway probably occurs at all times in the extravascular space. The coagulation proteins leave the vasculature, percolate through the tissues, and are found in the lymph roughly in proportion to their molecular size [20]. Thus, FVII is probably bound to extravascular TF even in the absence of an injury [21], and the extravascular FX and FIX can be activated as they pass through the tissues. This idea is consistent with the finding that low levels of the activation peptides from coagulation factors are present in the blood of normal individuals [21: Bauer 1990, Blood]. This has been called "basal" coagulation or "idling" [22] This process does not lead to clot formation under normal circumstances, because the really large components of the coagulation process, platelets and vWF-complexed Factor VIII (FVIII), are kept sequestered in the vascular space. Coagulation only proceeds when damage to the vasculature allows platelets and FVIII/vWF exposure into the extravascular tissues and to adhere to TF-bearing cells at the site of injury.

Amplification phase

The amplification phase represents the base for the subsequent large-scale formation of thrombin during the propagation phase.

Several important functions are exerted by the small amount of thrombin generated on TF-bearing cell in the initiation phase. One of these functions is the activation of platelets, resulting in an increase in phosphatidylserine exposure on the membrane outer leaflet [23], thus serving as a surface for assembly and activity of the coagulation complexes. Although platelets have already adhered at the site of injury and become partially activated, the addition of thrombin can induce a higher level of procoagulant activity than adhesive interactions alone [24]. Indeed, thrombin can move from the TF-bearing cells to platelets, where it binds to its high-affinity receptor GPIb [25]. As a result of full activation, platelets release partially activated forms of FV onto their surfaces. Another function of thrombin formed during the initiation phase is the activation of FV and FVIII on the activated platelet surface. In this process, the FVIII/vWF complex binds to platelets where is dissociated upon thrombin cleavage, allowing vWF to mediate additional platelet adhesion and aggregation at the site of injury (Fig. 1.1.2.2).

Thrombin also converts Factor XI (FXI) to its active form (FXIa), activated by the prekallekrein/kininogen/Factor XII cascade in the classic "intrinsic parhway", which enhances the amount of platelet surface FIXa, increasing the supply of platelet surface FX

and thereby acting as a "booster" of thrombin generation on the platelet surface [26]. This finding also strengthen the hypothesis that the intrinsic mechanism gives no contribute to in vivo coagulation process.

By the end of the amplification phase, the "stage" is set for the large-scale thrombin generation that occurs in the propagation phase.

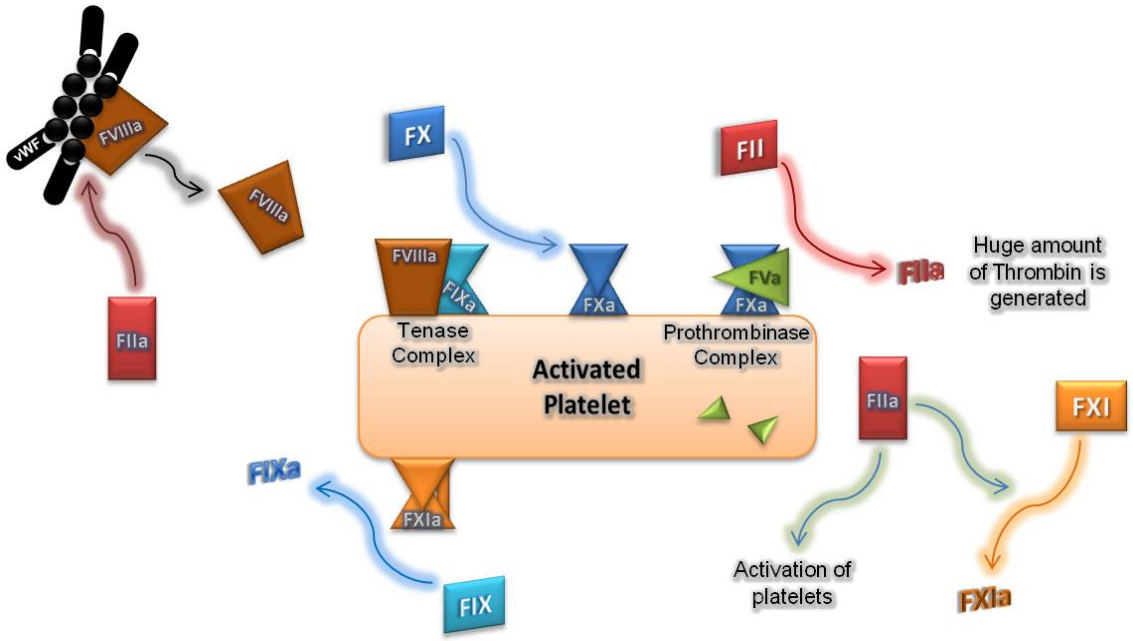

Figure 1.1.2.2: On the surface of an activated platelet, factor IXa formed on the TF-bearing cell can incorporate into a Xase complex. Additional factor IXa is formed by platelet-bound factor XIa. Factor Xa formed on the platelet membrane surface is channelled into IIase complexes, leading to a burst of thrombin generation. Because factor XI is activated on the platelet surface by thrombin, PK, HK, and factor XII are not required for thrombin generation in this model.

Propagation phase

The propagation phase of clot formation occurs on the surface of activated platelets. First, FIXa generated during the initiation phase can now bind to its activated cofactor, FVIIIa, on the platelet surface, thereby assembling in the so called "tenase-complex". Second, additional FIXa can be supplied by platelet-bound FXIa. Third, because FXa cannot move effectively from the TF-bearing cell to the activated platelets, FXa must be provided directly on the platelet surface by the FIXa/FVIIIa complex. Fourth, the FXa rapidly associates with FVa bound to the platelet during the amplification phase, producing a burst of thrombin generation of sufficient magnitude to clot fibrinogen [13,21,28]. Hence more

than 95% of the total amount of thrombin production takes place after initial clot formation, during propagation phase [29].

The burst of thrombin generated on the platelet surface produces a stable clot structure. Indeed, it has been proposed for thrombin additional actions responsible for clot stabilization: i) activation of Factor XIII (FXIII), the fibrin stabilizing factor [30]; ii) cleavage of the platelet protease- activated receptor-4 (PAR-4), that contributes to the full activation of human platelets [31]; iii) activation of thrombin activatable fibrinolysis inhibitor (TAFI) [32]. TAFI is a carboxypeptidase that removes terminal lysine residues from fibrin, thereby removing potential binding sites for fibrinolytic enzymes and enhancing clot resistance to fibrinolysis [33]. Greater levels of thrombin activity are needed to activate TAFI than to form a fibrin clot. Failure in TAFI activation is thought to contribute significantly to the bleeding tendency in hemophilia [34].

Termination phase

Once a fibrin platelet clot is formed over a damaged area, the clotting process must be limited to avoid thrombotic occlusion in other normal areas of the vasculature [21].

The TF/FVIIa activity is inhibited by the Kunitz-type inhibitor TFPI [35,37], secreted by endothelium. TFPI binds to FXa forming a quaternary complex with TF/FVIIa that quickly limits coagulation [38].

The serine protease inhibitor Antithrombin (AT) [39] neutralize enzymes of the coagulation system; its physiological role is to protect the circulation from free enzymes and limit the coagulation process to sites of vascular injury. Circulating AT is a relatively inefficient serpin, but its activity is stimulated by heparin and presumably by heparin-like molecules such as sulfated glycosaminoglycans that are synthesized and expressed by endothelial cells [40]. The increasing efficiency of AT by heparin is the molecular basis for the use of heparin as a therapeutic anticoagulant. Antithrombin is the major thrombin-inactivating protein [41].

While TF-bearing cells and platelets have procoagulant functions, vascular endothelial cells have anti-coagulant features.

The protein C (PC) anticoagulant system inhibits the procoagulant functions of FVIIIa and FVa, the cofactors involved the tenase and prothrombinase complexes, respectively [42,43]. The key component in the system is protein C (PC), a vitamin K-dependent zymogen (proenzyme) activated by thrombin bound to the membrane protein thrombomodulin (TM),

acting as a receptor for thrombin, located on the surface of intact endothelial cells. Upon binding to TM, the specificity of thrombin is changed, becoming more effective at activating PC than clotting fibrinogen or activating platelets [44]. By this way, thrombin effectively changes from a pro-coagulant to an anti-coagulant molecule when it is localized to an endothelial cell surface expressing TM.

Activated PC (APC) cleaves a few peptide bonds in each of the phospholipid membrane-bound cofactors FVa and FVIIIa, resulting in the inactivation of the cofactors [45]. APC can also cleave the intact form of FV. The consequence of APC-mediated cleavage of factor V is the generation of anticoagulant FV that functions in synergy with protein S as an APC cofactor in the degradation of FVIIIa. Thus, factor V can function as a procoagulant and an anticoagulant cofactor, procoagulant factor Va being formed after limited proteolysis by thrombin or factor Xa, whereas the anticoagulant FV activity is expressed by factor V that has been proteolytically cleaved by APC [46].

APC activity is enhanced by another vitamin K-dependent inhibitory cofactor, protein S (PS) (Fig. 1.1.2.3). In human plasma, about 30% of protein S circulates as free protein; the remaining is bound to the complement regulatory protein C4b-binding protein. Only the free form of PS functions as a cofactor to APC.

In addition to TM and heperan-like glycosaminoglycans on their surface, endothelial cells express a cell-surface ADPase (CD39) that metabolizes ADP release from activated platelets, thus blocking the aggregation when platelets are in close proximity to healthy endothelium.

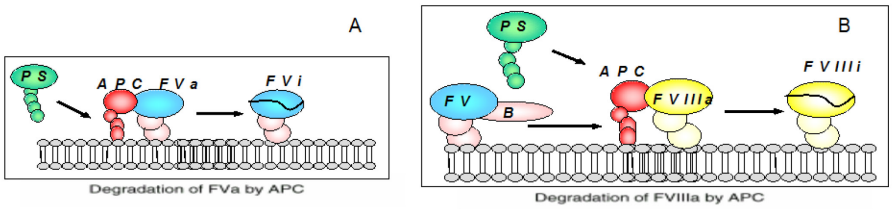

Figure 1.1.2.3. The protein C pathway
(A) Inactivation of FVa by Activated Protein C (APC) and protein S (PS)
(B) Inactivation of FVIIIa by APC, PS and FV

1.1.3 Macromolecular complexes

Activation of clotting factors does not occur in the solution phase, but within membrane-bound macromolecular complexes, each comprising a vitamin K-dependent serine protease, a non-enzymatic protein cofactor and a zymogen substrate, as well as Ca^{2+} ions [47] (Table 1.1.3.1).

Complex	*Enzyme*	*Cofactor(s)*	*Substrate(s)*
Initiation complex	FVIIa	TF	FIX, FX
Prothrombinase	FXa	FVa	PT
Intrinsic ten-ase	FIXa	FVIIIa	FX
Protein C-ase	Thrombin	TM, (EPCR)	PC

Table 1.1.3.1. Macromolecular complexes in blood coagulation. TM, thrombomodulin; EPCR, endothelial protein C receptor.

The protein-phospholipids and protein-protein interactions within the macromolecular complexes enhance reaction rates by several orders of magnitude, by affecting both the K_M for the substrate and the k_{cat} of the enzyme. Moreover, localization of different enzyme complexes on the same membrane surface allows product "channelling" between successive reaction centres, a circumstance that protects activated factors from inactivation by circulating inhibitors [47].

Finally, macromolecular complexes offer several opportunities for the control of coagulation reactions. In fact, complex assembly requires a number of simultaneous events: the conversion of a zymogen to the active serine protease, the activation of a procofactor to the active cofactor and the availability of negatively charged phospholipid membranes. The latter condition guarantees the confinement of the coagulation process to the site of injury [47] .

The catalytic domains of the coagulation serine proteases are highly homologous [48]. Despite the similarities, the coagulation proteases act on their substrates with narrow and distinctive specificity. It is assumed that the substrate specificity arises from specific interactions between the enzyme active site and distinctive sequences surrounding the scissile bond. In the case of FVIIa-TF, this assumption is supported by similarities between the residues preceding the scissile bonds in FIX and FX.

A series of studies have suggested a role for extended interactions between FX and surfaces in both FVIIa and TF during FX activation. Evidences support a direct interaction

between the N-terminal Gla domain in the FX light chain and regions of the FVIIa-TF complex near the membrane surface [49,50,51,73]. Since the activation peptide at the N-terminus of the heavy chain of FX is released upon cleavage, it is possible that the interactions between the substrate and the FVIIa-TF complex involve structural determinants common to FX and FXa. This finding would suggest the usage of a common region of TF in a dual role, as cofactor for FXa-mediated FVII activation and as cofactor for FVIIa-mediated FX activation. The same TF area would also contact FIX during its FVIIa-mediated activation [52].

Exosite-dependent substrate recognition implies that extended surfaces on the enzyme complex, distinct from residues surrounding the catalytic site, contribute to substrate affinity by favoring productive interactions between enzyme and substrate. The demonstration that an uncleavable form of FX can act as a competitive inhibitor of FX activation favors a role for exosite interaction in FVIIa-TF function [53]. New evidences showed that peptide binding to regions of the FVIIa distinct from the active site can lead to the inhibition of FX activation by the FVIIa-TF complex [54]. Peptide binding to this site led to non-competitive inhibition of FX activation by FVIIa-TF and partial inhibition of substrate cleavage.

The recognition of FX by Xase arises from a multistep reaction requiring an initial interaction at exosites, followed by active site interaction and bond cleavage. Exosite interactions determine substrate affinity, whereas the second binding step influences the maximum catalytic rate for the reaction [55]. Specific recognition of the substrate by interaction at exosites may represent a prevalent strategy by which specific cleavage is accomplished by the coagulation complexes. These complexes may have evolved their distinctive specificity not only through changes to residues surrounding the catalytic site but also by changes to residues in exosites that influence binding specificity for the substrate.

1.2 Coagulation factor VII

The first details about this protein were reported by Nemerson in 1966: coagulation FVII is a vitamin K-dependent plasma protein, which is able to activate FX in the presence of tissue factor and calcium [56]. While bovine FVII was isolated and extensively studied since the 1974 [57], purification of human protein in sufficient quantities for

characterization was not accomplished until 1980, due to its low plasma concentration [58].

1.2.1 FVII gene

The human Factor VII gene, localized through techniques of ibridization in situ, is a single copy gene on the long arm of chromosome 13, region q34-qter (Fig. 1.2.1.1), 2.8 kb downstream FX gene [59]. Steady state levels of Factor VII mRNA were only 6% of those of Factor X mRNA, correlating well with the 10-20fold difference in molar plasma levels (FVII: 0.009 μM; FX: 0.18 μM). Thus, the diversity in plasma concentrations of the two coagulation factors is due to a variation in rates if transcription or in RNA stability or in both [60].

The complete nucleotide sequence of the human gene coding for factor VII was reported by O'Hara et al. in 1987, one year after the isolation and characterization of cDNA clones coding for FVII by Hagen et al. in 1986 [61,62]. Clones coding for FVII were obtained from two different cDNA libraries prepared from poly(A) RNA from human liver and human hepatoma HepG2 cells.

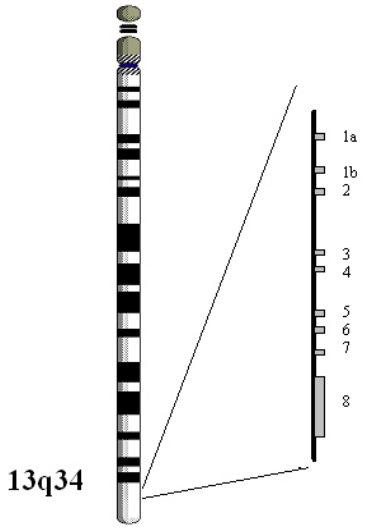

Figure 1.2.1.1. Representation of the human chromosome 13 and organization of FVII gene.

The FVII gene spread about 12.8 kb of genomic DNA and contained nine exons and eight introns (Table 1.2.1.1). The introns ranged in size from 68 nucleotides to nearly 2.6kb. The exons also varied considerably in size, ranging from 25 nucleotides to 1.6kb. Exons 1a, 1b

and part of exon 2 encode a prepro leader sequence that is removed during processing. The remainder of exon 2 and exons 3-8 encode the 406 amino acids present in the mature protein circulating in blood. Two different cDNAs coding for FVII were isolated by Hagen et al.: one clone coded for a prepro leader sequence of 38 amino acids and the second coded for a 60 amino acids leader sequence. The genomic sequence data revealed that the second clone contained an optional exon (exon 1b) that was absent in the first clone. cDNA clones representing the two mRNa in which exon 1b is either present or absent both give rise to functional transcripts which code for a biologically active FVII. In normal liver, however, mRNA lacking exon 1b is far more abundant than mRNA possessing this exon [63].

EXON	INTRON	CODING REGION	DIMENSION (bp)
1a		pre-proleader sequence	100
	1a		1068
1b		pre-proleader sequence	66
	1b		2574
2		pre-proleader, Gla domain	161
	2		1928
3		Gla domain	25
	3		70
4		EGF domain	139
	4		1716
5		EGF domain	141
	5		971
6		activation site	110
	6		595
7		catalytic domain	124
	7		817
8		catalytic domain	1622

Table 1.2.1.1. FVII gene organization in relation to exon/intron size

The –17 to –1 region of the vitamin K-dependent proteins functions as a γ-carboxylation signal domain. It is partially conserved in FIX, FX, PC, PS and PT and is present in the 38 or 60 amino acid leader of FVII. The 38 amino acid sequence resembles the other vitamin K proteins leader in sequence, size, hydrophobicity pattern, exon-intron structure, and predicted signal peptide cleavage more closely than the 60 amino acid leader. The predicted cleavage for the 60 amino acid leader would leave 16 amino acids encoded by exon 1b attached to the putative γ-carboxylase signal, but this is evidently tolerable.

As noted before for other vitamin K protein genes, exons in FVII gene encode discrete domains of the protein: prepro leader, γ-carboxylase region, growth factor domains, activation region and the serine protease domain. The conservation of domains, intron position, and intron phase (the conservation of phase preserves the reading frame) among the members of the vitamin K protein family supports the theory of differentiation by exon shuffling. The least conserved region is the activation domain. Unlike FIX, FX, PC and prothrombin, FVII does not release an activation peptide and the other proteins vary considerably in size and sequence in this region.

The striking degree of similarity between the exons of genes coding for vitamin K proteins contrasted with the lack of resemblance in the sequences or sizes of the introns of these genes [61]. For instance, introns in the human FIX and PC genes contain Alu repeats, whereas the FVII gene lacks such sequences. On the other hand, FVII gene contains five minisatellite imperfect tandem repeats (sequences that are repeated directly adjacent to each other) with monomer lengths ranging from 14 to 37 bp and copy numbers ranging from 6 to 52, FIX and PC lack minisatellite DNA [64]. These tandem repeats in FVII gene are often responsible for polymorphism due to allelic variation in the repeat copy number. Tandem repeats may evolve because of random crossover in DNA whose sequence is not maintained by selection. This suggests that much of the sequence information present in the introns and the 3' untraslated portion of FVII messenger may be dispensable.

An exception to the general dissimilarity of the introns of the vitamin K-dependent protein genes is intron 3 of protein C and FVII. The sequences of intron 3 of these two coagulation factors are not more dissimilar than the nucleotide sequences of exons 3 and 4 that flank this intron. It is possible that exons 3 and 4 of factor VII and protein C derived from an ancestral precursor that included an intact intervening sequence.

The gene coding for FVII contains copies of sequences that are typically associated with the regulation of transcription and translation. The sequence surrounding the ATG

initiation codon is TCATCATGG in which 7 of 9 nucleotides match with the translation initiation site consensus sequence of Kozak CCACCATGG. The sequence from nucleotide –366 to –260 exhibits limited homology with regions upstream of the putative transcription initiation sites of Factor IX and PC.

Promoter and silencer elements of the immediate 5' flanking region of the human FVII gene were identified and characterized [60,65,66]. Differently from the promoter regions of most eukaryotic genes, the 5' flanking sequence of FVII lack the typical TATA-box; the same feature has been found in promoters of other coagulation proteins like FIX, FX, FXII and prothrombin. Although a CAAT-box is critical in the promoters of Factor IX and X, no such element is apparent in the Factor VII promoter. Sequence alignment of FVII and FX promoters revealed similarity of 86% for a small 37-bp element, but the functional relevance, if any, of this sequence in unclear as its deletion did not alter significantly reporter gene expression. The major transcription start site has been identified within a strong initiator element (-57 CCCGTCAGTCCC -46), at position –51 upstream from the start site of translation (+1). Multiple other start sites have been recognized in the region surrounding the major transcription star site, a typical feature of constitutively expressed genes which lack TATA and CAAT sequences.

The major start site of FVII is only 8 bases downstream from a consensus sequence for the transcription factor hepatocyte nuclear factor-4 (HNF-4), which binds at nucleotide –63 to –58 (ACTTTG). This close proximity is similar to that of the FIX major start site, which is 18 bp from the HNF-4 sequence. HNF-4 binds FVII promoter with a lower affinity as compared to FIX and FX promoters and this may explain the large differences in steady-state mRNA levels and plasma concentrations of these coagulation proteins. HNF-4 has been found to play an important role in the transcription initiation of a number of genes expressed in the liver but it is not the only limiting factor in non-hepatic cells, so additional liver-specific factors are probably required to fully activate FVII promoter. To date, the proximal 185 base pairs upstream the ATG translation initiation codon were sufficient to confer liver-specific expression and maximal promoter activity in HepG2 cells.

The sequence from –101 to –94 (CCCCTCCC) was shown to be a binding site for the ubiquitous transcription factor Sp1 and a sequence with homology to a hormone responsive element has been detected at –227 to –213 (Fig. 1.2.1.2).

Functional studies of promoter deletions in the HepG2 cell line showed that deletion of sequences from -1601 down to -1212 increases expression about 2-fold, which suggests that a negative element is present upstream of the -1212.

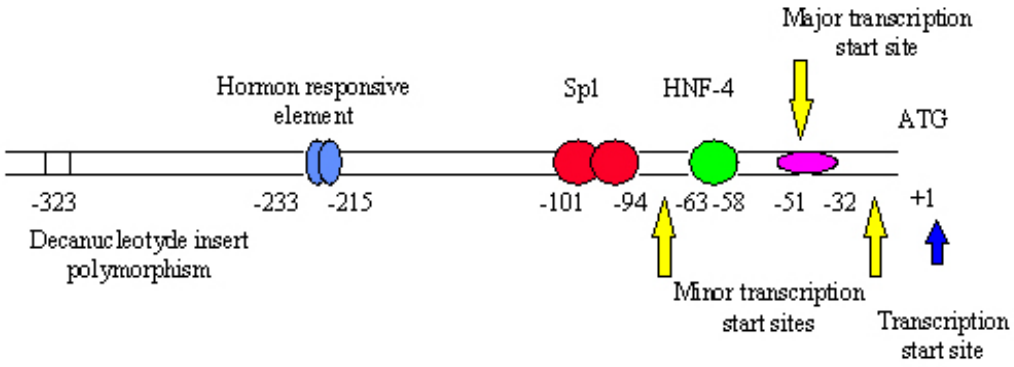

Figure 1.2.1.2. Schematic representation of regulatory sequences in factor VII gene 5'-flanking region

A comparison of different cDNA clones for Factor VII showed that alternative sites for polyadenylylation occurring downstream from the poly(A) signal of AATAAA were present in the 3'UTR. These multiple copies in FVII gene may direct polyadenylylation at more than one site [61].

1.2.2 Biosynthesis and post-translational modifications

FVII is synthesized by the hepatocytes and secreted as a serine protease precursor in plasma, where it reaches the concentration of about 10 nM (500ng/ml) [67].

The domain structures of the vitamin K-dependent coagulation factors FVII, FIX, FX, prothrombin, PC and PS, deduced from their cDNA sequences, demonstrate that they contain common structural features [68]. All contain a signal peptide, that is required for translocation into the lumen of the endoplasmic reticulum (ER), which is followed by a propeptide that directs vitamin K-dependent γ-carboxylation of the mature polypeptide. Upon transit through the trans-Golgi apparatus the propeptide is cleaved away. The amino terminus of the mature protein contains a γ-carboxy glutamic acid rich region (Gla) that includes a short α-helical stack of aromatic amino acids. Then there are two epidermal growth factor (EGF) like domains. The next region is the activation peptide that is glycosylated on an asparagine residue and presents the site of proteolytic cleavage. The

remainder of the vitamin K-dependent protein contains the serine protease catalytic triad (Fig. 1.2.2.1).

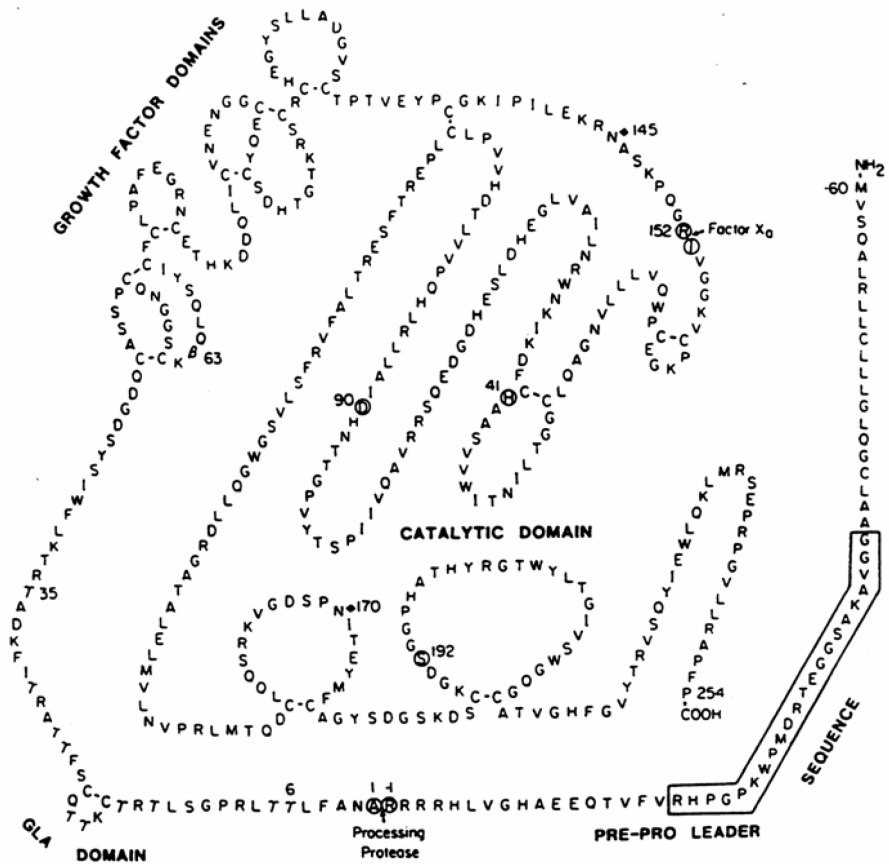

Figure 1.2.2.1 - FVII structure based on amino acidic sequence

Signal peptide cleavage. The precursor form of FVII that is translocated through the ER contains a hydrophobic signal sequence of 38 or 60 amino acids (Figure 1.2.2.1). The signal peptide mediates association of the nascent polypeptide with the cytosolic face of the ER. It is composed of three regions: 1) an amino terminal segment with a net positive charge, 2) a central hydrophobic core of 6-15 residues, and 3) a C-terminal region that often has a helix breaking residue (i.e Gly, Pro, Ser). The cleavage site for signal peptidase is marked by small amino acids (Ala or Gly) in the -3 and -1 positions relative to the cleavage site. Cleavage of the signal sequence by the signal peptidase then releases the mature amino-terminus into the lumen of the ER and is required for the translocation to the secretory pathway [68].

Disulfide bond formation. The vitamin K-dependent coagulation factors have some conserved disulfide bonds. Generally, three disulfide bonds occur within each EGF domain, and several disulfide bonds occur within the serine protease catalytic domain. In addition, in FVII, FIX, FX and PC a disulfide bond connects the amino terminal half with the carboxyl terminal half of the protein to prevent the dissociation of the two portions of the molecule after activation.

Disulfide bond formation occurs between cysteins belonging to the two different chains of activated Factor VII (Cys^{135}-Cys^{262}) and between cysteins of the same chains (Cys^{50}-Cys^{61}, Cys^{55}-Cys^{70}, Cys^{72}-Cys^{80}, Cys^{91}-Cys^{102}, Cys^{98}-Cys^{112}, Cys^{114}-Cys^{127}, Cys^{159}-Cys^{164}, Cys^{178}-Cys^{193}, Cys^{310}-Cys^{329}, Cys^{340}-Cys^{368}). Disulfide bond formation occurs in the oxidizing environment of the ER and it is possible that protein chaperones such as protein disulfide isomerase (PDI) are important to ensure proper disulfide bond [68].

Asparagine- and Serine-linked glycosylation. FVII presents two sites of N-glycosylation (Asn^{145} and Asn^{322}) and two sites of O-glycosylation (Ser^{52} and Ser^{60}). Addition of N-linked oligosaccharides is an obligatory event for the folding and assembly of newly synthesized polypeptides [69]. The presence of oligosaccharides is often required for the efficient transport of glycoproteins through the secretory pathway [70]. In addition, N-linked glycosylation frequently affects the plasma half-life and biological activity of glycoproteins. The ER lumenal enzyme oligosaccaryltransferase catalyzes the transfer of a preassembled high mannose containing an oligosaccaride core structure from a dolichol pyrophospahte precursor on to asparagine acceptor sites within the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. Transit out of the ER is the rate-limiting step in secretion for the majority of proteins and may vary from 15 min to days, depending upon the rate by which a polypeptide attains a properly folded conformation. Upon transit through the Golgi apparatus a series of additional carbohydrate, modifications occur that are separated spatially and temporarily. These reactions occur by specific glycosyltransferase that modify the high mannose carbohydrate to complex forms. Also within the Golgi apparatus, O-linked oligosaccharides are attached to the hydroxyl of serine residues through an O-glycosidic bond to N-acetylgalactosamine. O-glycosilation occurs in the Golgi complex concomitant with processing of complex N-linked oligosaccharides. Although the functional significance of these O-linked residues is not

known, this unusual structure may have some importance since the S52A FVII mutant possesses only 60% coagulant activity of wild type [71].

γ-Carboxylation of glutamic acid residues. The Gla residues are essential for vitamin K-dependent coagulation proteins to attain a calcium-dependent conformation and for their ability to bind phospholipid surfaces, an essential interaction for their function. The precursor of FVII contains a propeptide that directs γ-carboxylation of 10 glutamic acid residues at the amino-terminus of the mature protein (residues 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35). The propeptide (residues –17 to –1) of FVII shares amino acid similarity with other vitamin K-dependent proteins, by conservation of the γ-carboxylase recognition site and the cleavage site of the propeptide. NMR structural analysis of prothrombin identified that the propeptide is an amphipatic α-helix with the carboxylase recognition site N-terminal to the helix [72].

The function of Gla residues within the coagulation factors was studied by isolation of proteins from animals treated with inhibitors of γ-carboxylation, such as dicoumarol, by proteolytic removal of the Gla domain, and by site-directed mutagenesis of specific Gla residues. For example, des-γ-carboxy prothrombin binds Ca^{2+} much more weakly and is defective in procoagulant activity [73]. Analysis of partially carboxylated prothrombins demonstrated that their activation rates in coagulation assays are proportional to the number of Gla residues present. The cleavage of the Gla domain from vitamin K dependent factors is coincident loss of low affinity Ca^{2+} sites and with a greatly reduced biological activity.

Several moderate- to low-affinity calcium binding sites exist in the Gla domains of factors VII, IX, X, and protein C that are necessary for a conformational change requisite to phospholipids binding and as coordination sites for phospholipid binding.

In the absence of calcium ions, the Gla domain is disordered, whereas in the presence of calcium ions a unique structure is obtained.

The vitamin K-dependent γ-glutamyl carboxylase enzyme converts glutamate residues to Gla residues. In the presence of CO_2, O_2 and vitamin K hydroquinone (KH_2) the enzyme is able to carboxylate a peptide yielding Gla residues, vitamin K epoxide and H_20. The vitamin K epoxide formed is subsequently reduced by either a thiol or the enzyme vitamin K epoxide reductase to regenerate KH_2.

High expression levels of the vitamin K-dependent plasma proteins in transfected mammalian cells is limited by the ability of the host cells to efficiently perform γ-carboxylation of the glutamic acid residues as well as efficient cleavage of the propeptide.

β-hydroxylation. The unusual amino acid erythro-β-hydroxyaspartic acid, formed by post-translational hydroxylation of an aspartic acid residue, has been found in the EGF domain of FVII at position 63. Its function is unknown, as β-hydroxylation is unnecessary for high affinity calcium binding to the first EGF domain and inhibition of β-hydroxylation of factor IX expressed in mammalian cells did not reduce functional activity in factor IX [74,75]

Proteolytic processing. Propeptide cleavage occurs in the trans-Golgi compartment just prior to secretion from the cell. The localization of propeptide processing to this compartment ensures that the propeptide is associated with the mature polypeptide as proteins transit the secretory compartment. Characterization of the amino acid requirements at the propeptide cleavage site has identified that arginines at position 1 and 4 are important for processing. The enzymes candidates for this process are the subtilisin-like serine protease furin/PACE and PACE4, ubiquitously expressed but to a greater extent in the hepatocytes [68].

1.2.3 Activation

After purification of bovine FVII in 1974 Jesty and Nemerson asserted that FVII "apparently exists in plasma not as a zymogen, but in a partially active form" [57]. One year later Radcliffe and Nemerson better defined this characteristic of FVII [76]. In the presence of Ca^{2+} and phospholipids, single chain FVII is rapidly hydrolyzed by FXa and by thrombin to a two-chain form joined by disulphide bridges. This proteolysis is accompanied by an increase of at least 85-fold in the specific FVII coagulation activity with respect to the single chain species. In this report, the term "activated FVII" was used for the first time to depict the two-chain FVII form. The activation of Factor VII involves the cleavage of the single peptide bond located at Arg^{152}-Ile^{153} in the sequence Arg-Ile-Val-Gly-Gly [77].

In 1977 Kisiel *et al*. proved that FVII can be converted to FVIIa also by FXIIa [85], while in 1979 Seligsohn *et al*. demonstrated the important role of FIXa in FVII activation [78].

Several years later Nakagaki reported an autocatalytic mechanism of FVII activation following complex formation of FVIIa with TF, which may play a key role in the initiation of extrinsic coagulation in normal hemostasis [79]. Non physiological activators include hepsin [80], which proteolytically activates human FVII in a time- and calcium-dependent manner, as well as the prothrombin activator from the venom of Taipan snake [81].

A detailed kinetic estimate of FVII activation was performed in 1996, by Butenas and Mann, who studied the catalytic efficiency of several plasmatic enzymes and complexes towards FVII. A very sensitive fluorogenic substrate permitted the evaluation of FVIIa activity at nanomolar and subnanomolar concentration of this enzyme [82]. The FVIIa-TF complex was able to generate detectable levels of FVIIa only when high concentrations of the enzyme complex (0.1 nM) and of anionic phospholipids (Phosphatidylserine 25%/ phosphatistlcoline 75% vesicles, PCPS: 200 μM) were used. At physiologic concentrations, FXa was found to be a more effective activator (at least 15-fold better) of FVII than the FVIIa-TF complex. The FXa activation of FVII at plasma concentrations of the substrate was dependent upon phospholipids concentration. Previous publications on the influence of phospholipids on PT activation by FVa showed that in the presence of calcium ions, phospholipids increased the rate of thrombin [83]. Further increase in the concentration of phospholipids, however, decreased the ability of FXa to activate prothrombin. This effect is most likely caused by the dilution of enzyme and substrate on the phospholipid vesicles [84]. At a concentration of PCPS (5-20 μM) optimal for FXa binding to the phospholipid surface, the activation rate of FVII is only 21% of maximum. Increasing the PCPS concentration facilitates FVII binding to the membranes, but at the same time “dilutes” the enzyme FXa. At phospholipid concentrations optimal for FVII activation the loss in FVIIa generation rate due to the FXa-membrane dilution is compensated by increased FVII binding to PCPS. Therefore, for an efficient FVII activation both FXa and FVII must be located on a phospholipid vesicle [82]. FVII activation by FXa was not observed in the absence of PCPS/Ca^{2+}.

FVIIa, in the absence of TF, failed to activate FVII at detectable rates even if very high enzyme concentrations and long incubation times were tested. Tissue factor increased the ability of FVIIa to activate FVII to approximately 2-3% of that observed for FXa. No detectable activation of FVII was observed when thrombin, FIXa or FXIa were used as activators. FVIIIa in the presence of PCPS had no effect on the ability of FIXa to activate

FVII; FVa progressively decreased the FVII activation rate by FXa. These data suggested that the predominant physiological FVII activator is, most likely, membrane-bound FXa.
Basal *in vivo* levels of FVIIa are thought to be primarily generated by FIXa [85]. This statement is based on data showing that in patients with severe FIX deficiency the mean FVIIa level is markedly suppressed and the administration of full replacement doses of FIX led to a normalization [86].

1.2.4 FVIIa structure

Cleavage of the single peptide bond located at Arg^{152}-Ile^{153} generates the mature N-terminal residue Ile^{153}, and enables conformational changes that create the active enzyme. The structural modifications arise in a contiguous collection of four peptide segments collectively termed the "activation domain". Among these fragments the most important is the new N-terminus, which becomes buried with its non polar side chain in a hydrophobic environment and its charged α-amino nitrogen atom forms a salt bridge with the carboxyl group of Asp^{343} side chain. The three associated segments that undergo changes, creating the substrate binding cleft, are termed, based on chymotrypsin numbering, Loop 140s (142-152, in FVIIa 285-294), Loop 1 (186-194 in FVIIa 334-343) and Loop 2 (216-223 in FVIIa 365-372).
Blood coagulation FVIIa is a trypsin-like plasma serine protease and in its catalytic domains has strong primary sequence identity and tertiary structure similarity with trypsin and chymotrypsin. As in chymotrypsin, FVIIa catalytic domain folds into two domains of the antiparallel β-barrel type, each containing six β strands (Fig. 1.2.4.1). The active site is situated in a fissure between the two domains. The enzyme provides a general base, a His residue, which can accept the proton from the hydroxyl group of the reactive Ser thus facilitating formation of the covalent tetrahedral transition state. The His residue is part of a catalytic triad consisting of three side chains from Asp, His and Ser. One domain contributes two of the residues in the catalytic triad, His^{193} and Asp^{242}, whereas the reactive Ser^{344} is part of the second domain. Tight binding and stabilization of the transition state intermediate is facilitated by formation of hydrogen bonds between the enzyme and the substrate. These groups are in a pocket of the enzyme called oxyanion hole, while substrate specificity is dictated by the perfect fitting of the preferred side chains into pockets of the enzyme called specificity pockets. The substrate specificity pocket accommodates the side chain of the residue preceding the scissile bond.

A fine characterization of the crystal structure of FVIIa has centred the interest on the loops between the β strands that, for their variable length and composition, usually confer specificity to proteinases and are thought to participate in the mechanism of the enzymatic activity [87,88]. In fact, flexible loops seem involved in the direct recognition of substrates and in the transmission of the cofactor-induced effect from the interface to the catalytic domain [89,90] and they would also be privileged point of inactivation by degradation [87].

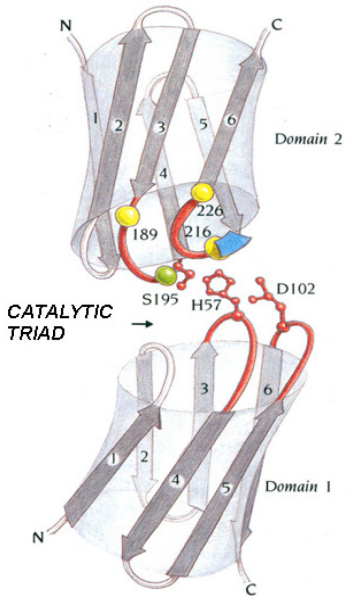

Figure 1.2.4.1. Representation of the catalytic domain of chymotrypsin

1.2.5 FVII structure

In 2001, trying to isolate crystals containing a shortened FVII construct (EGF2 plus protease domains) and the potent FVIIa/TF inhibitor peptide A-183, Eigenbrot *et al.* obtained crystals containing the zymogen instead of the enzyme [91]. Compared to the soluble TF-FVIIa complex structure, the key feature of this zymogen structure is a unique registration of the β strand B2 that permits Glu^{296} H bonds with residues near the scissile Arg^{152}-Ile^{153} peptide bond and precludes TF binding. Because the energetic cost of the transition between the two β strand B2 frames seemed small, it was suggested that there might be also a significant minority of FVII molecules that have a re-registered B2, resulting in loss of the Glu^{296} H bonds with residues 158 and 159 and a competent TF binding region. If TF binds to FVII, it would select for this species. When TF-FVII complex undergoes the activating cleavage reaction, the Ile^{153}-Asp^{343} salt bridge can be formed immediately, and a fully competent enzyme results. Alternatively, when FVII is

cleaved before association with TF, the equilibrium mixture would include a population in which H bonds between Glu^{296} and residues 158-159 prevent the formation of the Ile^{153}-Asp^{343} salt bridge. The catalytic activity of this equilibrium mixture would then be low due to the predominance of this form.

It has long been known that coagulation factor VII/VIIa is present in circulating blood but is largely inactive and even the nominally activated FVIIa retains zymogen character. Upon exposure to the extravascular environment, FVIIa forms a complex with the cell-surface bound TF and this combination activates downstream clotting factors. TF helps localize and orient FVIIa and guides substrates to productive interactions with the FVIIa active site.

1.3.1 FVIIa/TF complex

1.3.1 Tissue Factor

The concept and term of tissue factor, we have to look back to the late nineteenth century and early twentieth century. For over a century, studies have been directed at the clot-promoting activity of tissue or tissue fluids. This activity has long been known as thromboplastin or thrombokinase and more recently as “tissue factor”.

In 1845, Buchanan observed that the intravenous injection of various tissues extracts was able to accelerate clotting and to occlude the animal's blood vessels with clots. In 1862, Alexander Schmidt suggested that tissues provided a zymoplastic substance which converts prothrombin to thrombin and subsequently fibrinogen to fibrin. He described the substance as being thermostable and soluble in alcohol. Morawitz was the first to use in 1905 the term thrombokinase to describe the clot-promoting substance found in tissues and introduced it into his clotting theory. Later, in 1908, Nolf used the term tissue thromboplastin, and even later Howell introduced the term tissue factor. Morawitz and co-workers extracted thrombokinase with water, described it as thermolabile and concluded that the preparation contained proteins. The first attempts to purify tissue factor were undertaken by Howell in 1912 and Chargaff in 1944. Howell proposed that the thermolabile component was indeed a protein and that the thermostable component was a lipid. Chargaff demonstrated that tissue thromboplastin was associated with phospholipids. This clearly showed for the first time that tissue factor forms a complex with lipids [92].

The capacity for detergent solubilization of tissue factor lead ultimately to its purification from bovine and human brain [93,94].

In 1987 four different groups published the cDNA sequence of the Tissue Factor gene and its 5' and 3' flanking sequences, but its complete genomic sequence was published only in 1989 by Mackman *et al* [95]. TF is a transmembrane glycoprotein encoded by a 12.4 kb gene localized to human chromosome 1, specifically at p21-p22. The six exons are translated into a 295-residue precursor that included a leader sequence of 32 residues and a mature protein of 263 amino acids. In the mature protein, a remarkably hydrophilic extracellular domain of 219 amino acids precedes a hydrophobic 23-residue transmembrane region and a 21-residue cytoplasmic tail. Three potential N-linked glycosylation sites are available in the extracellular domain of human TF for assembly of carbohydrate moieties, which are responsible for the considerable heterogeneity of charge of TF. The cytoplasmic domain includes a single cystein residue that is not disulphide linked owing to the intracellular reducing power but is thioester bonded to stearate or palmitate [96], which may help anchor it into the plasma membrane. TF is readily transported to the cell surface upon synthesis and its turnover appears to be slow on cells that constitutively express TF.

The lung and the central nervous system are known to contain high levels of TF activity. Prominent expression of TF has been observed in cardiac myocytes, renal glomeruli, the granular layer of the epidermis, the epithelium of oropharynx and vagina, and in intestinal, urinary, bladder and respiratory mucosa [97]. TF is thus expressed at tissue barriers between the body and the environment and is also found at boundaries between organs, such as in fibrous organ capsules of liver, spleen and kidney, as well as in the adventitia of arteries and venules. The "envelope" pattern of TF expression is thus consistent with the function as an initiating molecule to arrest bleeding. Endothelial cells and monocytes are the only two cell types in the vasculature that can be stimulated to transiently express TF upon stimulation by certain inflammatory cytokines and by bacterial lipopolysaccharide (LPS).

TF has been classified as a member of the cytochine/hematopoietic growth factor receptor family. Molecules of this class are cell-surface proteins that are usually anchored through a single transmembrane domain followed by a cytoplasmic domain of varying length. The extracellular domain contains two tandem 7 β-strand sandwich-type modules that show structural similarities to the fibronectin III subclass of the immunoglobulin superfamily.

The extracellular domain of TF has been crystallized [98] and the structure solved simultaneously by two groups [99,100]. TF consists of two fibronectin III modules, which are characterized by two β-sheets formed either by three/four strands and are associated through an extensive interdomain region. The two modules are oriented with an angle of 125º and the solvent exposed surface of each has a similar number of charged residues. An interesting feature of the TF extracellular domain is the presence of stretches of α-helical structure. An extended finger-like region not only contains the α-helix, but also includes a short antiparallel β-sheet, which protrudes from the side of the molecule at the intermodule interface. This loop is highly conserved between mammalian sequences for TF and is particularly long. Interstrand loops from both modules interdigitate in a manner confering a high degree of rigidity to this receptor that will act as a scaffold for FVIIa on the membrane surface.

1.3.2 TF binding to FVII/FVIIa

One of the first attempts to measure the interaction between bovine TF and FVII/FVIIa was accomplished by Bach *et al*. in 1986 [101]. Homogeneous full-length tissue factor, purified from bovine brain, was reconstituted into phospholipid vesicles and its dissociation constant for FVII and FVIIa indicated that the one-chain zymogen binds to TF with slightly less affinity than the more active two-chain enzyme. The difference was judged significant but the magnitude of the change was small and could not account for the TF-induced FVIIa catalytic enhancement. Findings that the macromolecular substrates altered the interaction between FVIIa and TF suggested that this might be a complex phenomenon [102:]. These findings have led to the proposal of an ordered essential activation model in which the FVIIa undergoes two conformational transformations: one as a consequence of binding to TF, resulting in a species which binds to and hydrolyzes its natural substrates. The other conformational change in the FVII is induced by substrate, resulting in a species which binds more tightly to TF. Thus, the substrate induced a "conformational cage" which precludes the dissociation of FVIIa from TF when significant concentrations of substrate are present [102].

A few years later, a recombinant form of TF consisting of the extracellular domain (TF_{1-219}) was produced, resulting in a soluble TF form [49]. This truncated form of TF was exploited to characterize primary assembly of the TF-FVIIa complex and its catalytic function towards the substrates. These data provided evidence for the catalytic function of

TF-FVIIa independent of assembly on phospholipids and further demonstrated that the primary protein:protein interactions of FVIIa with the surface domains of TF alone are sufficient for marked enhancement of the catalytic function of FVIIa [103].

The interaction between FVII/FVIIa and TF has been extensively studied using extracellular domain of TF in a variety of experimental systems ranging from measurements of the binding interaction, using cell-associated TF [104] and TF immobilized on solid surfaces [49,105] to studies using intact TF in solution or reconstituted into synthetic membranes [101,106]. In all cases, the interaction between FVII or FVIIa and TF required the presence of calcium ions and was completely reversed by the presence of EDTA. A weaker interaction was observed using recombinant TF containing only the extracellular domain, thus membrane insertion of TF is not an absolute requirement for its interaction with FVIIa. A detailed investigation carried out by Krishnaswamy in 1992 led to the conclusion that i) the calcium dependent reversible interaction between FVIIa and TF is characterized by a dissociation constant of approximately 0.25 nM in the absence of FX, with approximately 1 mole of FVIIa combining per mole of TF at saturation. ii) The equilibrium parameters are independent of the nature of membranes, indicating that the enzyme complex assembles through protein-protein interactions that are not stabilized further by interactions between FVIIa or TF and the membrane surface. iii) The strong dependence of the rate of FX activation on the nature of the membranes used to reconstitute TF implies that the influence of membranes on the reaction is primarily exerted at the level of the substrate utilization and not complex assembly. iv) The independence of the equilibrium parameters for the binding of FVIIa to TF on ionic strength implies that this high affinity event is mediated by hydrophobic interactions [107].

1.3.3 TF-FVIIa structure

Numerous studies by several groups [49,108] consistently demonstrated that the binding of TF to FVIIa occurred via wide interface involving all four domains of FVIIa. This was subsequently confirmed by evidences from the crystal structure of the soluble TF-FVIIa complex [109], which showed that binding of FVIIa to TF occurs over an extended region of the FVIIa molecule. The complex is about 115 Å in length, has a diameter of 40-50 Å and involves three major contact sites with a total buried surface of 1800 $Å^2$ (Figure 1.3.3.1).

The C-terminal fibronectin type-III domain of TF near the membrane insertion binds the FVIIa Gla domain. Seven bound calcium ions were observed, six of which were arranged in a linear fashion. Binding of Gla^{6} and Gla^{7} to the row of calcium ions made a "W-like" structure in which the Phe^{4}, Leu^{5} and Leu^{8} protruded down towards the cell membrane. This interaction is mainly hydrophobic and involves mostly residues of the C-terminal helix of Gla domain (from Asp^{33} to Gly^{47}), a region commonly referred to as the hydrophobic or aromatic stack. Only one H-bond between FVIIa and TF has been clearly identified at this interface. The second contact site occurs between residues at the boundary between the C-terminal and the N-terminal fibronectin type-III domains of TF and the EGF1 domain of FVIIa. Binding at this interface is mediated by hydrophobic interactions as well as by a number of H-bonds that occurs mostly between the N-terminus of TF and the EGF1 domain of FVIIa. This domain in FVIIa binds a single calcium ion, as do the corresponding domains in FIX and FX. One sugar, may be identified on each of the two known O-glycosylation sites in EGF1, Ser^{52} and Ser^{60}. In the third site of contact, the EGF2 and the catalytic domains of FVIIa form a coherent structural unit that interacts with the top of the N-terminal domain of TF. Only two small separated hydrophobic interactions have been detected and several intermolecular H-bonds contribute to binding at this interface. In contrast to the other two contact regions, the centre of this interface contains water molecules that H-bond with FVIIa and TF side chains. The hydrophilic nature of this interface probably makes it more suitable in mediating the substantial conformational changes required to enhance enzyme activity. For the overall affinity of the TF-FVIIa interaction, the Gla and EGF domains contribute to more than 70% of the free binding energy. A high affinity calcium-binding site was found in FVIIa catalytic domain. Unlike trypsin, the loop Cys^{310}-Cys^{329} is five residues longer and is the only potentially mobile structure between the FVIIa active site and TF. This flexible region is therefore a major candidate for involvement in the TF-mediated enhancement of FVIIa activity, as this regional flexibility contributes to the inability of FVIIa to attain the active conformation and the interaction of TF with this flexible region may stabilize the structure in a conformation similar to that of the active state of FVIIa [110,111]. *In vitro* mutagenesis of amino acidic residues in both FVII and TF and the use of short peptide sequence inhibitors, has led to the identification of residues of the complex involved in the recognition [108]. Some of these residues in the catalytic domain of FVIIa directly participate in the formation of the complex (Arg^{277}, Met^{306}, Asp^{309}), while others, like Phe^{374} are involved in

the transmission of allosteric changes responsible for the TF-mediated catalytic enhancement of FVIIa [112,113].

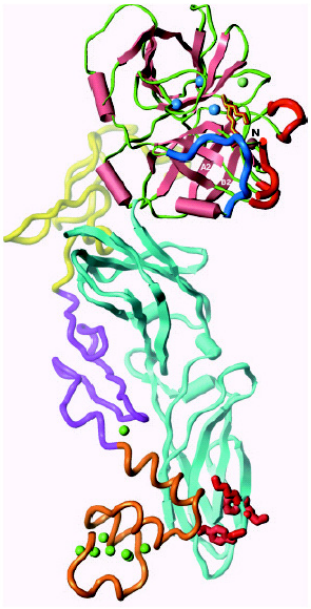

Figure 1.3.3.1. Overall view of the TF-FVIIa complex
The membrane is presumed to be at the bottom of this view. The three FVIIa light chain domains, Gla, EGF1, and EGF2, are colored orange, purple, and yellow, respectively, as they combine in a linear fashion with TF (light blue). The FVIIa protease domain (heavy chain) is at the top, depicted with salmon-colored ribbons and cylinders representing b-strands and α-helices, respectively. Protease domain loops are green, except for parts of three activation domain loops in red and the Loop 170s in blue. Green spheres in the Gla, EGF1, and protease domains represent calcium ions. Blue spheres in the protease domain denote the catalytic triad of Asp^{242}, His^{193}, and Ser^{344}. The protease domain N-terminus (dark sphere) is labeled "N." The red and yellow fragment near the protease domain represents part of a substrate bound in the active site cleft. The β-strands A2 and B2 are labeled. Tissue factor side chains depicted in red are where substrates Factor IX and Factor X contact TF

1.3.4 TF-induced enhancement of FVIIa activity

In trypsin the mechanism for zymogen activation requires limited proteolytic cleavage, insertion of the new Ile^{16} N-terminus into the domain core and salt bridge formation with the Asp^{194} side chain adjacent to active site. This strong interaction modifies the enzyme's specificity binding pocket for optimal substrate accommodation [114,115]. As stated before, coagulation FVIIa is present in circulating blood but is largely inactive. Even if FVIIa retains zymogen character, it would exist in equilibrium between dominant "zymogen-like" and fully active forms [87,110,111,116]. There is evidence that in FVIIa N-terminal insertion does not take place, or it is only partial [116,117], hence the

hypothesis that TF binding stabilises insertion and promotes protease activity. N-terminus insertion with an increase in FVIIa activity upon TF binding is supported by studies on TF binding influence on FVIIa activity.

Based on alanine scanning mutagenesis of the FVIIa protease domain surface, it has been proposed that TF may also function through an extended stabilization of the "activation domain", which would enhance catalysis also by influencing Asp^{338}, which form the bottom of the specificity pocket. This model would suggest that FVIIa may present itself in a zymogen-like state to TF and that conformational changes occur subsequent to docking of the protease domain with TF [118]. These conformational changes would be transmitted from the area of direct contact to the catalytic domain, through Loop1, Loop2, Loop 140s and Loop 170s in FVIIa heavy chain. Alternatively, TF may preferentially bind to the active conformation of FVIIa, in which the salt bridge is already formed. Assuming that FVIIa exists in equilibrium between minor active and dominant zymogen-like inactive conformational states, preferential binding of TF to the active state would lead to a shift in the equilibrium [116]. The strategies privileged by TF to enhance FVIIa catalytic efficiency are still unclear. Based on recent studies, soluble TF binding to FVIIa would alter the chemical environment of the FVIIa active site by protecting Ile^{153} from deprotonation in the free enzyme while deprotecting the catalytic triad when in complex with the substrate [119].

The structural determinants for the propensity of FVIIa to stay in a zymogen-like conformation have not been already clarified. From studies by Petrovan *et al*., it appears that Met^{298} contributes to the labile enzyme conformation of FVIIa. In fact, replacement of this residue with a Gln, the side chain found if FIX, had little effect on the activity of TF-bound FVIIa, while the free mutated enzyme had enhanced catalytic function towards macromolecular and small peptide substrates [120].

1.4 FVII activity

FVIIa catalyses the hydrolysis of peptide bonds within a polypeptide chain to produce two new smaller peptides. It is a trypsin-like enzyme, recognizing and cleaving peptide bonds after Arg or Lys side chains and the reaction proceeds in two steps. The first step produces a covalent bond between C1 of the substrate and the hydroxyl group of the reactive Ser of the enzyme. Production of this intermediate proceeds through a negatively charged

transition state intermediate. During this step the peptide bond is cleaved, one peptide product is attached to the enzyme in the intermediate and the other product rapidly diffuses away. In the second step, the intermediate is hydrolysed by a water molecule, releasing the second peptide with a complete carboxy terminus and restoring the Ser hydroxyl of the enzyme. This step proceeds through a negatively charged tetrahedral transition state intermediate. During the formation of the intermediate, the His of the catalytic triad accept a proton, first from the Ser and then from the water molecule and its positive charge is buffered by the Asp of the triad.

In 1964, FVIIa proteolytic activity was observed first against FX, but the methods used didn't reveal whether TF first reacted with FVII to form an intermediate that then activated FX or whether TF directly activated FX. A few years later, it was demonstrated that TF interacted with FVII and the formed intermediate was catalytically active [18]. The reaction product of TF and FVII is a potent activator also of FIX [121] (Fig. 1.4.1).

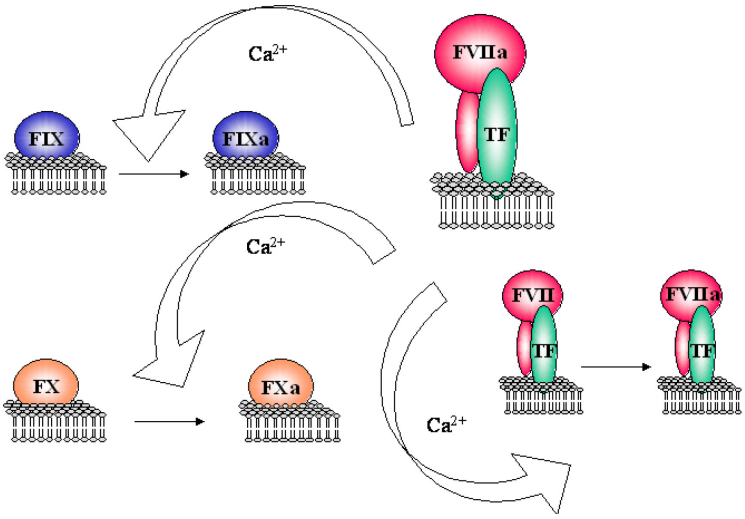

Figure 1.4.1. FVIIa activity

The ability of FVIIa to autoactivate FVII molecules was proved by Pedersen in 1989 [122]. thus leading to the concept that trace amounts of FVIIa could be responsible for the initiation of the coagulation cascade upon TF exposition in the vascular lumen, through the formation of trace concentrations of FXa that could then process FVII bound to TF [123].

The kinetics of substrate hydrolysis by FVIIa were extensively investigated [124]. Binding of FVIIa to TF increases the catalytic efficiency of FVIIa of more than 100 fold. It was suggested that TF might induce an alteration in the catalytic site of FVIIa, which allows a

more efficient hydrolysis of the small fluorescent substrate. Measurements conducted using various phospholipids and detergents demonstrated that the increase in the catalytic efficiency of FVIIa, when complexed to TF, is independent of the supporting surface [125].

The interaction of FVIIa with TF is Ca^{2+}-dependent. Ca^{2+} saturation of the Gla domain is likely responsible for this increase in affinity, since deletion of Gla in FVIIa results in a loss of affinity for TF. Ca^{2+} may stabilize energetically important hydrophobic contacts of Gla with TF [126]. In addition, Ca^{2+} increases FVIIa affinity for FX by conformational changes in FVIIa and FX that are essential for the interaction of these proteins with phospholipids [127]. This conclusion is reasonable considering the membrane-binding capability of FX and the great enhancement in productive collisions between substrate and enzyme realized by an initial interaction of the substrate with the membrane surface.

1.4.1 Half-life and degradation

Regulation of FVIIa activity is a key step and it includes inactivation by plasmatic inhibitors such as antithrombin and TFPI, internalization and degradation. TFPI directly inhibits FVIIa-TF complex and FXa activity and the formation of the quaternary complex promotes its internalization by about 3-fold [128]. Alternatively, TFPI anchored to glycosyl phosphatidylinositol can mediate a transient down regulation of the quaternary complex through its translocation of the to glycosphingolipid-rich microdomains, unfavorable for FVIIa-TF activity [129]. Hansen *et al*. also described a clathrin-independent mechanism of FVIIa-TF internalization not affected by the presence of TFPI [130]. Internalized FVIIa can return to the cell surface, as recycled fully active FVIIa, or associate with nuclear fractions. Whether FVII/FVIIa is degraded in plasma prior to internalization is not known. *In vitro* degradation of FVIIa occurs through cleavage after Arg^{290} and Arg^{315} [131]. These residues belong to Loop 140s and Loop 170s, respectively, solvent-exposed structures that in the activated form of FVII present a high degree of flexibility.

The circulating half-life of the zymogen FVII in humans has been reported to be approximately 5 hours [132], while it was about 2.5 hours for the activated form [133]. Compared to other vitamin K-dependent coagulation proteases, the circulating half-life of FVIIa is extremely long.

1.5 FVII deficiency

Inherithed FVII deficiency, first described by Alexander *et al*. in 1951 [134], is the most frequent of the rare congenital coagulation disorders with an estimated prevalence of 1 in 300.000-500.000 individuals [135]. It is usually transmitted in an autosomal recessive fashion and it is frequently associated with consanguinity. Triplett *et al.* have classified FVII deficiency in CRM^- (activity and antigen proportionally reduced), CRM^+ (reduced activity, antigen normal) and CRM^{red} (antigen is reduced but not as much as activity) [136].

There is a considerable phenotypic [136] and molecular heterogeneity [137,138] in the congenital FVII deficiencies. The clinical bleeding tendency ranges in severity from lethal to mild, or even asymptomatic forms. Some individuals experience mild mucous membrane bleeding, menorrhagia, and post-surgical bleeding, but more significant events such as hemarthroses and soft tissue bleeds are documented. A higher prevalence of females was found among symptomatic subjects and in particular among moderate bleeders: much of the excess of bleeding tendency can be attributed to menorrhagia, the most frequent symptom in this gender. Life-threatening gastrointestinal and central nervous system bleeds are well recognized (GI or CNS, 20% of patients) and are characterized by early presentation and association with lower FVIIc levels. Life-threatening bleeds occur most frequently (70% of the cases) during the first 6 months of life and are associated to high morbidity and mortality rate. The greatest risk factor for CNS hemorrhage is trauma related to the birth process [139].

The potential severity of the clinical phenotype of FVII deficiency reflects its pivotal role in the initiation of coagulation. Mice with targeted disruption of their FVII gene show lethal hemorrhage in the peri-partum period: 70% suffered fatal intra-abdominal bleeding within the first 24 hours and most of the remaining neonates died from intracranial hemorrhage before the age of 24 days [140]. Interestingly, the FVII deficient embryos develop to term and do not exhibit the developmental lethality at mid-gestation experienced by TF deficient embryos. In fact, TF is also implicated in a variety of biological processes, from angiogenesis and tumor metastasis to vascular remodelling and signal trasduction [141-144]

In human neonates that are homozygous for a Factor VII null allele development in utero is normal but mortality occurs shortly after birth due to intracranial haemorrhage. The

minimal FVII level able to interact with TF to prevent lethal bleeding in human subjects has not yet been defined.

The clinical phenotype in patients with FVII deficiency correlates poorly with FVII coagulant activity (FVII:C) measured in vitro. This lack of correlation probably reflects the fact that only trace amounts of FVIIa are required to initiate coagulation in vivo, and in vitro tests fail to differentiate between a 'true' null mutation and one that results in very low but not-zero FVII:C levels, capable of initiating coagulation in vivo and resulting in a mild/moderate bleeding phenotype. Furthermore, FVII:C levels were usually measured using a non-human source of TF and this could generate values discrepant with those obtained with human TF.

1.5.1 FVII levels

Plasma levels of FVII protein and procoagulant activity vary significantly in the general population (18% and 26% respectively) [145] and are influenced by different environmental factors including sex, age, body mass index and diabetes [146]. In women, in whom the increase in FVII with age appears to be greater than for men [147], levels of FVII have also been linked to use of oral contraceptives [146], reproductive status [147], and use of oestrogen hormone replacement therapy [148]. Variations in plasma FVII levels can also be attributed to genetic factors as demonstrated for several FVII polymorphisms.

-402 and –401 polymorphisms. The G to A substitution at position –402 and the G to T substitution at position –401 are two common, nonrelated, functional polymorphisms in the promoter region of the FVII gene. Both polymorphisms strongly influence the binding properties of nuclear proteins. The rare –401T allele is associated with a reduced basal rate of transcription of the FVII gene in human hepatoblastoma cells and with reduced plasma concentrations of total FVII and FVIIa molecules. In contrast, the rare –402A allele confers increased transcriptional activity and is associated with increased plasma FVII levels [145].

Decamer insertion at -323. Studies of FVII levels in healthy individual have shown that insertion of the sequence CCTATATCCT at position –323 in the 5' UTR of the FVII gene is associated with a decrease of about 25% in FVII levels. The allele with the decanucleotide insertion is called the A2 allele while the one lacking the insertion is called

the A1 allele. Clear evidence concerning the effect of this decanucleotide was provided through the examination of promoter strength by transfection experiments in HepG2 cells in which it was shown that the insertion reduced promoter activity by 33% compared with the allelic sequence which lacks the decanucleotide [60]. The decamer insertion at –323 and the R353Q polymorphism have been shown to be in strong allelic association with each other [149,150].

G73A polymorphism. This polymorphism is located in intron 1a of the FVII gene and caused by the nucleotide change G to A at position 73. It is often associated with the promoter decamer insertion and the Q353 alleles, thus impairing the understanding of the A73 allele per se contribution to lowering FVII levels in plasma. The concomitant presence of A73 allele with both the decamer insertion and the Q353 alleles was associated with the lowest factor VII levels and might confer protection against myocardial infarction in the young [151].

Arg353Gln polymorphism. This polymorphism results from a G to A transversion at position 10976 in exon 8 [152]. The Arg353 allele is referred as M1 allele, while the Gln353 allele is called M2. The M2 allele is associated with a decrease of about 25% in FVII:C and FVII:Ag levels [149,150,152]. The conformation of the Gln 353 molecule may be different from that of the Arg 353 protein, affecting its intracellular processing, secretion, turnover in plasma, or activity. In vitro expression studies in COS-1 cells have demonstrated that the Q353 variant was secreted with a significantly reduced efficiency [153]. Analysis of the crystal structure of the soluble TF-FVIIa complex reveals a peripheral location for Arg^{353}. It has been proposed that this residue may be involved in interaction between triglyceride-rich lipoproteins and FVII and the substitution to Gln may therefore alter the strength of this interaction that limits or slows cleavage to the active two-chain form, or rate of removal from the circulation [154].

The Gln variant occurs with a frequency of about 10% in various populations and this high frequency could indicate that the variant confers some benefit, for example protection against thrombosis or myocardial infarction [153].

Variable number tandem repeat polymorphism (VNTR) in intron 7. This polymorphism spans the exon 7-intron 7 boundary and is due to a variation in repeat copy number of a 37 bp element. Four different alleles with 5 to 8 monomer repeats have been reported and the

most common are those containing 6 and 7 repeats [149,150], designated as b and a, respectively. The allelic forms with a lower number of repeats was found to be associated with a decrease in FVII levels [150], very likely caused by reduced efficiency of mRNA splicing [155].

His115His. The polymorphism is located within exon 5 and results from a C to T change at position 7880 (codon 115). This change is silent at the amino acid level [156]. The most common C allele is generally referred as H1 and the rare T allele is called H2.

1.5.2 FVII mutations

A considerable number of mutations have been reported to date in FVII gene [137]. The majority of individuals with mutations in their FVII gene are either asymptomatic or the clinical phenotype is unknown and have come to notice through pre-operative clinical tests.

Missense mutations were the most frequent and occurred in the 68% of subjects, followed by splicing-site (13%), promoter (8%) and nonsense (6%) mutations, small insertions and deletions (6%) (Figure 1.5.2.1). Many of these mutations have been identified as the cause of FVII deficiency but only a few of them have been expressed and characterized. In some cases, naturally occurring FVII mutants constitute valuable tools to investigate single residues or to define regions important in the structure-function relationship in FVII and in the formation of macromolecular complexes responsible for coagulation initiation.

Among FVII deficient patients, the most severe cases are all either homozygous or doubly heterozygous for deleterious mutations resulting in FVII:C levels less than 2% of normal. The majority are mutations that disrupt appropriate expression: promoter, splice-junction or frameshift mutations caused by deletions. Only a few missense mutations have been described that result in a severe phenotype [137].

Cases of mild/moderate FVII deficiency have in vitro FVII:C levels which range from <1% to 52% and these levels don't correlate with the reported clinical severity. Thus it's impossible to differentiate between the severe and mild/moderate cases only on the bases of FVII:C or FVII:Ag in plasma.

The asymptomatic cases have FVII:C ranging from 4% to 61% and FVII:Ag levels from 5% to 113% of normal. The mutations are all missense.

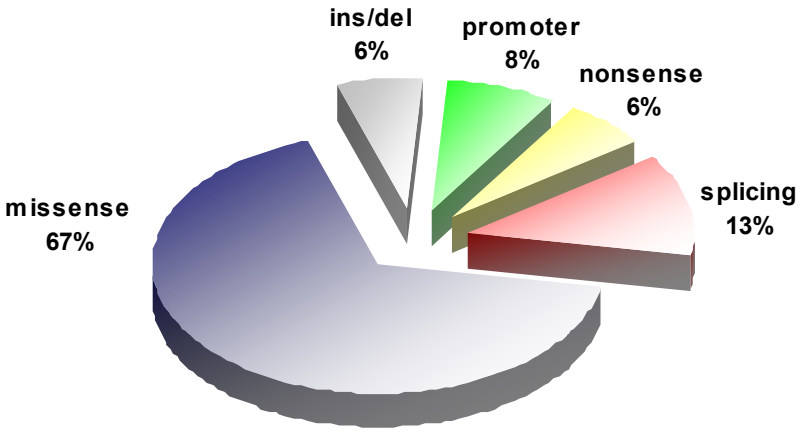

Figure 1.5.2.1. Pie chart showing the type of mutations reported in the International Registry of FVII Deficiency (IRF7)

1.5.3 Conventional treatment strategies in FVII deficiency

As for the hemophilias, replacement of the deficient coagulation factor is the mainstay of treatment for FVII deficiency, but safe and efficacious products are fewer and experiences on their optimal use much more limited [157].

Intermediate Purity Factor IX Concentrates and prothrombin complex concentrates (PCCs): their main advantages are the small volume of infusion, fewer allergic reactions, and the adoption of virus-inactivation procedures during manufacturing.
These products are not calibrated for FVII concentrations and as the half- life of FVII is much shorter than that of other coagulation factors present, multiple doses of PCCs may result in a build-up of other factors, increasing thrombotic risk.

Plasma-derived Factor VII concentrates: FVII concentrates are prepared from pooled plasma. They are used for prophylactic treatment, as well as for controlling serious bleeding episodes, and bleeding during surgery. However, plasma-derived concentrates carry the risk of potential transmission of blood-borne pathogens

Fresh frozen plasma (FFP): single-donor FFP, that contains all coagulation factors, is relatively inexpensive and widely available. However, because of the very short half-life of FVII the risk of volume overload is real when repeated infusions are administered to raise and keep the deficient factor at hemostatic levels. Hence, concentrates should be preferred for major surgical procedures or when the severity of the clinical manifestations predicts a

long-lasting treatment. Most importantly, infectious complications with viruses as the hepatitis viruses or human immunodeficiency virus (HIV) are still perceived as a threat of FFP.

Recombinant activated FVII (rFVIIa): rFVIIa is indicated for the treatment of bleeding episodes and for the prevention of bleeding in patients with congenital FVII deficiency undergoing surgery procedures. It is free of human plasma and albumin, so there is no risk of human viral transmission, but it is very expensive and not available for all patients.

1.6 Coagulation Factor X

Human coagulation Factor X (FX) is a vitamin K-dependent serine protease playing a crucial role in the clotting cascade, as the convergence point of intrinsic and extrinsic pathways of the coagulation system [158,159]. FX, also known as Stuart Factor, was discovered by Graham, Barrow and Hougie in 1959 [160].

1.6.1 FX activation

Factor X circulates in plasma as an inactive two-chain zymogen protein, until activated by proteolytic cleavage of the peptide bond between Arg^{194} and Ile^{195} in the N-terminal region of the heavy chain of FX, giving rise to the activated Factor X (FXa), and a 52-aminoacid glycopeptides [161,162]. It has been proposed that the activation peptide serves primarily as negative self-regulation mechanisms to prevent spurious activation of FX, and secondarily in cofactor dependence and activator specificity [163]. Activation of FX to FXa occurs through multiple pathways. In the presence of Ca^{2+} and phospholipids, FX can be activated by both the extrinsic FVIIa/TF complex, in the initiation phase of coagulation, and the intrinsic FIXa/FVIIIa complex, in the subsequent propagation phase [159,164]. In both pathways, FX activation requires specific interactions of the enzyme with the cofactor/activator complex, leading to the formation of a "tenase" complex on the phospholipidic surface (Fig. 1.6.1.1).

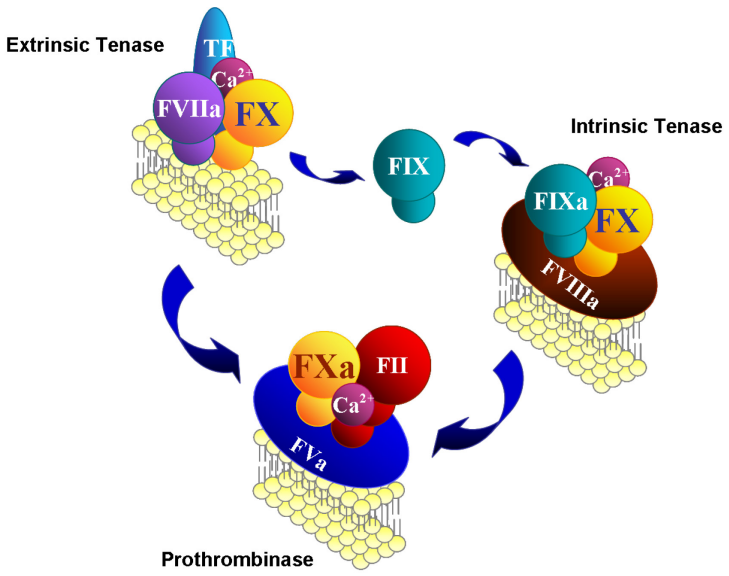

Figure 1.6.1.1. Macromolecular complexes in blood coagulation. Each serine protease is shown in association with the appropriate cofactor on the membrane surface.

In vitro FVIIa alone is able to directly activate FX in absence of its cofactor [165], whereas *in vivo* the TF binding to FVIIa in the complex TF/FVIIa is necessary to activate FXa to trigger coagulation cascade [166], enhancing the FVIIa proteolytic activity to FXa [167].
Also FX activation by FIXa in the intrinsic complex is $2*10^2$-enhanced by its association with FVIIIa [168], and requires binding of FIXa, FVIIIa and FX to the phospholipidic membrane, as result of multiple protein-protein and protein-phospholipids interactions [169].
Russell's viper venum (RVV) represents a potent non-physiological FX activator [162,170,171]. This metallo-protease (79 kDa) activates FX only in the presence of Ca^{2+}, without assembling into a ternary complex on the surface membrane.

1.6.2 FXa procoagulant activity

FXa reversibly associates with its cofactor FVa on a cellular negatively charged phospholipidic membrane surface, in the presence of Ca^{2+} ions, to form the prothrombinase complex (Fig. 1.6.2.1), which catalyze the conversion of prothrombin into thrombin, leading to the formation of the fibrin clot [2].

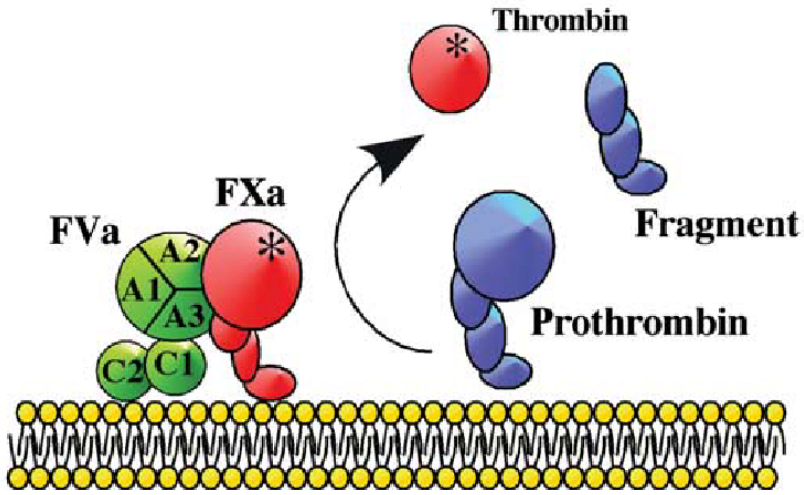

Figure 1.6.2.1. Schematic representation of prothrombin activation by the prothrombinase complex.

Although FXa alone catalyzes prothrombin activation and generates traces of thrombin, they are not sufficient to initiate fibrin polymerization and the macromolecular interactions which stabilize prothrombinase lead to a substantial enhancement in catalytic efficiency,

indicating that assembly of this complex is an important step for rapid and localized thrombin generation. While FXa catalyzes prothrombin cleavage, the macromolecular interactions that stabilize prothrombinase lead to a profound enhancement in catalytic efficiency (~10^5-fold), indicating that prothrombinase, not FXa, is the physiologically relevant enzyme leading to explosive thrombin generation [47].

The process of formation of the prothrombinase complex on a membrane surface proceeds through a mechanism in which FVa and FXa initially bind independently to the membrane surface and subsequently rearrange to form the active prothrombinase complex. FVa and FXa are both bound to the membrane surface when assembled in the prothrombinase complex. These proteins mutually exclude each other for the initial binding reactions with membrane combining sites on synthetic phospholipid vesicles, suggesting that saturation of the membrane surface with one component would substantially reduce the maximum concentration of complex formed. However, equilibrium binding studies of complex assembly indicate that the protein-membrane interactions are linked to the protein-protein interactions within prothrombinase, such that the affinity of the individual interactions with the membrane surface is increased approximately 100-fold [47].

FVa functions as a non-enzymatic cofactor of FXa in the conversion of prothrombin into thrombin, and its presence in the prothrombinase complex enhances the rate of prothrombin activation by several orders of magnitude [172]. The prothrombinase complex is structurally and functionally homologous to the intrinsic tenase complex (FIXa, FVIIIa, phospholipids and Ca^{2+}), responsible for FX activation (Fig. 1.4).

FXa in the prothrombinase complex rapidly converts prothrombin to thrombin via proteolysis of two internal peptide bonds (Arg^{320}-Ile^{321} and Arg^{271}-Thr^{272}) [47,173,174].

This propagation step allows the generation of sufficient amounts of thrombin resulting in the generation of a fibrin clot, and, importantly, this propagation step is independent from the TF/FVIIa complex, which is rapidly inhibited by TFPI.

FXa also cleaves other protein substrates involved in blood coagulation. These include the proteolytic activation of FVII, FIX, FV and PC.

The activation of FV by FXa is required at the beginning of the clotting cascade, when thrombin, the physiological activator of FV, has not been generated yet [18,175]. FXa catalyzes the activation of FVII, in complex with TF, amplifying the coagulation cascade with a positive feed-back mechanism [176]. Furthermore FXa is able to activate PC *in vitro*, in the presence of Ca^{2+}, phospholipids and the cofactor thrombomodulin [177].

1.6.3 FXa autocatalytic activity

In the presence of Ca^{2+} and phospholipids, the FXa (FXa-α) catalyzes the cleavage of at least two peptide bonds in its own heavy chain. The faster cleavage, between Arg^{429} and Gly^{430}, accelerated on phospholipid surfaces, liberates a 19-residue glycopeptide (2.3-kDa) from the C-terminus of the heavy chain. This reaction evidently results from autoproteolysis by FXa and leads to the conversion of FXa from the α to the β-form. No difference in function has been observed yet for the α and β forms of the protease [178]. A second autoproteolytic cleavage within the Arg^{326}-Arg^{336} autolysis loop releases a peptide containing the active-site serine residue from the C-terminal region of the heavy chain, resulting in the FXa γ-form, a species that has little or no catalytic activity [179,180].

The role of the autolysis loop in FX function has been investigated by site-specific mutagenesis [181]: the introduction of a new glycosylation site at position 333 of FX might prevent degradation within the autolysis loop and impair catalytic activity, based on the proximity of the region to the active site of FX [180,182,183]. Even the Ca^{2+} binding loops of the catalytic domain play an important role preventing FX autoproteolysis and might enhance the amidolytic activity of the FXa-β form of approximately 1.6-fold [180].

1.7 Factor X protein and gene

1.7.1 Structure

FX is a vitamin K-dependent plasma glycoprotein, with a molecular weight of 58.8 kDa, which circulates in plasma at a concentration of 8 μg/ml, with a 32-48 hours half-life.

The mature form of the protein (448 amino acids) exists of a light chain (139 residues, 16.9 kDa) and a heavy chain (306 residues, 42.1 kDa), linked by a disulfide bond between residues Cys^{132} and Cys^{302} [184]. The light chain contains a γ-carboxyglutamic acid domain (GLA) and two epidermal growth factor-like domains (EGF-1 and EGF-2), whereas the heavy chain contains the serine protease domain (Fig. 1.7.1.1).

The amino terminus of the mature protein light chain contains the GLA domain, very well conserved within the family of vitamin K-dependent coagulation serine proteases (Fig. 1.7.1.2, A), which allows the anchorage of the protein onto the membranes in the presence of Ca^{2+} ions [2,185]. The N-terminal domain of all vitamin K-dependent coagulation serine proteases, that is about 45 amino acid residues long, contains 9-12 glutamic acid residues that undergo post-translational carboxylation at the γ-carbon [186]. These γ-carboxyglutamic acid (Gla) residues are necessary for Ca^{2+} binding, which induces a

conformational change in the GLA domain required for membrane interaction and biological activity [7,187-189]. Comparison of GLA domains among coagulation serine proteases has indicated the presence of three conserved pairs of Gla residues: 6 and 7, 19 and 20, 25 and 26.

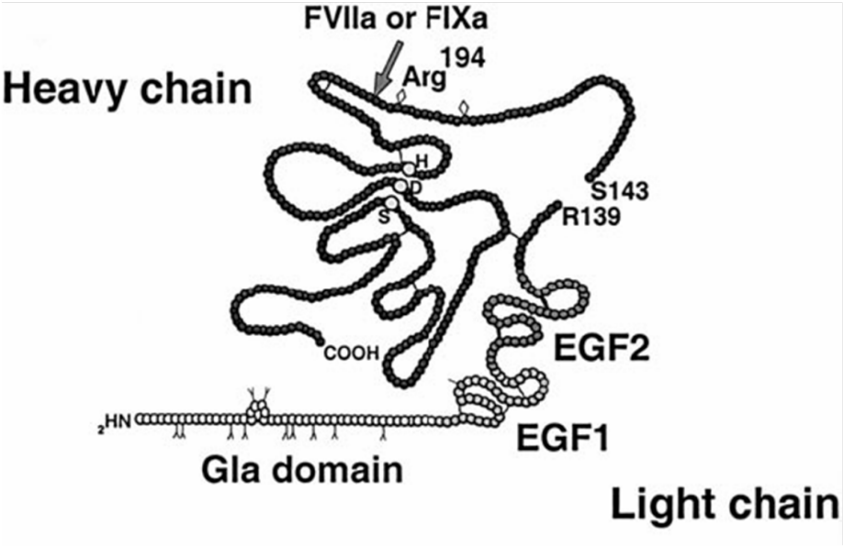

Figure 1.7.1.1. Schematic representation of the primary sequence and domain structure of Factor X. Each circle represents an amino acid residue. The structure is shown without the pre-pro leader sequence and without the tripeptide (Arg^{140}-Lys^{141}-Arg^{142}), which connects the heavy chain to the light chain.

The three dimensional structure of the GLA domain (Fig 1.7.1.2, B) [190] suggests that Gla residues are in a dynamic state in the absence of calcium but fold in the presence of calcium into a tightly packed structure, leading to the exposure of solvent-accessible hydrophobic residues that are available for membrane or protein interaction [186,189].

The GLA domain of FX, which contains 11 Gla residues, has been reported to be also an important recognition site for TF, suggesting to be implicated in the formation of the ternary FVIIa/TF/FX and FVII/TF/FXa complexes, but the specific FX residues involved in the macromolecular interactions have not been clearly identified [51,52,191,192].

The GLA domain is followed by a short linking segment of aromatic amino acids known as the aromatic stack (region Phe^{40}-Lys^{45}), which participates in stabilization of the protein. This hydrophobic stack is followed by two Epidermal Growth Factor (EGF-1 and EGF-2) like domains, containing three conserved disulfide bonds each one, which are considered important for interdomain or protein-protein interactions. EGF domains in FX seem to be important recognition sites for TF in the extrinsic complex, in particular EGF-1 [193], and

for FV in the prothrombinase complex [194]. Moreover, the N-terminal EGF-like domain contains an high affinity Ca^{2+} binding site,which in the presence of Ca^{2+} induces a conformational changes in the GLA domain, which help its binding with membranes [195].

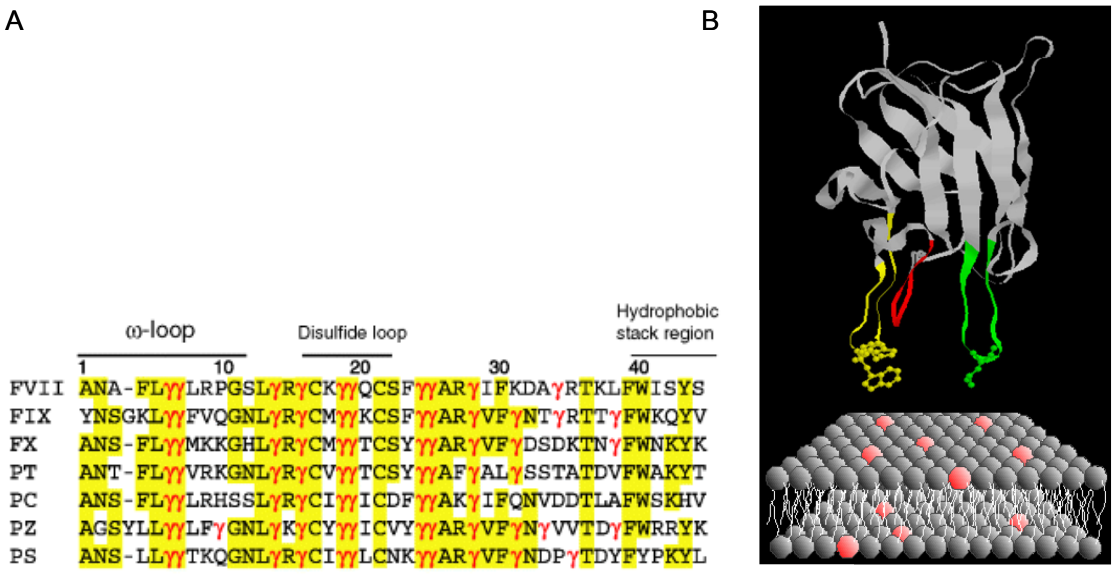

Figure 1.7.1.2. A) Amino acid sequences of the GLA domain of human vitamin K-dependent plasma proteins. Amino acid residues that are identical in at least four of the proteins are marked in yellow. γ indicates the γ-carboxyglutamic acid residues. Residues that form the ω-loop, the disulfide loop, and the hydrophobic stack region are marked. B) Crystallographic structure of FX GLA domain. Loops of interaction with membrane are shown.

The heavy chain contains the activation peptide and the catalytic domain.

Proteolysis at the Arg^{194}-Ile^{195} peptide bond in the N-terminal region of the heavy chain of FX leads to the formation of the serine protease FXa, and the generation of a 52-residue glycosylated activation peptide [162]. Analogous to chymotrypsin, trypsin and thrombin, the new N-terminal isoleucine in the heavy chain of FXa folds into the interior of the protein and forms a salt bridge with the aspartic acid residue adjacent the serine of the active site [196]. This conformational change leads to exposition of the active site, necessary for FXa catalytic activity [183,197]. The activation peptide region may have an important role in the recognition and cleavage of FX by the extrinsic tenase complex [161]. Other studies have also indicated an important role for the activation peptide mediating

interactions between FX and FIXa in a carbohydrate-dependent manner within the intrinsic tenase complex [198].

The catalytic domain of FX starts with the consensus sequence IVGG at the N-terminus and presents a conserved structure, which is common to all others serine proteases, consisting of 12 β-sheets separated by loops folding in two β-barrels, containing the catalytic triad (His^{57}, Asp^{102} and Ser^{195}; chymotrypsin numbering) at their interface (Fig. 1.7.1.3). The two β-barrels domains delimit the specificity pocket (Asp^{373}, Gly^{400}, Gly^{410}) for the substrate, in which a residue of arginine takes place in all coagulation serine proteases [199]. In the FX the residues His^{236}, Asp^{282} and Ser^{379} constitute the catalytic triad.

In the serine protease domain of FX interaction sites for FV/FVa in the prothrombinase complex have been identified [200]; in particular FX residues Arg^{347}, Lys^{351}, Lys^{414} seem to be important exosites for FVa binding [201,202].

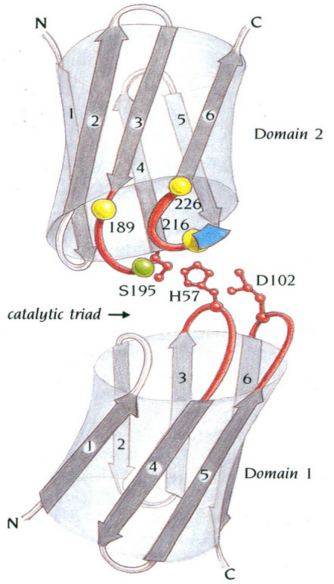

Figure 1.7.1.3. Chymotrypsin catalytic domain. The two β-barrels contain the catalytic triad (red residues), with the His^{57} and Asp^{102} in the domain 1 and Ser^{195} in the domain 2. In yellow are represented residues of the substrate specificity pocket.

The catalytic domain of FX contains also an high affinity Ca^{2+} binding site, within the conserved loop Asp^{250}-Glu^{260} [203,204]; and a Na^{2+} binding site, within the loop Cys^{403}-Gly^{409} [205]: the site-specific binding of these ions to FX could modulate the structure and function of the circulating protein.

1.7.2 Biosynthesis and post-translational modifications

FX is synthesized in the liver as inactive precursor, in a single chain form, and is secreted into plasma as a two-chain form. The precursor protein contains a pre-proleader sequence of 40 aminoacids [206], followed by a 448 aminoacidic chain.

The pre-proleader region of FX contains the signal peptide (from residue -37 to -22) followed by the pro-peptide (from residue -18 to -1). The signal peptide targets the protein for translocation during the secretion pathway in the endoplasmic reticulum, where is cleaved off by a signal peptidase [207]. The pro-peptide is essential for the recognition by the hepatic carboxylase which catalyses the γ-carboxylation of glutamic acid residues and is released from the N-terminus of the protein in the Golgi apparatus by cleavage between Arg^{-1} and Ala^{+1}, just prior to secretion of the protein from the cell.

In the *trans*-Golgi compartment the single chain FX precursor is processed in the two-chain form by two proteolytic cleavages at Arg^{139} and Arg^{142} [208], with the removal of an internal tripeptide (Arg^{140}-Lys^{141}-Arg^{142}), and this intra-chain cleavage can precede the release of the propeptide [209].

As all vitamin K-dependent proteins, the biosynthesis of FX involve several co- and post-translational modifications, including formation of disulfide bonds, γ-carboxylation of glutamic acid residues, β-hydroxylation of aspartic acid, and N- and O-linked glycosylation. These post-translational modifications are required for protein secretion, folding and functional activity [185,210].

Gamma-carboxylation of glutamic acid residues

The γ-carboxylation of glutamic acid is the first important post-translational modification during the biosynthesis of vitamin K-dependent coagulation factors and γ-carboxyglutamic acid (gla) residues are required for Ca^{2+}-induced interaction of these proteins with membrane surfaces [211].

The γ-carboxylation of 11 glutamic acid residues within the GLA domain of FX occur in the endoplasmic reticulum by a vitamin K-dependent carboxylase. After cleavage of the signal peptide, the FX precursor binds to the carboxylase *via* its γ-carboxylation recognition site [188.212,213]. Also the γ-carboxylase has a specific regognition site in the propeptide of FX, which is the consensus sequence ZFZXXXXA, highly conserved among the vitamin K-dependent coagulation serine proteases (where Z indicates a hydrophobic residue; F a phenylalanine; A the residue of alanine and X each residue) [214].

The vitamin K-dependent γ-carboxylase, an integral membrane protein resident in the endoplasmic reticulum [215-217], converts glutamic acid residues to γ-carboxyglutamic in the presence of CO_2, O_2 and vitamin K hydroquinone (KH_2), which is converted in epoxide during the reaction (Fig. 1.7.2.1). The requirement for vitamin K as cofactor of the reaction is unique to the vitamin K-dependent carboxylase and the biosynthesis of γ-carboxyglutamic acid [188], but the mechanism by which vitamin K participates as a cofactor with the γ-carboxylase remains unknown.

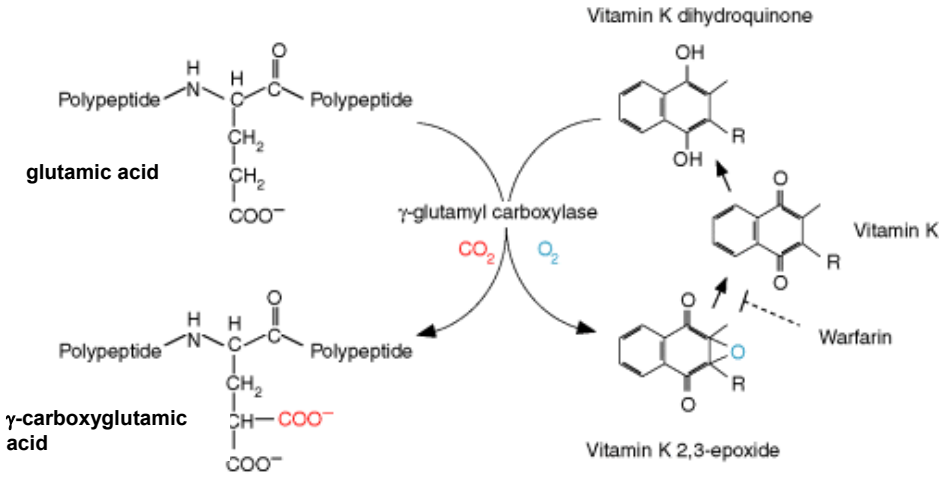

Figure 1.7.2.1. Post-translational γ-carboxylation of a glutamic acid to a γ-carboxyglutamic acid residue by γ-glutamyl carboxylase.

Warfarin, a dicoumarinic anticoagulant largely employed, is a vitamin K antagonist and is known to affect the γ-carboxylation reaction by inhibiting the vitamin K-epoxide reductase, the enzyme responsible for the recycling of the reduced form of vitamin K (hydroquinone), which functions as cofactor for the γ-carboxylase. Inhibition of the γ-carboxylation reaction by antagonists of vitamin K leads to decreased γ-carboxylation, resulting in defective Ca^{2+} binding of the GLA domain and loss of ability of vitamin K-dependent proteins to interact with the phospholipid membrane, thus supporting the importance of this post-translational modification for protein biosynthesis and function. Warfarin treatment results in the synthesis of uncarboxylated or partially carboxylated forms of the vitamin K-dependent proteins [218,219], inducing a reduction of their biologic activity in blood plasma [220]. For example, warfarin can reduce the total concentration of prothrombin in plasma by about 30%, presumably because of an increased intracellular degradation of under-carboxylated forms of the protein.

Beta-hydroxylation of aspartic acid

The modification of Asp^{63} to β-hydroxyaspartic acid in the first EGF domain of FX occurs within the endoplasmic reticulum. The function of β-hydroxyaspartic acid at residue 63 in FX is not completely clear, but it seems to mediate Ca^{2+} binding to the N-terminal EGF domain, being important to orient the adjacent GLA domain in a manner that is commensurate with FX biologic activity [75,189].

Asparagine- and threonine- linked glycosylation

Glycosylation of transmembrane and secreted proteins is an essential process in eukaryotic cells. The presence of oligosaccharides is often required for the efficient transport of glycoproteins through the secretory pathway, and is an obligatory event for the folding and assembly of newly synthesized polypeptides. The carbohydrate moieties not only stabilize folded domains but also provide polar surface groups that prevent aggregation of folding intermediates and allow newly synthesized polypeptide chains to interact with chaperones and enzymes in the endoplasmic reticulum [185]. Glycosylation also plays a significant role in determining the plasma half-life and biologic activity of many proteins [210]. Almost all of the proteins that transit the secretory pathway of eukaryotic cells acquire one or more oligosaccharide units by the action of enzymes of the endoplasmatic reticulum and Golgi apparatus. Sugars are attached to the protein through either the side chain amide nitrogen of an asparagine residue (N-linked glycosylation) or the oxygen atom in the side chain of serine or threonine residues (O-linked glycosylation). The majority of N- and O-linked glcosilation on the vitamin-K dependent coagulation factors occurs within the activation peptide, suggesting that glycosylation plays a role in regulating the activation of these proteins. In FX N-linked glycosylation occur at residues Asn^{181} and Asn^{191} [184,221] whereas O-linked glycosylation occur at residues Thr^{159} and Thr^{171} [222,223]. Whether the carbohydrates in the activation peptide domain of FX are important for its activation is not fully understood. Initial studies suggested a role in the recognition and cleavage of FX by the extrinsic tenase complex during FX activation [222,224], but this could not be corroborated by more recent studies [161].

Disulfide bond formation

The vitamin K-dependent coagulation factors have conserved disulfide bonds. In FX one disulfide bond occur in the GLA domain (Cys^{17}-Cys^{22}), three within each EGF domain

(Cys^{50}-Cys^{61}, Cys^{55}-Cys^{70}, Cys^{72}-Cys^{81} in EGF-1 and Cys^{89}-Cys^{100}, Cys^{96}-Cys^{109}, Cys^{111}-Cys^{124} in EGF-2) and four occur in the serine protease catalytic domain (Cys^{201}-Cys^{206}, Cys^{221}-Cys^{237}, Cys^{350}-Cys^{364}, Cys^{375}-Cys^{403}). Furthermore, a disulfide bond links the light chain with the heavy chain (Cys^{132}-Cys^{302}).

1.7.3 The FX gene

The gene encoding FX is located on chromosome 13 at position 13q34, 2.8 kb downstream the FVII gene (Fig. 1.7.3.1) [225,226].

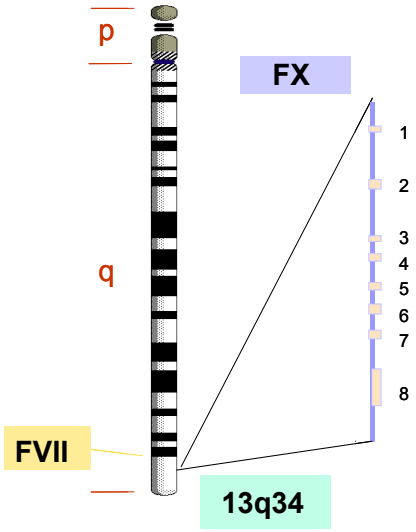

Figure 1.7.3.1 Schematic representation of chromosome 13 and organization of FX gene.

Recombinant bacteriophages containing overlapping DNA inserts coding for the gene for human FX have been isolated and characterized [208]. These DNA inserts code for almost the entire gene for FX, extending from the prepro leader peptide through the 3' noncoding region of the transcription product.

The organization of the gene for FX was established by DNA sequencing to identify the location of the introns and exons in the gene. As the other vitamin K-dependent coagulation proteases, the FX gene (27 kb) consists of seven introns and eight exons, each of these encoding a specific functional domain of the protein (Table 1.7.3.1). Exon 1 encodes the signal peptide. Exon 2 encodes the pro-peptide and the γ-carboxyglutamic acid-rich domain (GLA). Exon 3 encodes a short linking segment of aromatic aminoacids known as the aromatic stack. Exons 4 and 5 encode two regions which are homologous to Epidermal Growth Factor (EGF-like domains), exon 6 encodes the activation peptide,

which is at the amino-terminus sequence of the heavy chain. Exons 7 and 8 encode the serine protease catalytic domain containing the catalytic triad His^{57}, Asp^{102} and Ser^{195} (numbered according to the conventional chymotrypsic labelling).

EXON	INTRON	PROTEIN REGION	DIMENSION (bp)
1		signal peptide	9
	A		?
2		Pro-leader region, GLA domain	163
	B		7400
3		aromatic stack	25
	C		950
4		EGF-1 domain	114
	D		1800
5		EGF-2 domain	132
	E		2900
6		Arg-Lys-Arg tripeptide, activation peptide	245
	F		3400
7		catalytic domain	118
	G		1700
8		catalytic domain	612

Table 1.7.3.1 Factor X gene organization and protein functional domain encoded by each exon. The base pairs extension of each exon (1-8) and intron (A-G) is shown.

The seven introns interrupt the coding sequence at essentially identical locations in the amino acid sequence as the introns in the genes for human FIX and PC, suggesting that the vitamin K-dependent proteins present in plasma have evolved from a single, common ancestral gene, which arose through a process that involved the assembly of small protein coding units of DNA into a single gene [227]. Moreover, a comparison of the amino-acid sequences of human and bovine FX shows high sequence identity, especially around the calcium-binding regions and catalytic regions, but low sequence identity around the non-functional regions [206].

Studies on a recombinant λ phage containing the 5'-flanking region of the human FX gene showed that the FX gene was linked to and was located at the 3' end of the FVII gene: the initiation codon of the FX gene was 2823 bp downstream from the polyadenylation site of the FVII gene. This 2.8-kilobase intergenic region, and progressively deleted fragments of it, was fused to the chloramphenicol acetyltransferase gene, and transient expressions in HepG2 cells, human fibroblasts, and Chinese hamster ovary cells were measured. A liver-specific promoter element, FXP1-binding site, essential for hepatocyte-specific transcription was identified. This promoter sequence, further localized to -63 to -42 bp in DNase I footprint studies, was homologous to LF-A1 or hepatic nuclear factor-4 recognition sequence and was equally functional in the normal and inverse orientations. FXP1 site bound to nuclear protein(s) from HepG2 cells and complex formation was partially abolished by the presence of duplex oligonucleotides containing liver factor-A1 or hepatic nuclear factor-4-binding sequences. Two additional positive elements located upstream of the promoter region, spanning from -215 to -149 bp (FXP2 site), and -457 to -351 bp (FXP3 site), were also established by reporter gene assays [225].

1.7.4 Factor X deficiency

FX deficiency, described for the first time by Telfer *et al* in 1956 [228] and Graham *et al* 1957 [229], is one of the rarest coagulation disorder, with a prevalence of 1 to 1.000.000 in the general population, inherited as an autosomal recessive trait and characterized, in the homozygous or doubly heterozygous conditions, by considerable clinical heterogeneity, which poorly correlates with FX levels and usually presents with variable bleeding tendency [230-232]. A severe bleeding phenotype is usually associated with homozygous or doubly heterozygous conditions [233]. Heterozygous FX deficiency is generally asymptomatic, and in most cases is identified incidentally during pre-operative screening.

Generally, FX deficiency presents either early in life as a severe hemorrhagic syndrome or later in life as a rather mild phenotype. In comparison to other coagulation factor deficiencies, patients with a severe FX deficiency tend to present more severe bleeding diathesis [231]. FX deficient patients frequently present with haematomas, hemarthroses, gastro-intestinal bleeding [233]. Next to the more common bleeding symptoms, some FX deficient patients suffer from bleeding in the central nervous system.

As a first classification system, FX deficiency is classified based on both activity and antigen levels. In type I deficiency (CRM-) both functional activity and antigen levels are decreased, which is a characteristic hallmark of defective secretion or reduced stability of

the protein. In type II deficiency (CRM+), low coagulant activity contrasts with normal or low borderline antigen levels, indicating the presence of a dysfunctional protein. Type III deficiency (CRM^{red}) is characterized by concomitantly decreased coagulant activity and antigen levels, but the antigen level is above the activity level. Type III defects probably result from a combination of mutations leading to type I and type II defects.

Molecular genetic analysis is beginning to elucidate the mutational spectrum of FX deficiency [230,234,235], which shows similarities to that observed in FVII deficiency [230,236,237].

In FX deficiency, the large majority of the causative mutations identified so far are missense mutations (Fig. 1.7.4.1). Among all the reported missense mutations for the FX gene, only 18 are responsible for severe FX deficiency associated with hemorrhagic syndrome: 3 have been reported in the GLA domain, 2 in the EGF-2, and 13 in the catalytic domain of FX [233]. No promoter mutations associated with severe bleeding diathesis have been reported for the FX gene, and only 5 splice site mutations, affecting the splice sites of introns 1 and 2, have been identified [230,234,235,238,239]. Several short deletions (1 to 3 nucleotides) and a 17-bp deletion have been found to be associated with severe bleeding diathesis. A single 1-bp gene insertion has been described and two large gene deletions (more than 5 kb) have also been reported [230,233,234].

Most mutations are unique and present only in members of the proband family but some specific mutations are recurrent in unrelated families with apparent different ethnic origin. These recurrent mutation are sometimes located in CpG dinucleotides, which are known to be hot-spot sites for mutation [240-242], and probably result from independent events.

Only a few naturally occurring variants have been characterized [207,243-253], therefore the structural and functional consequences of the majority of reported FX mutations remain largely unexplained. The limited number of homozygous FX deficient patients [230,234,235] and the incomplete characterization of the few severe CRM+ patients have precluded definition of the relationship between residual FX activity and bleeding tendency. The diagnosis of heterozygous FX deficiency and an estimate of the prevalence of this deficiency can be furher complicated by FX levels borderline to the normal range, which may represent a general problem for the diagnosis of both hemorrhagic and thrombotic risk conditions [236,253].

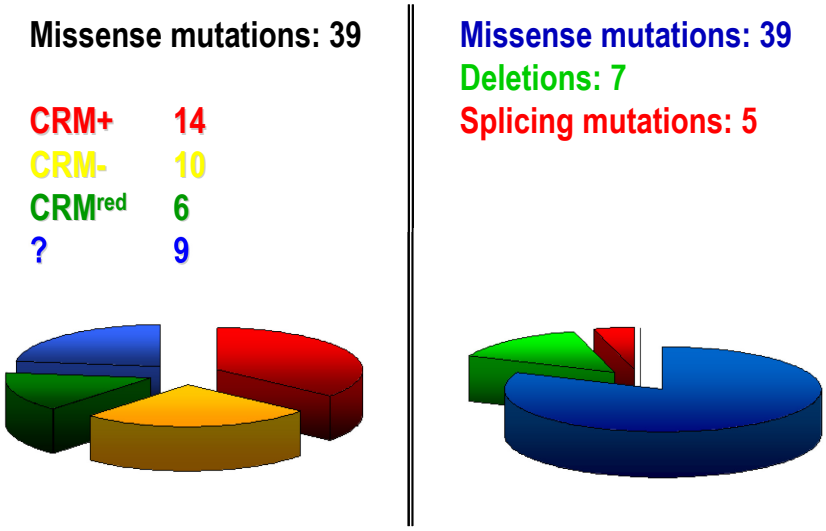

Figure 1.7.4.1. FX molecular defects associated with FX deficiency

1.8 Evolution of chymotrypsin-like serine proteases

1.8.1 Evolution of serine proteases in vertebrates

Serine proteases of the chymotrypsin-like family share a common fold and participate in key physiological functions like digestion, blood coagulation, fibrinolysis and complement [48,254]. Differently from the wide specificity of digestive enzymes, like trypsin, and their presence also in organisms as primitive as eubacteria, proteases involved in more specialized functions like blood coagulation, fibrinolysis and complement have a narrow specificity and are found only in vertebrates [255-258]. Indeed, enzymes involved in more specialized functions often carry additional modules that confer more stringent specificity and localize the proteolytic function in space [259].

Among serine proteases, one of the most studied is represented by thrombin, for which a change in substrate specificity has been shown to be related to exosite-mediated interactions. Indeed, thrombin activity and specificity are allosterically regulated by binding of Na^+ [260], which is related to a thrombin Na^+-bound fast form with enhanced activity toward fibrinogen and thus procoagulant, and a thrombin Na^+-free slow form which cleaves protein C and thus with an anti-coagulant function [261]. In addition, Na^+ has been found as a monovalent cation necessary for optimal catalytic activity of some serine proteases, whereas more primitive proteases involved in digestive function do not require this cation for their function. The residue 225 (chymotrypsin numbering) of serine

proteases, typically proline or tyrosine, has been shown as a determinant for the Na^+-induced regulation of catalytic activity. Proteases with Y225, like thrombin, are involved in highly specialized functions as blood coagulation and complement and their activity is enhanced allosterically by Na^+, whereas proteases with P225, like trypsin, are involved in digestive functions without the requirement for Na^+ and are found in more primitive organisms [262]. Interestingly, among procoagulant serine proteases, FVII represents one exception, bearing phenylalanine at position 225 instead of tyrosine.

The Na^+-induced allosteric regulation bears on the molecular evolution of serine proteases. Noticeably, a strong correlation exists between residue 225 and the codon used for serine 195 of the active site, indicating a different meaning of these molecular features for different classes of proteases. Indeed, proteases with P225 typically use a TCN codon for S195, while proteases with Y225 use an AGY codon. Thus, an evolution model has been proposed in which TCN/P225 proteins evolved from a lineage arisen from a trypsin-like ancestor and AGY/Y225 from a lineage with a thrombin-like ancestor [262].

Binding of Na^+, the most abundant cation in the extracellular fluids where most serine proteases act, might have been evolutionarily advantageous. It has been hypothesized that a transition from the primitive P225 to Y225, or F225, has occurred through an intermediate aminoacid, and serine (coded by a TCN codon) has been identified as a safer and flexible choice on the pathway leading to the most active derivatives tyrosine and phenylalanine [263]. In addition, for serine residues coding, a primitive usage for the TCN codon in respect of the AGY, which appeared later during evolution, has been proposed [264].

Together with the conserved geometry of the catalytic site, containing the Ser-His-Asp triad, and the serine 214 residue, which has been shown to contribute to the substrate binding pocket, the residue in position 225 represents a molecular marker of serine proteases evolution [265].

It has been postulated that procoagulant proteins have evolved in vertebrates through the classical route of gene duplications, point mutations and divergence of the general defense system that protects against infection and injury [257].

The phylogenetic analysis of the Gla–EGF1–EGF2–SP proteins supports evolution through both global and local gene duplications, and based on the isolation and characterization of cDNAs from several organisms with sequence identity to FV, FVIII, FIX and FX, it has been suggested that these haemostatic proteins are present in all jawed vertebrates [266,267].

An ancestral vitamin K-dependent serine protease was initially duplicated, giving rise to the ancestral PC gene and the ancestral FVII/FIX/FX gene. The next gene duplication generated FVII and the ancestral gene that would ultimately give rise to FIX and FX through a further duplication event. Since genes for FVII and FX are tandemly linked in all jawed vertebrates, this suggests that the second gene-duplication event was a local tandem duplication generating the ancestral FVII and FIX/FX genes. Finally, the FVII, FIX/FX and PC genes were further duplicated in a second global-duplication event, generating FIX [268].

In this scenario, a model for two coagulation states has been proposed: one before the global gene duplication events and one between the two global gene-duplication events. In the first 'primitive' coagulation network, the ancestral Gla–EGF1–EGF2–SP protein forms a complex with TF, leading to the conversion of prothrombin to thrombin and eventually to the formation of fibrin. After the first global duplication, the subsequent tandem duplication allows the ancestral FVII protein to retain the function of initiating coagulation by interacting with TF, thus activating the ancestral FX protein, which in turns activates prothrombin with the enhancement due to the formation of a complex with ancestral FV/FVIII cofactors. This ancestral FX–FV complex is inactivated by the ancestral PC generated from the first global duplication. Ultimately, the final global duplication originates the genes for the FIX–FVIII protein complex [268].

1.8.2 The carboxyl-terminal region of coagulation factors

The protease domain is both necessary and sufficient to specify function and evolution. The carboxyl-terminal segment of the protease domain plays a dominant role in substrate recognition and has been involved in all of the evolutionary decisions pertaining to serine proteases. This segment contains most of the determinants of function and evolution and is sufficient to produce a coherent segregation of function in the phylogenetic tree [269].

The indication that the carboxyl-terminal amino acids play important roles in the secretion of many proteins has previously been reported [270-272.

1.8.3 Factor IX carboxyl-terminal region

Factor IX is a vitamin K-dependent serine protease of liver origin that circulates in plasma as a single chain inactive zymogen with 415 amino acids [273]. FIXa plays a key role in hemostasis, and its deficiency or reduced functional level is associated with an abnormal

bleeding disorder, hemophilia B, with various levels of severity [274,275]. Upon activation by proteolytic cleavage, the fully active enzyme consists of a light and a heavy chain held together by a single disulfide bond [121,276]. The resulting N-terminal light chain of FIXa contains the non-catalytic γ-carboxyglutamic acid and two epidermal growth factor-like domains, while the carboxyl-terminal heavy chain contains the trypsin-like catalytic domain [195].

The presence in the FIX carboxyl-terminal region (residues 403-415) of several natural mutations responsible for mild to severe hemophilia B [275,277] might indicate a potential implication of this region in FIX physiology or about its fate at the cellular level. Most of these mutations display severely reduced FIX antigen and activity levels in the circulation.

A study from Kurachi and coworkers indicated that the carboxyl-terminal region of FIX is essential for its secretion. In a mutagenesis study, a series of both natural and artificial mutants bearing different missense mutation at the carboxy-terminus, indicate that the major observation was the decrease to various extents in the intracellular and secreted factor IX protein levels. Interestingly, secreted factor IX mutants showed specific activities very similar to that of the normal factor IX, thus strongly supporting a fundamental role for the carboxyl-terminal region of FIX in the cellular secretion process [278].

1.8.4 Protein C carboxyl-terminal region

Protein C is a vitamin K-dependent glycoprotein and plasma serine protease precursor that acts as an anticoagulant and plays an important role in hemostasis [279]. The native human protein C molecule is a disulfide-linked heterodimer composed of light and heavy chains and is synthesized in the liver as a 461-amino acid precursor protein that undergoes extensive cotranslational and post-translational modifications [280].

A first indication for the importance of the carboxyl-terminal region of protein C came from the observation that an elongated PC (protein C Nagoya) failed to be secreted at normal levels. The characterization of this naturally occurring mutation evidenced a frame shift at codon 381 with replacement of the carboxyl-terminal 39 downstream residues by 81 abnormal amino acids. This deletion was found to be caused by a deletion of a single guanine residue (8857 G) among four consecutive guanine nucleotides [281], resulting in an elongated variant mostly retained and degraded within the endoplasmic reticulum [282].

The most important indication for the carboxyl-terminal region of PC to be fundamental in its physiology became from deletion scanning studies of wild-type PC that strongly

evidenced that the carboxyl-terminal region of protein C is essential for its secretion. The deletion scanning approach indicated that secretion of the truncated PC was affected at different extents by the removal of terminal residues, whereas the specific activity observed was similar to that of the wild-type protein C [283].

1.9 Aim of the present work

Coagulation serine proteases belong to a protein family characterized by high homology in structure and sequence, both at the gene and the protein level, due to their evolution from a common ancestor that drove the narrow specialization of these proteins, indispensable for the tight regulation of a complex network as the coagulation.

Among coagulation serine protease family members such as Factor VII (FVII), Factor X (FX), Factor IX (FIX) and protein C (PC), the carboxyl-terminal region is extremely variable. Noticeably, the alignment of their primary sequences show remarkable differences, both in extension and aminoacid composition.

Previous works [278,283] showed the fundamental role of the carboxyl-terminal region for the biosynthesis and secretion of FIX and PC, but little is known about FVII and FX.

The aim of this work was to determine the importance of the carboxyl-terminal region for biosynthesis/secretion and activity of two closely related serine proteases as FVII and FX.

In the study we took advantage of the characterization of i) a natural variant of FVII characterized by a nonsense mutation (R402X) leading to a slightly truncated protein (-4 residues), and ii) natural anti-FVII inhibitory antibodies developed in a patient with an altered carboxyl-terminal region.

The study was approached both by studies in patient's plasma and by expression of the recombinant FVII variants in eukaryotic cells.

To address the issue of the role of the carboxyl-terminal region of FX, a panel of progressively truncated FX variants has been expressed and characterized.

The information stemming from these studies, and the knowledge of the role of the carboxyl-terminal region of highly homologous members of the serine protease coagulation family could contribute understand the mechanisms underlying the evolution of these proteins from a common ancestor.

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MATERIALS AND METHODS

2.1 Coagulation Assay

2.1.1 Measurement of Factor X antigen

FX antigen levels in plasma or conditioned medium were determined by the enzyme-linked immunoadsorbent assay (ELISA) using a sheep anti-human FX polyclonal antibody as the capture antibody, and a peroxidase-conjugated goat anti-human FX polyclonal antibody, as the detecting antibody (CEDARLANE, Burlington, Ontario, Canada).

The principle of "sandwich-style" ELISA is here described. The FX coating antibody was 1:1000 diluted in coating buffer (50 mM carbonate/bicarbonate, pH 9.2). 70 µl of coating antibody were coated into a 96-well microplate and incubated overnight at 4°C. Prior to adding samples, wells were blocked with 80 µl of blocking buffer (PBS, 6% BSA, 0.05% Tween-20, pH 7.4) and, after an incubation of 1 hour at room temperature, 50 µl of samples containing FX were applied on each well and incubated for 1 hour at room temperature. Samples were properly diluted in diluent buffer (100 mM Hepes, 100 mM NaCl 1% BSA, 0.1% Tween, pH 7.4), also used to prepare the standard curve. Finally, 50 µl of a 1:1500 dilution of the detecting antibody in diluents buffer were incubated for 1 hour at room temperature. At every experimental step, wells were washed 3/4 times with wash buffer (PBS, 0.1% Tween-20, pH 7.4) to remove the excess of unbound BSA (blocking), samples or antibodies (coating and detection).

The addition of 50 µl of a solution composed by a 5 mg tablet of the chromogenic substrate ortho-phenylenediamine (OPD, Sigma, St. Louis, MO) dissolved in 12 ml of substrate buffer (0.05 M citrate-phosphate buffer, pH 5) triggers a colour-forming reaction due to the presence of HRP-containing antigen-antibody immunocomplexes. Finally, 50 µl of 2.5 M H_2SO_4 was added to stop colour formation. The colour produced was quantified using the SUNRISE microplate reader (Tecan, Salzburg, Austria), measuring the absorbance at 492 nm. A standard curve using serial dilutions of pooled normal plasma was used to determine FX concentration.

2.1.2 Measurement of Factor VII antigen

The quantitative determination of FVII antigen levels in plasma or culture medium was performed using a commercially available ELISA kit (Affinity Biologicals™ Inc., Canada).

The FVII coating antibody was 1:100 diluted in coating buffer (50 mM carbonate, pH 9.6) and 100 µl of coating antibody were incubated overnight at 4°C in a 96-well microplate.

Wells were blocked with 150 µl of blocking buffer (PBS, 0.05% Tween-20, BSA 6%, pH 7.4) and, after an incubation of 1.5 hour at room temperature, 100 µl of FVII (from plasma or in conditioned media) were added to each well and incubated for 1 hour at room temperature. Samples were diluted in sample diluent buffer (PBS, 0.1% Tween-20, pH 7.4), used also to prepare the reference plasma curve. Finally, 100 µl of the secondary antibody 1/100 diluted in conjugate diluent buffer (100 mM Hepes, 100 mM NaCl 1% BSA, 0.1% Tween, pH 7.4) were incubated for 1 hour at room temperature. At every step, wells were washed 3 times with the same buffer used for sample preparation (PBS, 0.1%Tween-20, pH 7.4).

Finally, 50 µl of 2.5 M H_2SO_4 were added to the coloured reaction developed upon the addition of 100 µl of OPD solution (in 0.05 M citrate-phosphate buffer, pH 5), and the absorbance was read at 492 nm on SUNRISE microplate reader (Tecan, Salzburg, Austria).

2.1.3 Measurement of Factor IX antigen

Quantification of FIX antigen levels in plasma samples was performed using a commercially available ELISA kit (Affinity Biologicals^{TM} Inc., Canada).

The FIX coating antibody was 1:100 diluted in coating buffer (50 mM carbonate, pH 9.6) and 100 µl of coating antibody were coated overnight at 4°C in a 96-well microplate. Then, without a blocking reaction as indicated by the manufacturer,100 µl of FIX from plasma samples were added to each well and incubated for 1.5 hour at room temperature. Samples were diluted in sample diluent buffer (100 mM HEPES, 100 mM NaCl, 2.5 mM Na_2EDTA, 1% BSA, 0.1% Tween, pH 7.2). The same buffer was also used to prepare serial dilutions of PNP as the standard curve. Finally, 100 µl of the secondary antibody 1:100 diluted in sample diluent buffer were incubated for 1.5 hour at room temperature. At every step, wells were washed 3 times with a wash buffer (PBS, 0.1% Tween-20, pH 7.4).

Finally, 50 µl of 2.5 M H_2SO_4 were added to the coloured reaction developed 5-10 minutes after the addition of 100 µl of OPD solution (in 0.05 M citrate-phosphate buffer, pH 5), and the absorbance was read at 492 nm on SUNRISE microplate reader (Tecan, Salzburg, Austria).

2.1.4 Factor VII activity

The activity of plasma and recombinant FVII was evaluated by its ability to activate its physiological substrate Factor X in specific FXa generation assays [1].

Generation of activated Factor X (FXa), the natural substrate of FVII, was monitored over time using the specific CH_3-SO_2-D-CHA-Gly-Arg-AMCAcOH FXa fluorogenic substrate (American Diagnostica, Greenwich, CT, USA) diluted in reaction buffer (20 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% w/v PEG-8000, pH 7.5) and the fluorescence (360 nm excitation, 465 emission) was measured on SpectraFluor Plus microplate reader (TECAN, Salzburg, Austria) at 37°C. Activity assays were performed into 96-well microplates (Costar, Corning, NY, USA).

Plasma

Plasma samples, PNP and FVII-deficient plasma (George King, Bio-Medical Inc., USA) were 1:20 diluted in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5). Generation of FXa was triggered by the addition of a solution containing the commercially available Innovin (Dade Behring, Marburg, Germany), as a source of Ca^{2+}, phospholipids and TF, added with FXa fluorogenic substrate (500 μM).

Optimized FIIa generation in the presence of anti-FIX antibody

This assays was optimized in order to avoid the participation of FIX in the generation of thrombin. Plasma samples, PNP and FVII-deficient plasma (George King, Bio-Medical Inc., USA) were 1:20 diluted in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5). FIIa generation was triggered by the addition of a solution containing 300 μM FIIa fluorogenice substrate (Thrombin Substrate III, Fluorogenic, EMD Biosciences Inc., La Jolla, CA, USA). The activity was measured either in the presence or in the absence of a commercially available anti-human FIX antibody (Affinity BiologicalsTM Inc., Ancaster, Ontario, Canada). The anti-human FIX antibody was 1:100 diluted in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5) and then incubated with PNP and plasma samples for 2 hours at 37°C.

Recombinant FVII

Factor VII in conditioned medium samples was incubated for 10 minutes at 37°C with an activation mixture containing 0,5 nM human Factor Xa (hFXa) (Haematologic Technologies Inc., Essex Junction, VT, USA) and Innovin. Then, a second mixture containing 100 nM hFX (Haematologic Technologies Inc.) and 500 μM FXa fluorogenic substrate was added to activated samples. Fluorescence (360 nm excitation, 465 nm

emission) was measured on SpectraFluorPlus microplate reader (TECAN, Salzburg, Austria).

Recombinant FVII in FVII-deficient plasma

Medium samples were 1:1 mixed with FVII-deficient plasma 1:20 diluted in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5). Then, samples were loaded into a 96-well plate and a mixture of 500 µM FXa fluorogenic substrate and Innovin was added to samples and emitted fluorescence weas measured on SpectraFluorPlus microplate reader (TECAN, Salzburg, Austria)

FXa generation assays in plasma were standardized using serial dilution of PNP in FVII-deficient plasma. Differently, the assays in media were standardized using serial dilution of recombinant Wt-FVII and a negative control was inserted by using medium from cells transfected with the gutted pCDNA3, not encoding FVII. Finally, for FXa generation assays in FVII-deficient plasma, the standard curve was represented by serial dilutions of the rWt-FVII:FVII-deficient plasma mixture diluted in medium from cells transfected with the empty pCDA3 mixed 1:1 with 1:20 FVII-deficient plasma. in the three different assays, the negative control consisted of FVII-deficient plasma alone, medium from cells transfected with the empty pCDA3 and medium from cells transfected with the empty pCDA3 mixed 1:1 with 1:20 FVII-deficient plasma, respectively.

To permit a more reliable estimate of the specific activity of FVII variants that were secreted at very low levels, FVII in conditioned medium was concentrated approximately 10-fold by using Amicon® Ultra Cenntrifugal Filter Devices (30 KDa cut-off, MILLIPORE, Bedford, MA). 4 ml of conditioned medium were spun 2-3 times at 7,000×g for 5 minutes at 4°C up to a final volume of 400 µl. Subsequently, concentrated medium was collected and used for protein assays.

2.1.5 Measurement of thrombin generation activity

Plasma samples were centrifuged at 23,000 g at 4◦C for 1 hour before testing. Calibrated automated thrombin activity measurement was conducted according to Hemker *et al.* [41,42] in a microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using the Thrombinoscope software (Synapse BV, Maastricht, The Netherlands). The assay was carried out at 37°C essentially as previously reported by

Regnault *et al.* [1]. Coagulation was triggered in platelet poor plasma by recalcification in the presence of 5 pM recombinant human tissue factor and 4 µM phospholipids. Thrombin generation was then evaluated overtime by exploiting a specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Thrombin generation measurement was conducted in parallel in plasma samples after the addition of a thrombin calibrator provided by the manufacturer (Synapse BV). The software enables the estimate of the following parameters: a) the Lag Time of thrombin generation, b) the time to reach the maximum concentration of thrombin (time to Peak), c) the maximum concentration of thrombin (Peak), d) the total duration of thrombin generation activity (Start Tail), and e) the total amount of thrombin activity assessed as the area under the curve, i.e. the endogenous thrombin potential (ETP). All experiments were carried out in duplicate.

2.1.6 Factor X activity

Activity of Factor X was assessed both by activation via the non-physiological activator RVV and in FX-deficient plasma. These assays were performed to measure the amidolytic activity and the FIIa (Thrombin) generation activity, respectively, of recombinant FX in conditioned medium.

Recombinant FX

Factor X in conditioned medium was incubated for 15 minutes at 37°C with 0.5 nM RVV (RVV-X, Haematologic Technologies Inc., Essex Junction, VT, USA) and then a solution containing 500 µM FXa fluorogenic substrate was added to activated samples. Fluorescence (360 nm excitation, 465 nm emission) was measured on SpectraFluorPlus microplate reader (TECAN, Salzburg, Austria).

Optimized FIIa generation assays

FX-deficient plasma (George King, Bio-Medical Inc., Overland Park, Kansas, USA) was added in a 1:10 rate to medium samples. Then, samples were loaded into a 96-well plate and a mixture of 500 µM FIIa fluorogenic substrate (Thrombin Substrate III, Fluorogenic, EMD Biosciences Inc., La Jolla, CA, USA) and Innovin was added and emitted fluorescence was measured on SpectraFluorPlus microplate reader (TECAN, Salzburg, Austria).

2.2 DNA analysis and recombinant DNA techniques

Mutation search in FVII gene was conducted by PCR amplification and direct sequencing of all coding regions, the exon/intron boundaries and the promoter region [2, 3]. When the mutation created or disrupted a restriction endonuclease recognition site, its presence in patients or in expression vectors was confirmed by restriction analysis of PCR products.

2.2.1 Extraction of genomic DNA from whole blood

In order to obtain genomic DNA from blood, it is necessary to lyse blood cells (in particular leukocytes, which have a nucleus), to get rid of phospholipid membranes and to purify DNA from contaminating proteins. DNA extraction from blood was performed with the "salting out" tecnique.

3 ml whole blood were mixed with 9 ml lysis solution (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA) and incubated on ice for 20 min. The solution was then centrifuged at 1500 rpm for 10 min at room temperature to separate the DNA-protein complex from phospholipid membranes. After discarding the supernatant, the precipitate was washed a couple of times with 5 ml distilled water to eliminate the excess of heme. Then it was subjected to proteolytic digestion by adding 20 µl 20 mg/ml pronase (Boehringer Mannheim GmbH, Mannheim, Germany), 900 µl SE buffer (75 mmol/L NaCl, 25 mmol/L EDTA) and 90 µl 10% SDS (a detergent) and incubating overnight at 37°C. On the following day, 300 µl of a saturated (6 mol/L) solution of NaCl were added to the tube, the mixture was vortexed and then centrifuged at 2500 rpm for 15 min at 4°C to precipitate the digested proteins. The supernatant was then transferred to a fresh tube and one volume of chloroform was added to extract residual proteins. After mixing gently by inverting the tube several times, the material was centrifuged again at 4500 rpm for 5 min at room temperature. The supernatant (aqueous phase) was brought to a fresh tube, to which two volumes of ice-cold absolute ethanol were added. Delicate mixing caused the DNA to "condense" into a tangle of whitish filaments, which were recovered with a glass pipet, washed in 70% ethanol, dried in the open air and resuspended in 300 µl pure water. Concentration and purity of the extracted DNA was evaluated by reading the absorbance of the DNA suspension at 260 nm (DNA), 280 nm (proteins) and 415 nm (heme) with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). An OD_{260}/OD_{280} ratio equal or greater than 1.8 indicates pure DNA. A rough estimate of the

quality and quantity of the DNA was obtained by running a few microliters on a 0.8% agarose gel. DNA samples were stored at –20 °C.

2.2.2 Polymerase chain reaction (PCR)

DNA amplification by PCR [2] takes advantage of a natural enzyme (the thermostable DNA-polymerase I of the thermophilic bacterium Thermus aquaticus, known as Taq polymerase) to produce a large number of copies of the same DNA fragment. The DNA polymerization reaction requires a template (usually genomic DNA extracted from whole blood), two primers (single-stranded oligonucleotides that frame the target sequence) and all four deoxyribonucleoside 5'-triphosphates (dNTPs) in the presence of $MgCl_2$. The amplification reaction is performed via 25-30 DNA replication cycles, each comprising three steps: 1) denaturation (separation of the two strands of template DNA); 2) annealing (hybridization of the primers to their complementary sequences on the template DNA); 3) extension (elongation of the primers by DNA-polymerase activity). These steps require different temperatures (denaturation: 95°C; annealing: 45-60°C, according to the characteristics of the primers; extension: 72°C) that are provided automatically by a programmable thermal cycler.

Primers for the PCR-amplification of all exons of the FX gene (Table 5.1) were designed on the basis of the published FX gene sequence using the computer programme OLIGO 4.1 Primer Analysis Software. Amplification reactions (25 µl total volume) were carried out using 1 unit of *Taq* polymerase (BioTherm GenCraft, Germany) in the buffer provided by the supplier (10 mmol/L K-phosphate buffer pH 7.0, 100 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, 0.01% Tween 20, 50% glycerol v/v). Reaction conditions were as follows: 0.1 ng genomic DNA (template), 7 pmol each primer, 200 µmol/L each nucleotide precursor, 1.5-2.0 mmol/L $MgCl_2$ and, in some cases, 4% DMSO. A negative control (*i.e.* a reaction carried out in the absence of template DNA) was always included to check for reagent contamination with template DNA. Thermal cycles comprised 5 min initial denaturation at 95°C, 10 min hot start at 65°C (during which the enzyme was added to the reaction mixtures), 30 cycles of denaturation, annealing, extension as described above, and 10 min final extension at 72°C.

The qualitative and quantitative outcome of the amplification reaction was checked by running 3-5 µl of PCR product on agarose gel, in parallel to an appropriate molecular weight marker.

Primer	Location	Primer Sequence
PreproF	5' UTR	5'- $^{-35}$TGAACAGGCAGGGGCAGC $^{-18}$-3'
PreproR	5' UTR	5'-^{3876}CTTGCACTCCCTCTCCAG ^{3859}-3'
I4F	Intron 4	5'-^{7686}GCAGAACACCACTGCTGACC ^{7705}-3'
110F	Exon 5	5'-^{7847}CCACACGGGCACCAAGCGCTCCTGTCGGCC ^{7878}-3'
F1	Intron 5	5'-^{8870}GCATCTTTCTGACTTTTGTT ^{8889}-3'
6FA	Exon 6	5'-^{8948}AAACCCCAAGGCCGAATTG ^{8966}-3'
R1A	Intron 6	5'-^{9270}GTGAGGACGACGTGACAACT ^{9251}-3'
F2	Intron 6	5'-^{9567}CAATGTGACTTCCACACCTCCT ^{9388}-3'
F2a	Exon 7	5'-^{9672}ACCCTGATCAACACCATCTGG ^{9692}-3'
R7	Exon 7	5'-^{9709} GTTCCTCCAGTTCTTGATTTTGTCG ^{9685}-3'
R2	Exon 7	5'-^{10010}GATGTCTGTCTGTCTGGA ^{9990}-3'
F1	Intron 7	5'-^{10494}TGAGGTGGCAGGTGGTGGAAA ^{10514}-3'
R1a	Exon 8	5'-^{10608}GGATGATGACCTGCGCCAC ^{10590}-3'
R1c	Exon 8	5'-^{10652}GCAGCGCGATGTCGTGGTT ^{10634}-3'
F3	Exon 8	5'-^{10831}TGATGACCCAGGACTGCCT ^{10849}-3'
R1	Exon 8	5'-^{10908}CGGCACAGAACATGTACTCC ^{10889}-3'
R3	3' UTR	5'-^{11203}GGGATTTGGTGCCAGGACA ^{11185}-3'

Table 2.2.1. Oligonucleotides used to sequence all exons and splicing junctions of the FVII gene. Nucleotide numbering according to FVII DNA sequence by O'Hara et al. [3]

2.2.3 Gel electrophoresis

Due to their numerous phosphate groups, nucleic acids are negatively charged at neutral pH and tend to migrate towards the anode if subjected to an electric field. Their migration rate is inversely proportional to the logarithm of their length in bp. These properties make

it possible to separate DNA fragments according to their size. At the end of the electrophoretic run, the positions of the DNA fragments in the gel are visualized by ethidium bromide, an intercalating dye that fluoresces when bound to DNA.

Agarose gels (able to separate fragments ranging from 200 bp to 50 kb) were prepared by dissolving the desired amount of agarose in 1× TAE buffer (40 mmol/LTris-acetate, 1 mmol/L EDTA) and heating this mixture in a microwave oven till complete clarification. A 1% agarose gel contains 1 g agarose in 100 ml buffer. Ethidium bromide was added directly to the melted gel before casting, in the proportion of 5 µl of a 10 mg/ml stock to 100 ml gel. Agarose gels were run horizontally in 1× TAE buffer, by applying a voltage of 5 V/cm.

Polyacrylamide gels (able to resolve fragments ranging from 5 bp to 500 bp) were prepared by mixing the desired volume of an acrylamide stock (40% 1/19 N,N'-methylenebisacrylamide/acrylamide) in 1× TBE buffer (90 mmol/L Tris-borate, 2 mmol/L EDTA). Immediately before pouring the gel, appropriate amounts of ammonium persulfate (700 µl of a 10% solution in 100 ml) and TEMED (35 µl in 100 ml) were added to allow matrix polymerization. Polyacrylamide gels were run vertically in 1× TBE buffer, by applying a voltage of 1-8 V/cm. At the end of the run, the gel was recovered and stained by soaking it in 1× TBE buffer containing 2 µg/ml ethidium bromide for about 15 min.

Stained gels were viewed under UV transillumination at 254 nm, the picture was imported with a GelDoc 1000 UV-gel camera (Bio-Rad Laboratories, Hercules, CA) and stored on a computer as an image file. Gel images were manipulated with the software Molecular Analyst (Bio-Rad Laboratories, Hercules, CA).

2.3 Cloning

2.3.1 Site-directed mutagenesis

Mutagenesis of FVII (1.7 Kb) and FX (1.6 Kb) cDNAs cloned in the pCMV4-FX (Fig. 2.3.1, a) and pCDNA3-FVII (Fig. 2.3.1, b) plasmids, was performed by using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

The basic procedure utilized supercoiled, double-strand DNA vector and two synthetic oligonucleotide primers containing the desire mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of Pfu DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. On incorporation of the

oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with DpnI, an endonuclease (target sequence 5'-G^{m6}ATC-3') which is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is *dam* methylated and therefore susceptible to DpnI digestion. The nicked vector DNA incorporating the desire mutation is then transformed into *E. coli*.

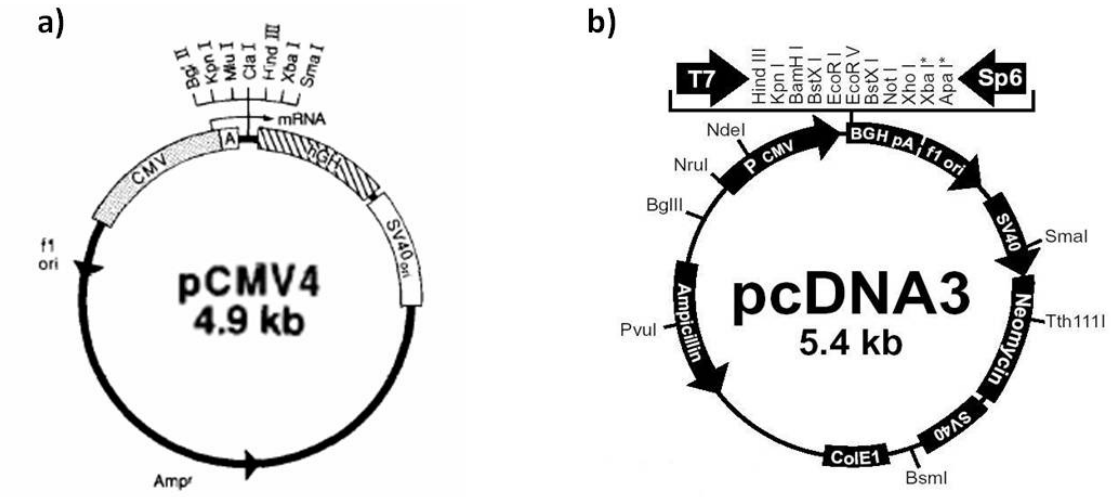

Figure 2.3.1 – Plasmids for FX (a) and FVII (b) cloning and expression

The mutagenesis reaction was carried out in a final volume of 25 µl, containing 10-50 ng of cDNA template, 1 µl (125 ng) of mutagenized oligonucleotides, 0.5 µl dNTPs mix, reaction buffer 1X and 0.5 µl PfuTurbo DNA-polimerase ($1.3x10^{-6}$ error rate). After 30 sec at 95°C, reactions were subjected to 12, 16 or 18 cyclesm depending on the type of mutation to insert (point mutations, single aminoacid changes, multiple aminoacid deletions or insertions). Cycles were as follows: 30 seconds at 95°C, 60 seconds at 55°C, and 68°C for 1 min/Kb of plasmid. The endonuclease DpnI (5U; 1 hour digestion at 37°C) was added at the end of cycles to digest the metilated (parental) plasmids. An aliquot of the reaction (5 µl) was then used to transform 50 µl of *E. coli* competent cells. After incubation on ice for 30 min, the transformation reaction was heat shocked for 45 seconds at 42°C. Placed on ice for 2 min, competent cells were grown with 200 µl of LB broth (shaking at 37°C for 1 hour). The reaction was then plated on LB-ampicillin agar plate.

For each mutant, two complementary oligonucleotides were used to insert the appropriate mutations. Primers were designed on the basis of the FVII and FX cDNA sequence [4,5] and sequences are reported in Table 2.3.1 and Table 2.3.2.

Clones were screened for the desired mutations by colony PCR. Colonies were picked and used as template in a canonical PCR, preceded by an initial heating cycle at 100°C for 15 minutes to lyse bacteria and by a hot-start cycle of 10 minutes at 65°C, at which point the *Taq* DNA polymerase was added. Positive clones were identified by restriction analysis and separation by gel electrophoresis. The presence of the correct mutation was confirmed by DNA sequencing of the plasmid DNA template. For the P406X, F405X, P404X, A 403X and R402X FVII mutant plasmids, positive clones were found upon direct sequencing.

Mutation	**Primer Sequence**
F418X	5'-CCAAGGTCACCGCC**TAG**CTCAAGTGGATC-3' 5'-GATCCACTTGAG**CTA**GGCGGTGACCTTGG-3'
K427X	5'-GACAGGTCCATG**TAG**ACCAGGGGCTTG-3' 5'-GAAGCCCCTGGT**CTA**CATGGACCTGTC-3'
T428X	5'-CAGGTCCATGAAA**TAG**AGGGGCTTGCCC-3' 5'-GGGCAAGCCCCT**CTA**TTTCATGGACCTG-3'
P432X	5'-CCAGGGGCTTG**TAG**AAGGCCAAGAGC-3' 5'-GCTCTTGGCCTT**CTA**CAAGCCCCTGG-3'
A434X	5'-GGCTTGCCCAAG**TAG**AAGAGCCATGCC-3' 5'-GGCATGGCTCTT**CTA**CTTGGGCAAGCC-3'
A438X	5'-GCCAAGAGCCAT**TGA**CCGGAGGTCATAACG-3' 5'-CGTTATGACCTCCGG**TCA**ATGGCTCTTGGC-3'
P439X	5'-GCCAAGAGCCATGCC**TAG**GAGGTCATAACG-3' 5'-CGTTATGACCTCC**TA**GGCAGTGCTCTTGGC-3'
P439A	5'-GAGCCATGCC**G**CGGAGGTCATAACG-3' 5'-CGTTATGACCTCCG**C**GGCATGGCTC-3'
E440X	5'-GAGCCATGCCCCG**T**AGGTCATAACG-3' 5'-CGTTATGACCT**A**CGGGGCAGTGCTG-3'
I442X	5'-GCCCCGGAGGTC**TAG**ACGTCCTCTCC-3' 5'-GGAGAGGACGT**CTA**GACCTCCGGGGC-3'
S444X	5'-GAGGTCATAACGT**GA**TCTCCATTAAAG-3' 5'-CTTTAATGGAGA**TC**ACGTTATGACCTC-3'
FX-FVIIter	5'-GCCAAGAGCCATGCCCCG*TTTCCCTAG*GAGGTCATAACGTCC-3' 5'-GGACGTTATGACCTC*CTAGGGAAA*CGGGGCATGGCTCTTGGC-3'

Table 2.3.1 Oligonucleotides used to mutagenize pCMV4-FXwt expression vector. Mutated bases are in bold and underlined font, while insertions are indicated in italic and underlined font.

Mutation	Primer Sequence
P406X	5'-CTGCGAGCCCCATTT**TAG**TAGCCCAGCAGCCC-3' 5'-GGGCTGCTGGG**CTA**CTAAAATGGGGCTCGCAG-3'
F405X	5'-CTCCTGCGAGCCCCA**TGA**CCCTAGCCCAGCAGC 5'-GCTGCTGGGCTAGGG**TCA**TGGGGCTCGCAGGAG-3'
P404X	5'-CCTCCTGCGAGCC**TAG**TTTCCCTAGCCCAGC-3' 5'-GCTGGGCTAGGGAAA**CTA**GGCTCGCAGGAGG-3'
A403X	5'-GGAGTCCTCCTGCGA**TAG**CCATTTCCCTAG-3' 5'-CTAGGGAAATGG**CTA**TCGCAGGAGGACTCC-3'
R402X	5'-GGAGTCCTCCTG**TGA**GCCCCATTTCGCTAG-3' 5'-CTAGCGAAATGGGGC**TCA**CAGGAGGACTCC-3'
R402A	5'-GAGTCCTCCTG**GCA**GCCCCATTTCCCTAG-3' 5'-CTAGGGAAATGGGGC**TGC**CAGGAGGACTC-3'
R492A/A403X	5'-GAGTCCTCCTG**GCATAG**CCATTTCCCTAG-3' 5'-CTAGGGAAATGG**CTATGC**CAGGAGGACTC-3'
FVII-FXterEVITS	5'-CCTCCTGCGAGCCCCA*GAGGTCAT* *AACGTCC*TTTCCCTAGCCCAGCAGC-3' 5'-GCTGCTGGGCTAGGGAAA*GGACGT* *TATGACCTC*TGGGGCTCGCAGGAGG-3'
FVII-FXterEVITSSPLK*	5'-GAGGTCATAACGTCC*TCTCCATTAA* *AGTGA*TTTCCCTTGCCCAGCAGC-3' 5'-GCTGCTGGGCAAGGGAAA*TCACTT* *TAATGGAGA*GGACGTTATGACCTC-3'

* this plasmid was obtained using the FVII-FXterEVITS as the template

Table 2.3.2 Oligonucleotides used to mutagenize pCDNA3-FVIIwt expression vector. Mutated bases are in bold and underlined font, while insertions are indicated in italic and underlined font.

2.3.2 Restriction analysis

Restriction enzymes are endonucleases of bacterial origin able to recognize and reproducibly cut specific sequences of DNA (recognition sites). Single-nucleotide substitution can create/disrupt a restriction endonuclease recognition site, making it possible to identify the substitution by restriction analysis of PCR products spanning the mutation (Table 2.3.3).

Restriction enzymes were purchased from Amersham Life Science Inc., Cleveland, OH; New England BioLabs, Inc., Beverly, MA; Fermentas AB, Vilnius, Lithuania; Boehringer Mannheim GmbH, Mannheim, Germany. 100 ng amplified DNA were incubated with 3-5 units restriction enzyme in the appropriate buffer for 1 hour or longer at the recommended temperature. The products of DNA digestion were then analyzed by agarose or polyacrylamide gel electrophoresis.

Mutation	Restriction Enzyme	Sequence (5'-3')
FX		
F418X	Mnl I	CG↓GC
K427X	Acc I	GT↓MKAC
T428X	Mva I	CC↓WGG
P432X	Eco130 I	C↓CWWGG
A434X	Hae III	GG↓CC
A438X	Bcn I	CC↓SGG
P439X	FspBI	C↓TAG
P439A	BstUI	CG↓CG
E440X	Bcn I	CC↓SGG
I442X	Xba I	T↓CTAGA
S444X	Mbo I	↓GATC
FX-FVIIter	Bsl I	CCNNNNN↓NNGG
FVII		
FVII-FXterEVITS	HpyCH4IV	A↓CGT
FVII-FXterEVITSSPLK	BanI	C↓GYRCC
R402A	Bsp1286I	GDGCH↓C
R402A/A403X	Bsp1286I	GDGCH↓C

Table 2.3.3 Detection of FX and FVII mutations by restriction analysis. The symbol ↓ indicates the cleavage site of the endonuclease. D: not C (A or G or T); H: not G (A or C or T); K: G or T; M: A or C; R: G or A; N: A,,C,G or T; S: G or C; W: A or T; Y: C or T.

2.3.3 Plasmid DNA purification

Rapid isolation of plasmid DNA from 5 ml of E. coli culture grown over night was achieved by using the GenElute^{TM} HP Plasmid Miniprep (SIGMA).

High-quality DNA for transfection experiments was obtained from 70-100 ml of E. coli culture grown over night by using the GenElute™ HP Plasmid Midiprep Kit (SIGMA, St. Louis, MO).

2.3.4 Automated sequencing

Sequencing was performed according to the dideoxy-mediated chain termination method [6].

PCR fragment purification. PCR products were purified from contaminating primers, free nucleotides, Taq polymerase and salts using the Montàge PCR device filters (MILLIPORE, Bedford, MA), which is based on the property of glass fibres to specifically bind nucleic acids. 50 µl PCR reaction were mixed well with 350 µl distilled water and spun at 1000×g for 15 seconds. Subsequently, the filter tube was inverted and applied to a clean collection tube, and filled with 20 µl distilled water, before spinning again at 1000×g for 2 minutes.

Cycle sequencing. The sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Forster City, CA). This kit makes use of a genetically modified Taq polymerase that does not discriminate di-deoxynucleotides and includes a set of terminators labeled with high-sensitivity dyes. 10 µl reaction mixture were prepared by mixing 3.5 µl purified PCR product (corresponding to 30-90 ng, template DNA), 0.5 µl 6.8 µM primer (corresponding to 3.4 pmol), 3.0 µl Terminator Ready Reaction Mix from the kit (comprising A-dye, C-dye, G-dye, T-dye, deoxynucleoside triphosphates, the thermostable DNA polymerase, $MgCl_2$ and Tris-HCl buffer, pH 9.0) and 3.0 µl distilled water. Cycle sequencing was carried out by subjecting this mixture to 25 cycles of denaturation (10 sec at 96 °C), annealing (5 sec at 45-60 °C, according to the primer used), extension (4 min at 60 °C).

Sample preparation. Prior to electrophoresis, extension products were subjected to ethanol/sodium acetate purification to remove unincorporated dye-labeled terminators. 2 µl 3 M sodium acetate (pH 4.6) and 50 µl 95% ethanol were added to the sequencing reaction (10 µl) and mixed well. Following centrifugation at 16,000 rpm for 30 min, the supernatant was discarded and 250 µl cold 70% ethanol were added to wash the precipitate, which was vortexed gently and spun again at 18,000 rpm for 5 min. After discarding the supernatant, the pellet was dried in the vacuum pump and finally resuspended in 6 µl loading buffer (5 volumes of deionized formamide and 1 volume 25 mM EDTA pH 8.0 with 50 mg/ml blue dextran). The sample was denatured at 95°C for 2 min before 1.5-2 µl were loaded on the gel.

Electrophoresis. The extension products were run on a 4% polyacrilamide gel in 1× TBE buffer, containing 6 M urea as the denaturing agent. To prepare 30 ml gel, 10.8 g urea were dissolved in 12 ml distilled water, and 3.12 ml 40% 1/19 N,N'-methylenebisacrylamide/acrylamide stock solution (PagePlus 40% Concentrate, Amresco, Solon, OH) and 0.3 g amberlite (Sigma, St. Louis, MO) were added. The mixture was

stirred at room temperature until complete clarification, filtered and deaerated by applying vacuum. Then, 6 ml 5× TBE buffer were added and the solution was brought to the final volume with distilled water. Before pouring the gel between the assembled glass plates, 150 µl 10% ammonium persulfate and 21 µl TEMED were added to allow acrylamide polymerization. Electrophoresis was performed with an ABI PRISM 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA) for 7 hours.

2.4 Expression of recombinant proteins and protein assays

2.4.1 Eukaryotic cell cultures and transfection

Two cell lines of kidney origin, Baby Hamster Kidney 21 (BHK) cells and Human Embryonic Kidney 293 (HEK 293) cells, capable to correctly process coagulation factors by post-translational modifications, were used for FVII and FX expression.

BHK and HEK cells were grown in Dulbecco's modified Eagle's medium (DMEM) or DMEM//F12 (1:1) medium (Lonza, Verviers, Belgium), respectively, in the presence of 10% fetal bovine serum (FBS, BioWhittakerTM FBS, Lonza, Verviers, Belgium), 2 mM L-glutamine (Gibco, Gaithersbur, MD), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Gaithersbur, MD).

Factor X expression

Recombinant FX proteins, in the mammalian expression plasmid pCMV4, were expressed in HEK 293 cells using the LipofectamineTM 2000 Reagent (Invitrogen, Carlsbad, CA, USA), a formulation suitable for the transfection of nucleic acids into eukaryotic cells. Cells in OptiMEM medium (Gibco, Gaithersburg, MD) added with 5 µg/ml vitamin K were transfected with 4 µg of plasmid DNA in 6-well dishes and in the presence of a 1:1 ratio of LipofectamineTM 2000 Reagent. In the first step, 4 µl of LipofectamineTM 2000 were added to 150 µl of OptiMEM medium and incubated for 5 minutes at room temperature; in the second step, 4 µg of plasmid DNA were diluted into 150 µl of OPTI-MEM medium and then combined with the Lipofectamine-containing solution. The obtained mixture was incubated for 20 minutes at room temperature. The DNA-Lipofectamine complexes (300 µl) were then added directly to each well of cells in the presence of OptiMEM medium and cells were grown at 37°C and 5% CO_2 for 72 hours.

Factor VII expression

The same protocol was used for recombinant FVII expression in BHK cells, even if 3 μg plasmid and 3 μl Lipofectamine™ were used. In addition, the concentration of vitamin K was 6 μg /ml.

2.4.2 Western blotting analysis on Factor VII

Western blotting is a technique for protein analysis which comprises three steps: i) electrophoresis on a polyacrylamide gel (SDS-PAGE); ii) blotting to nitrocellulose; iii) immunostaining.

Samples (plasma or conditioned medium) for western blotting were prepared in a non-reducing 1X sample buffer (NuPage® LDS Sample Buffer, Invitrogen, Carlsbad, CA, USA) containing SDS and bromophenol blue, denaturated for 5 minutes at 95°C and loaded on precast gels (NuPage® Novex® 4-12% Bis-Tris gels, Invitrogen).1X MOPS buffer (NuPage® MOPS SDS Running Buffer, Invitrogen) was used as running buffer. The SeeBlue® Plus2 Prestained Standard (Invitrogen) was loaded in parallel to samples as a molecular weight marker. After 2 hours separation at 160 V by vertical electrophoresis, samples were transferred to pre-hydrated PROTRAN® 0.2 μm nitrocellulose membrane (Whatman®, Dassel, Germany) by incorporating the gel into the blotting sandwich and using a tris-glycine-SDS-methanol (25 mM Tris, 190 mM glycine, 0,02% SDS (w/v), 20% methanol) transfer buffer. Nitrocellulose filter was then saturated by an overnight incubation at 4°C in a blocking buffer containing PBS (Sigma-Aldrich) added with 5% (w/v) dry milk (Bio-Rad, Hercules, CA, USA) and 0,1% Tween-20 (Sigma-Aldrich).

The nitrocellulose membrane was successively incubated for 4 hours at room temperature with a 1:2500 dilution in blocking buffer of a HRP-conjugated sheep polyclonal anti-human FVII antibody (2 mg/ml, Pierce®, Thermo Scientific, Rockford, IL, USA). After incubation with the antibody and washes in blocking buffer, the detection was performed using chemioluminescent substrates as either ECL (Pierce ECL Western Blotting Substrate) or Femto (Supersignal® West Femto) reagents (Thermo Scientific, Rockford, IL, USA), depending on the amount of sample initially loaded or the sensitivity required. The result was visualized on chemiluminescence films (Amersham Hyperfilm™ ECL (GE healthcare).

2.4.3 FVII assays with anti-FVII inhibitory antibody

ELISA-based assays, Western blot analysis and Bethesda-like FXa generation assays were carried out to study the interaction between rFVIIa, plasma-derived zymogen FVII and recombinant FVII proteins with an anti-FVII antibody developed in a patient homozygous for a frequent FVII mutation.

ELISA-based assays – Interaction studies

70 µl of rFVIIa (NOVO Nordisk®, Denmark) or plasma-derived zymogen FVII (Haematologic Technologies Inc., Essex Junction, VT, USA) were diluted in a coating buffer (50 mM carbonate, pH 9.2) and coated overnight at 4°C in a 96-well microplate. Prior to adding samples, wells were blocked for 1.5 hours with 80 µl of blocking buffer (PBS, 6% BSA, 0.05% Tween-20, pH 7.4) at room temperature. 50 µl of antigen, represented by the plasma containing the anti-FVII antibody, were applied on each well and incubated at 37°C for 1 hour upon dilution in sample diluent buffer (100 mM Hepes, 150 mM NaCl 1% BSA, 0.1% Tween, pH 7.4). Finally, 50 µl of a 1:10,000 dilution of a goat HRP-conjugated anti-human IgG (1 mg/ml, Bethyl Laboratories, Montgomery, TX, USA) were incubated for 1 hour at room temperature. The addition of 50 µl of a solution composed by a 5 mg tablet of the chromogenic substrate ortho-phenylenediamine (OPD, Sigma, St. Louis, MO) dissolved in 12 ml of substrate buffer (0.05 M citrate-phosphate buffer, pH 5) triggers a colour-forming reaction due to the presence of HRP-containing antigen-antibody immunocomplexes. Finally, 50 µl of 2.5 M H_2SO_4 was added to stop colour formation. The colour produced was quantified using the SUNRISE microplate reader (Tecan, Salzburg, Austria), measuring the absorbance at 492 nm. The rate of binding of the anti-FVII antibody to either rFVIIa and plasma-derived zymogen FVII was indicated as measured OD at the wavelength of 492 nm.

ELISA-based assays - Quantification of specific anti-FVII IgG in patient's plasma

Quantification of the specific anti-FVII IgG content was carried out by using serial dilutions of a purified human IgG as a standard curve. 70 µl of equal molar concentrations of anti-human IgGs and either rFVIIa or plasma-derived zymogen FVII were diluted in coating buffer (50 mM carbonate, pH 9.2) and coated overnight at 4°C.

Wells were blocked by the addition of 80 µl of a blocking buffer (PBS, 6% BSA, 0.05% Tween-20, pH 7.4). Serial dilutions of a purified human IgG (1 mg/ml, Bethyl

Laboratories) were prepared in sample diluent buffer (100 mM Hepes, 150 mM NaCl 1% BSA, 0.1% Tween, pH 7.4) and added to wells coated with the anti-human IgG, while anti-FVII antibody-containing plasma was added to either coated rFVIIa or plasma-derived zymogen FVII at different dilutions. 50 µl of both human IgG and plasma with anti-FVII antibody were incubated for 1 hour at 37°C. 50 µl of 1:10,000 dilution of goat HRP-conjugated anti-human IgG (1 mg/ml, Bethyl Laboratories, Montgomery, TX, USA) were added and incubated for 1 hour at room temperature. Detection of the immunocomplexes was carried out as described above and the standard curve served as the reference for IgG content evaluation.

ELISA-based assays - Interaction studies with mimicking peptides

In the same experimental set up as that described above, dilutions of anti-FVII antibody were added to coated rFVIIa and incubated for 1 hour at 37°C either in the absence or in the presence of two synthetic peptides. Peptides and the anti-FVII antibody-containing plasma were mixed 1:1 and incubated for 1 hour at 37°C prior to the addition to coated rFVIIa. Upon the experimental steps described above, the interaction rate between coated rFVIIa and the plasma anti-FVII antibody was evaluated by OD measurement. The effectiveness of peptides was measured as a decrease in the measured OD values.

Western blotting – Recognition of rFVIIa and FVII by anti-FVII antibody

rFVIIa, plasma-derived FVII (Haematologic Technologies Inc., Essex Junction, VT, USA) and hFXa (Haematologic Technologies Inc., Essex Junction, VT, USA) were prepared in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5) and then mixed with 1X sample buffer (NuPage® LDS Sample Buffer, Invitrogen, Carlsbad, CA, USA). rFVIIa was also reduced by adding 1 µl beta-mercaptoethanol to the sample mixed with 1X sample buffer. Upon denaturation at 95°C for 5 minutes, SDS-PAGE on precast gels (NuPage® Novex® 4-12% Bis-Tris gels, Invitrogen) was carried out using 1X MOPS buffer (NuPage® MOPS SDS Running Buffer, Invitrogen) as running buffer. The SeeBlue® Plus2 Prestained Standard (Invitrogen) was loaded in parallel to samples as a molecular weight marker. After 2 hours separation at 150 V by vertical electrophoresis, samples were transferred to nitrocellulose membrane (Whatman®, Dassel, Germany) using a tris-glycine-SDS-methanol (25 mM Tris, 190 mM glycine, 0,02% SDS w/v, 20% methanol) transfer buffer. Nitrocellulose filter was then saturated by an overnight

incubation at 4°C in a blocking buffer containing PBS (Sigma-Aldrich) added with 5% (w/v) dry milk (Bio-Rad, Hercules, CA, USA) and 0,1% Tween-20 (Sigma-Aldrich).
The nitrocellulose membrane was successively incubated for 2 hours at room temperature with a 1: 500 or 1:1000 dilution in blocking buffer of the plasma containing the anti-FVII antibody. Upon washes in blocking buffer to remove the exceed of unbound antibody, a a goat HRP-conjugated anti-human IgG (1 mg/ml, Bethyl Laboratories, Montgomery, TX, USA) 1:5,000 diluted in blocking buffer was used as the secondary antibody.
After incubation with the antibody and washes in blocking buffer, the detection was performed using chemioluminescent substrates as either ECL (Pierce ECL Western Blotting Substrate) or Femto (Supersignal® West Femto) reagents (Thermo Scientific, Rockford, IL, USA), depending on the amount of sample initially loaded or the sensitivity required. The result was visualized on chemiluminescence films (Amersham Hyperfilm^{TM} ECL (GE healthcare).

Bethesda-based FXa generation with plasma anti-FVII antibody

This assay was optimized using experimental conditions based on our protocols for measurement of FVII activity (FXa generation) and a modified Bethesda assay, known to be widely used for assaying inhibitory antibody content of plasma samples [7,8].
Recombinant FVII in conditioned medium was 1:1 mixed with FVII-deficient plasma diluted in dilution buffer (20 mM Hepes, 150 mM NaCl, 0,1% w/v PEG-8000, pH 7.5). Plasma containing the anti-FVII inhibitory antibody was serially diluted in FVII-deficient plasma. The mixture containing FVII added with FVII-deficient plasma was incubated for 2 hours at 37°C with the anti-FVII antibody. After the incubation, activation of plasma samples was triggered by Innovin and activity was measured adding 250 μM FXa fluorogenic substrate. The emitted fluorescence (360 nm excitation, 465 emission) was measured on SpectraFluor Plus microplate reader (TECAN, Salzburg, Austria) at 37°C.
Residual activity of each recombinant FVII variant in the presence of the inhibitory antibody was calculated and normalized considering the activity in the absence of antibody as 100%. In these Bethesda-based assays, negative controls were: i) a 1:1 mixture of medium from cells transfected with the empty plasmid and FVII-deficient plasma; ii) 1:1 mixture of i) with the highest concentration of inhibitory antibody; iii) the highest concentration of inhibitory antibody itself.

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- 3 -

RESULTS AND DISCUSSION

PART I

Characterization of the R402X nonsense mutation in the carboxyl-terminal region of coagulation FVII

We investigated the naturally occurring nonsense mutation R402X in coagulation FVII as a peculiar model to investigate the role of the carboxyl-terminal region in biosynthesis/secretion and activity of this serine protease. The study was approached by investigations in patient's plasma and by characterization of the truncated FVII protein upon expression in eukaryotic cells.

3.1 Patients and detection of the nonsense mutation

During a routine preoperative coagulation screening, a 6-year old baby, presented with a prolonged prothrombin time and FVII coagulant activity (FVIIc) of 3-5% of normal. The girl experienced bleeding manifestations.

Direct sequencing of all exons, intron-exon junctions and promoter region in FVII gene led to the identification of a C to T transition in the homozygous condition at position 11121 (nucleotide numbering according to FVII DNA sequence [1]), responsible for the premature stop codon TGA in the terminal tract of exon 8, and resulting in the R402X nonsense mutation (Fig. 3.1.1).

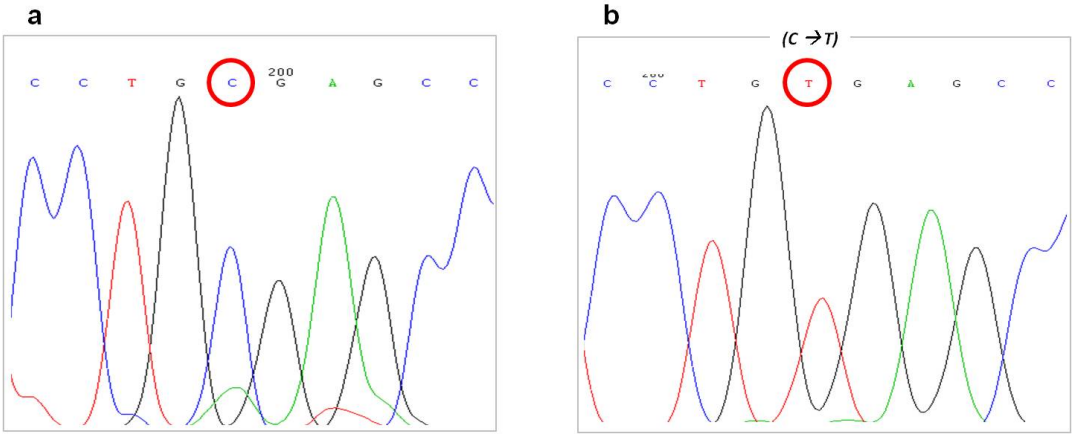

Figure 3.1.1. Chromagrams showing the a) normal nucleotide sequence (CGA) coding for arginine 402 and b) the premature stop codon TGA identified in the proposita. The nucleotide change is reported within the red circle.

The 402X nonsense mutation was found in the heterozygous state in the father and the mother, and was associated with FVIIc values of 54-72% and 24-48%, respectively. The pedigree of the family is shown in Fig. 3.1.2.

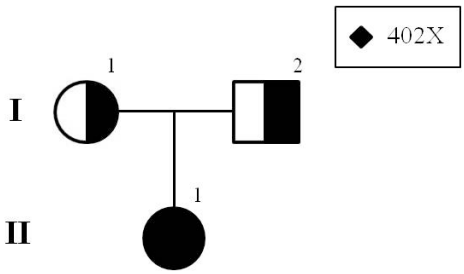

Figure 3.1.2. Pedigree of the family.

3.2 Characterization of FVII levels in plasma

After the first analysis of coagulant activity, the quantification of the FVII mutant protein in the patient's plasma was performed. ELISA assays indicated very low protein FVII levels in the proposita (3.9±1.3 ng/mL), corresponding to 0.78±0.23% of pooled normal plasma (PNP). The presence of trace amounts of the protein in the patient's plasma was confirmed by western blotting analysis performed on two independent plasma samples (Fig. 3.2.1).

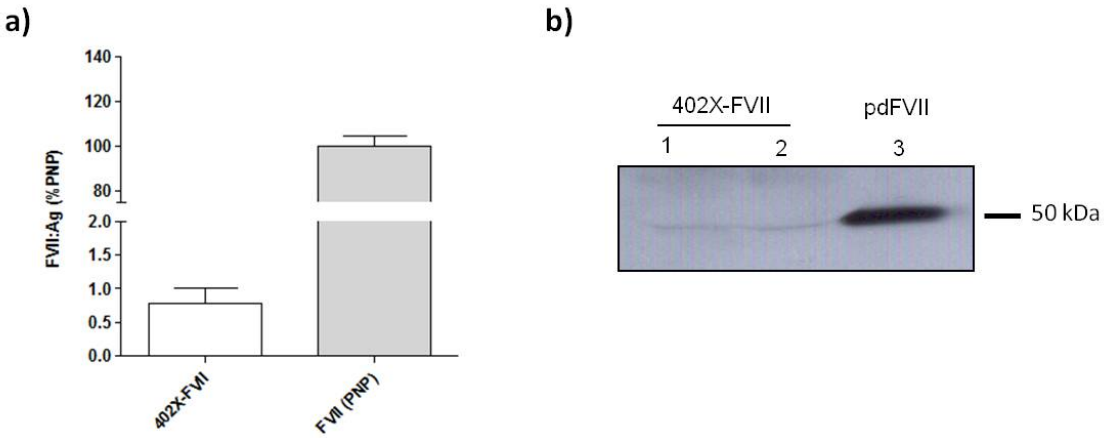

Figure 3.2.1. Antigen levels and trace amounts of FVII in patient's plasma.
a) Antigen levels (0.78%±0.23% of PNP) from at least five independent assays on two different plasma samples.
b) Trace amounts of FVII detected by western blotting on two independent plasma samples; lanes 1,2: patient's plasma; lane 3: 1 nM plasma-derived FVII (control).

To further evaluate the coagulant activity in patient's plasma we also performed a thrombin generation assay which enables us to monitor, upon extrinsic coagulation

triggering, the generation of Thrombin over time. Overall, the thrombin generation activity in the proposita's plasma appeared to range from that of 1% and 2% of PNP. Since FVII is the trigger of the coagulation process, we first considered the variations in the lag time that should reflect the efficiency of the initiation phase. Considering the lag time values from the standard curve, the activity of the 402X-FVII from patient's plasma was 1,5%±0,1% when compared to normal plasma (Fig. 3.2.2).

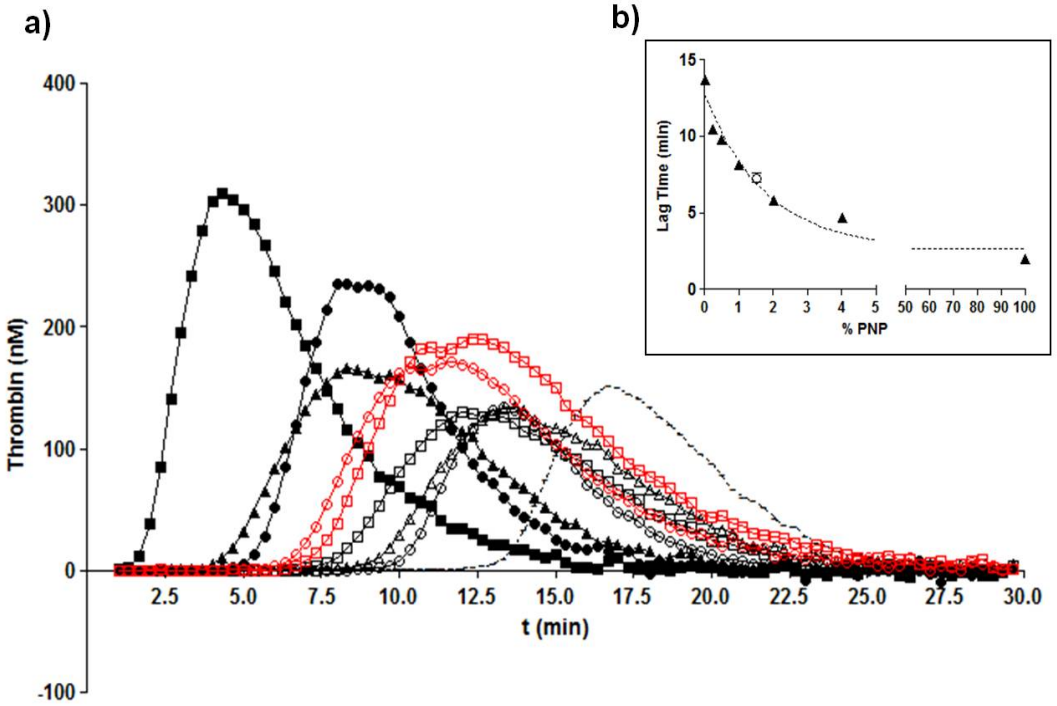

Figure 3.2.2. a) Thrombin generation assay in the proposita's plasma. Activity is expressed in the y-axis as concentration of Thrombin (nM) generated per minutes (x-axis). Black squares: 100% PNP; black triangles: 4% PNP; black circles: 2% PNP; open squares: 1%; open triangles: 0,5%; open circles: 0,25% PNP; dashed line: 0% PNP; red squares and circles: 402X-FVII from patient's plasma. The standard curve was prepared by dilution of the PNP in FVII-deficient plasma; the 0% of the curve was FVII-deficient plasma. b) Lag time values (y-axis, expressed in minutes) derived from thrombin generation assay in function of activity (expressed as %PNP, x-axis). 402X-FVII is indicated by the white circle and PNP (0-0,25-0,5-1-2-4-100%) standard curve by black triangles.

Moreover, the ETP (Endogenous Thrombin Potential), the Peak and the Time-to-peak values in patient's plasma were 1589.2±193.4, 180.5±14.3 and 12.0±0.4, respectively. Noticeably, with the exception of the 100% of PNP, the ETP value measured in patient's plasma was greater than that observed for each tested PNP concentration point (Table 3.2.1).

	Lag time (min)	ETP (nM FIIa)	Peak (nM FIIa)	Time to Peak (min)
omo402X-FVII	7.3 ± 0.3	1589.2 ± 193.4	180.5 ± 14.3	12.0 ± 0.4
PNP 100%	2.0	1700.5 ± 12.5	308.9 ± 2.4	4.5 ± 0.2
PNP 4%	4.7	1278.5 ± 53.5	165.6 ± 8.9	8. ± 0.3
PNP 2%	5.8 ± 0.2	1325.5 ± 2.5	236.8 ± 6.9	8.8 ± 0.2
PNP 1%	8.2 ± 0.2	1048.5 ± 85.5	129.9 ± 23.5	12.3 ± 0.3
PNP 0,5%	9.8 ± 0.2	1021.1 ± 36.1	133.7 ± 4.1	13.6 ± 0.3
PNP 0,25%	10.5 ± 0.8	727.0 ± 49.4	134.4 ± 26.4	13.6 ± 1.0
PNP 0%	19.1 ± 5.5	986.5 ± 62.9	117.8 ± 34.4	22.7 ± 5.9

Table 3.2.1. Main FIIa generation parameters (Lag time, ETP, Peak and Time-to-Peak) measured in plasma from the proposita.

To further detail the activity of FVII in patient's plasma we evaluated its activity toward factor X, the physiological substrate in the TF/FVIIa activation pathway. These FXa generation assays are very sensitive since they exploit a synthetic fluorogenic substrate specifically recognized by the activated form of FX (FXa). In independent assays, the activity in plasma from R402X homozygote was 1.8±0.5% (Fig. 3.2.3), which further strengthen the hypothesis that the truncated FVII-402X variant possesses a higher specific activity than the normal FVII protein.

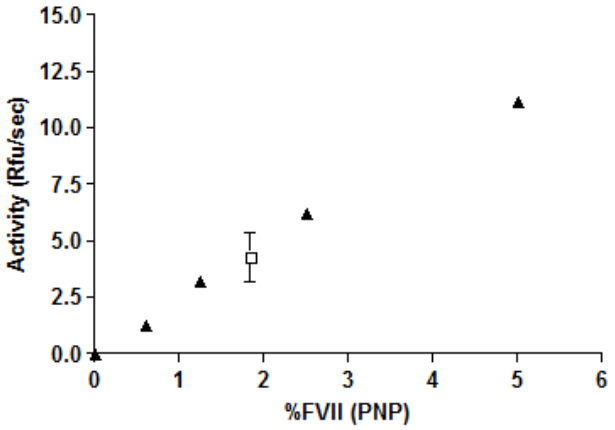

Figure 3.2.3. FXa generation in patient's plasma. Activity is expressed as Relative Fluorescence Units (Rfu) per second (Rfu/sec). Black triangles: standard curve of PNP (serial dilutions in FVII-deficient plasma); white square: 402X-FVII variant from patient's plasma.

Taking into consideration the data from FXa generation, the specific activity of FVII in PNP was 1268,6±361.0 Rfu/min/nM FVII, while that observed for the 402X-FVII variant was found to be 3296,7±115,8 Rfu/min/nM FVII, thus indicating a 2.5-fold increase in

specific activity of the truncated variant in respect of the normal full-length FVII (Fig. 3.2.4).

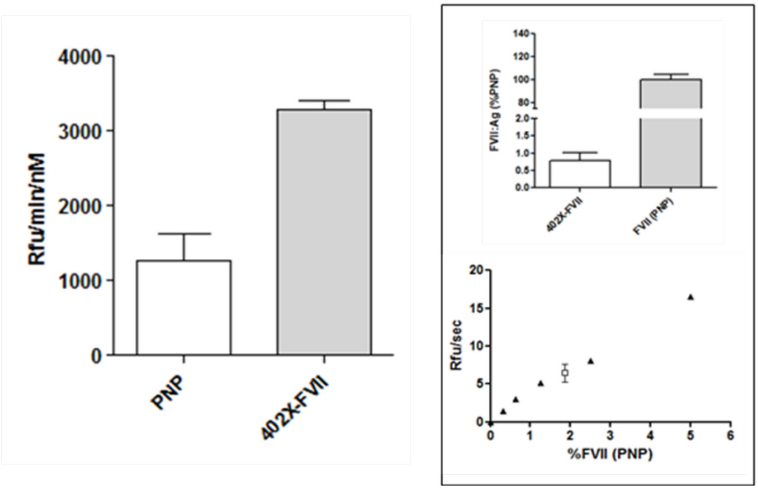

Figure 3.2.4. Specific activity of FVII in the proposita's plasma and in PNP, expressed as Relative Fluorescence Units (Rfu)/min/nM FVII.

All these data suggested that the patient's plasma retained a coagulant capacity higher than that of normal FVII, despite of the low levels of circulating 402X-FVII variant. Since the augmented activity observed could be due to amounts of other pro-coagulant factors higher than normal, we determined the circulating levels of FX and FIX, which are both activated by the TF/FVIIa pathway. As shown in Table 3.2.2. the levels of FX and FIX from patient's plasma were within the physiologic range, thus not supporting their impact on coagulation phenotype of the proposita.

	ng/ml	**% PNP**
FVII	3.6 ± 1.2	0.78 ± 0.2
FX	7903.1 ± 1409.6	98.79 ± 17.6
FIX	6218.1 ± 1381.8	123.05 ± 38.4

Table 3.2.2. Plasma levels of coagulation FX and FIX, together with FVII, in patient's plasma expressed as ng/ml and the corresponding % of PNP.

Since in the coagulation cascade both FX and FIX are natural substrates of the TF/FVIIa pathway of activation, after fluorogenic substrate-based FXa and Thrombin generation assays, we checked whether the FVII in the proposita's plasma possess increased activity toward FIX. To address this issue, our optimized FIIa generation assay was used in the

absence and in the presence of a commercially available anti-human FIX antibody incubated either with two PNP concentrations (2 and 1%) diluted in FVII-deficient plasma or 402X-FVII from patient's plasma (Fig. 3.2.5). Considering the thrombin generation activity of 2% PNP, 1% PNP and the 402X-FVII in the absence of anti-FIX antibody as 100%, we observed a residual activity of 67.4±0.3%, 76.2±3.2% and 70.2±4.5%, respectively.

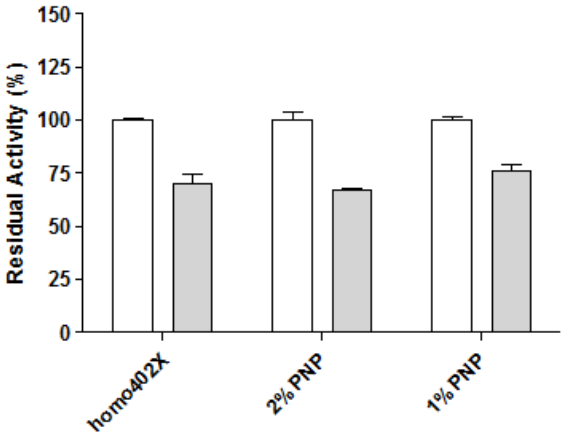

Figure 3.2.5. Fluorogenic FIIa generation in the absence (white columns) and in the presence (grey columns) of a commercially available anti-FIX antibody (1/100 dilution). The activity is expressed as mean percentage ± standard deviation of residual activity, considering the activity measured in the absence of the anti-FIX antibody as 100%. 2% and 1% samples were prepared by serial dilutions of PNP in FVII-deficient plasma.

Our results showed a residual activity for 402X-FVII, due to inhibition by the anti-FIX antibody, similar to that of PNP, indicating that neither a higher concentration of FIX, as previously shown by ELISA, nor a higher capacity of the 402X-FVII variant to convert FIX to FIXa, were responsible for the higher pro-coagulant capacity found for the homozygous patient bearing the 402X nonsense mutation.

3.3 Characterization of the recombinant 402X-FVII variant

The naturally truncated 402X-FVII variant was transiently expressed in BHK21 cells to elucidate the contribution of the FVII last carboxyl-terminal residues to biosynthesis and function. After a 72-hour transient transfection of BHK cells with the p402X-FVII and pWt-FVII plasmids, ELISA and functional assays were performed to evaluate expression and function of the r402X-FVII variant in conditioned media. Our results showed that the r402X-FVII, as compared to wild-type FVII (120 ng/ml), was secreted at levels below the lower sensitivity limit of the ELISA assay (1 ng/ml), thus mirroring the observations shown in the patient's plasma. Furthermore, functional FXa generation assays indicated

that the activity of the poorly secreted r402X-FVII variant ranged from barely to not detectable when assessed in the FVII-deficient plasma and reconstituted systems, respectively.

To better estimate the activity of the r402X-FVII variant in FXa generation assays, conditioned media were concentrated approximately 10-fold by using Millipore devices, thus permitting a reliable evaluation of the specific activity of this protein (expressed as Relative Fluorescent Units (RFU)/min/nM FVII) and allowing us to better quantify and detect this protein by ELISA and to confirm its presence in conditioned medium also by western blotting. Indeed, after concentration of conditioned media from three independent transfections, the 10-fold concentration permitted us to obtain a concentration of 2.5±0.2 ng/ml and to observe a difference in detection also in western blotting (Fig. 3.3.1). As control, the wild type FVII was concentrated by the same devices and results obtained in ELISA showed a 10-fold increase in concentration also for the rWt-FVII.

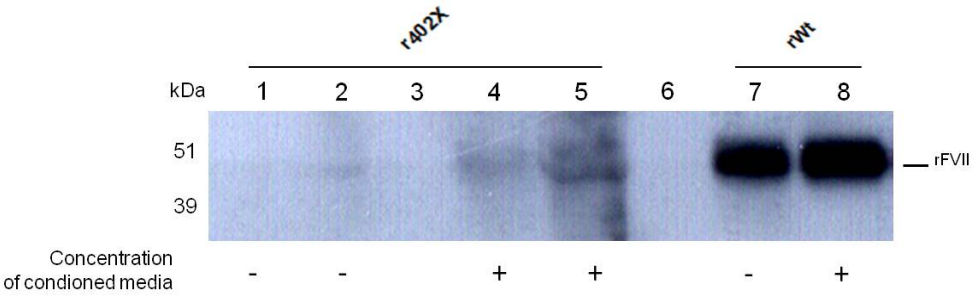

Figure 3.3.1. Detection of the very low amounts of r402X-FVII in conditioned medium by western blotting. Media were checked for the presence of FVII before and after a 10-fold concentration of mediua. Lane 1: not diluted r402X-FVII; lane 2: 1/2 dilution of r402X; lane 4: not diluted r402X-FX after concentration; lane 5: 1/2 dilution of r402X-FX after concentration; lane 7: 40 ng/ml rWt-FVII; lane 8: 60 ng/ml of rWt-FVII (1/10 dilution) after the 10-fold concentration; lane 3,6: buffer only.

Then, the 10-fold concentration of r402X-FVII in conditioned media allowed us to perform a FXa generation in the FVII-deficient plasma system. The FXa generation activity observed for the r402X-FVII upon media concentration was 9.7±0.3 ng/ml when compared to a standard curve prepared by serial dilutions of wild-type FVII (Fig. 3.3.2).

In these experiments, the specific activity observed for the rWt-FVII was 650.6±62.0 RFU/min/nM FVII, while that measured for the r402X-FVII variant was 2519.7±70.1 RFU/min/nM FVII, thus remarkably higher than that of rWt-FVII protein, showing a 3- to 4-fold increase in the specific activity of the r402X-FVII variant.

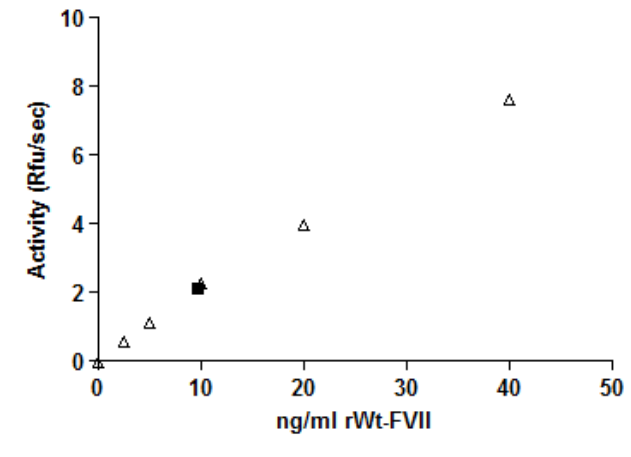

Figure 3.3.2. FXa generation activity of r402X-FVII in FVII-deficient plasma. Activity is expressed as Relative Fluorescence Units (Rfu)/sec. Open triangles: standard curve of wild-type FVII; black square: r402X-FVII variant in conditioned medium.

These results, together with observations in the patient's plasma, indicated that the deletion of the 402 residue and downstream aminoacids was responsible for the very low amounts of secreted protein but conferred to the truncated FVII protein a specific activity higher than that of the normal full-length FVII.

The results previously obtained from patient's plasma and in vitro by transient expression of the recombinant r402X-FVII protein, showed a potential functional role of the arginine 402 in the activity of this variant, even if the amount of secreted protein was very low, according to data from the natural variant, indicating that the carboxyl-terminal region of FVII is essential for its normal secretion, as for other coagulation factors such as FIX and PC [2,3].

To study the contribution of the R402 residue to biosynthesis and function, the arginine residue at this position was substituted with an alanine (r402A-FVII), or replaced by an alanine residue in a construct bearing a premature stop codon at position 403 (r402A/403X-FVII). These recombinant FVII variants were transiently expressed in BHK cells and antigen and activity levels were evaluated in conditioned media (Fig. 3.3.4).

Considering the biosynthesis and thus the amount of protein secreted in the conditioned media, our results showed that the deletion of the carboxy-terminus has more detrimental effects than substitution of the 402 residue. This suggestion is given by the different expression of the recombinant variants observed after transient expression in BHK cells. Indeed, the r402A-FVII variant showed antigen levels comparable with the 50% of the rWt protein, while the double mutant r402A/403X-FVII, bearing the same arginine to alanine substitution but truncated in the 402 position, was barely detectable. The different outcome

for substitution and deletion was observed also for the activity. Substitution of the Arg402 in the full-length protein (r402A-FVII) led to a FXa generation activity in FVII-deficient plasma corresponding to the 50% of rWt, while for r402A/403X-FVII the activity was not detectable.

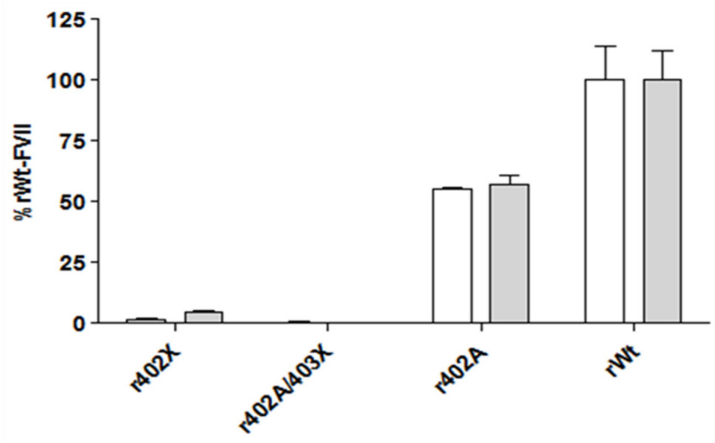

Figure 3.3.4. Transient expression of r402X, r402A/403X, r402A and rWt-FVII in BHK cells. The antigen and activity levels of recombinant variants are expressed in the y-axis as percentage of rWt-FVII ± standard deviation. White columns: antigen levels; grey columns: FXa generation activity in FVII-deficient plasma.

Together, these results indicated that the r402A-FVII protein showed a specific activity similar to that of the Wt protein, while a detectable activity for the double mutant r402A/403X-FVII was absent, and, furthermore, these experiments confirmed the higher specific activity of the r402X-FVII variant, in this case used as control for both secretion and protein assays.

DISCUSSION

FVII deficiency (OMIM 227500) is a rare haemorrhagic disease, but the most common among the coagulation disorders, with an autosomal recessive inheritance pattern and an incidence of 1 affected individual in 500.000 in the general. This defect is associated with a variable bleeding tendency [4], and clinical phenotypes range from mild to severe, even if cases associated with asymptomatic phenotypes have been found [5], even in the homozygous condition. The large heterogeneity of the mutational spectrum observed for FVII deficiency provides complexity to the genotype–phenotype relationship. For instance, mutations that interrupt protein production (deletions, insertions, splicing mutations, stop codons), together with missense mutations responsible for low or very low activity levels have been found to be associated with severe clinical phenotypes.

While a large number of mutations in FVII gene, and associated to FVII deficiency, has been described [6-8] only a limited number of them has been functionally characterized, thus preventing the elucidation of the relationship between molecular lesions and coagulation and clinical phenotype. The severe cases are either homozygous or double heterozygous for mutations that disrupt appropriate expression and cause FVIIc levels less than 2% of normal. Cases of FVII deficiency with mild or moderate clinical phenotype, for which the measured in vitro FVIIc levels can range from <1% to 52%, are either homozygous or double heterozygous. and in this group the majority of mutations is represented by missense changes. The asymptomatic cases have FVIIc ranging from 4% to 61% of normal and almost all mutations found are missense [6].

As for other coagulation factor deficiencies [9,10], the mutational spectrum of FVII deficiency is mainly represented by missense changes, and the majority of substitutions associated to symptomatic phenotypes is responsible for a parallel reduction of antigen and activity. Therefore, the classification of FVII deficiency is based on antigen and activity levels. Indeed, FVII deficiency can be classified as type 1 or type 2 on the basis of the absence (cross-reacting material negative, CRM^-) or the presence (CRM^+) of a disparity between antigen and activity levels, respectively. Typically, in type I deficiency both functional activity and antigen levels are decreased, which is a characteristic hallmark of defective secretion or reduced stability of the protein. In type II deficiency, low coagulant activity contrasts with normal or low borderline antigen levels, indicating the presence of a dysfunctional protein.

Considering the total number of causative mutations in FVII deficiency, the most frequent alteration is represented by missense mutations (about 70%), followed by other alterations as deletions, splice site mutations, insertions and mutations in the promoter region, while nonsense mutations range from few cases to about 7% of all identified mutations when only homozygous or also heterozygous and double heterozygous mutations are considered [6,11]. A study by Mort *et al* [12] indicated that nonsense mutations account for approximately 11% of all described gene lesions causing human inherited diseases and approximately 20% of disease-associated single-base pair substitutions affecting gene coding regions. In addition, the most frequent substitution responsible for disease-associated stop codons in human genome is the CGA→TGA change (21% of total), while C→T transitions account for the majority (46%) of all nonsense mutations. Overall, together with the CAG (Gln)-TAG transition, the CGA (coding for arginine) change to TGA is one of the most frequent nonsense mutation reported in the Human Gene Mutation Database (HGMD). The high proportion of C-T transitions that result in a TGA stop codon (24% of all nonsense mutations) is explicable in terms of the methylation-mediated deamination of 5-methylcytosine (5mC) within the CpG dinucleotides [13].

The fatal phenotype resulting from the complete absence of FVII was demonstrated in a mouse model knock-out for this coagulation factor [14]. Thus, even if nonsense mutations responsible for FVII deficiency are mainly associated with undetectable levels of both antigen and activity levels of circulating FVII, it must be assumed that a residual function is present.

Consistent with the dramatic detrimental effect of nonsense mutations, this mutation type has been described in homozygous FVII deficient patients in only few cases (Table D1), while a slightly greater number of mutations was found in the heterozygous or double heterozygous condition [15-23].

In the FVII gene sequence these homozygous nonsense changes are localized at codon 52 (Ser52X, [24]), 72 (Cys72X, [25]), 227 (Gln227X, [26]), 316 (Lys316X, [23]) and 382 (Gln382X, [26]), corresponding to the first EGF-like (Ser52X and Cys72X) and the protease (Gln227X, Lys316X and Gln382X) domains. With the exception of the Cys72X, in patients carrying these mutations very low levels of FVII (below 1% or barely detectable) were observed, and clinical phenotypes were classified as severe due to symptoms such as umbilical stump bleeding, hemarthroses and central nervous system haemorrage. The Cys72X mutation was diagnosed as a moderate bleeding tendency since

recurrent epistaxis, mouth bleeding and spontaneous hematoma after minor trauma were found, even if both FVII antigen and activity were very low (≤1%).

Mutation	Nucleotide change	Exon	Protein Domain	Genetic condition	Reference
Ser52X	TCA → TGA	Exon 4	EGF 1	Homozygous (Severe)	[22]
Cys72X	TGC → TGA	Exon 4	EGF 1	Homozygous (Moderate)	[23]
Gln227X	CAG → TAG	Exon 8	Catalytic	Homozygous (Severe)	[24]
Lys316X	AAG → TAG	Exon 8	Catalytic	Homozygous (Severe)	[21]
Gln382X	CAG → TAG	Exon 8	Catalytic	Homozygous (Severe)	[24]

Table D1. Nonsense mutations in FVII gene carried in the homozygous condition. The nucleotide substitution is underlined. The clinical phenotype associated with each nonsense mutation is indicated in parentheses.

The mutational pattern observed for nonsense mutations in the FVII gene and the resulting phenotypes indicate for homozygous patients a severe bleeding tendency probably due to the presence of truncated proteins with severely compromised coagulant activity or protein products more unstable than the normal full-length FVII. However, in spite of their great interest, the molecular mechanisms underlying the presence of residual FVII activity in plasma of these patients has not been investigated. One of the processes that might account for traces of functional FVII molecules is the occurrence of the ribosome readthrough at premature nonsense triplets, a phenomenon documented through reporter gene assays by Manuvakhova *et al* [27].

In our study, for the first time, we describe a nonsense mutation that in homozygous condition is associated to well appreciable FVII coagulant levels (3-5%) and thus to a completely asymptomatic clinical phenotype. The nonsense mutation is localized at position 11121 in the terminal tract of exon 8, where in a CpG site at codon 402 the CGA, normally coding for an arginine, to TGA change was found to be responsible for the R402X nonsense mutation. Patients with the same mutation, either in the heterozygote [28] or in the double heterozygote [29] condition have been previously reported. In the double heterozygote patient, the 402X mutation, together with the missense mutation Thr359Met, was found to be responsible for very low antigen levels (below 1%), a prolonged prothrombin time and a reduced FVII activity (less than 3% of normal). Contrarily, the

patient heterozygous for the 402X nonsense mutations showed a slightly prolonged prothrombin time and a FVIIc of 24%, thus indicating a mild phenotype.

The association between the homozygous 402X nonsense mutation and FVIIc levels typical of asymptomatic or mild-to-moderate phenotypes led us to investigate FVII levels in the patient's plasma. Intriguingly, ELISA assays showed very low FVII levels (below 1%) despite of the activity measured by routine laboratory tests. This discrepancy led us to hypothesize the presence in patient's plasma of a truncated FVII variant with increased specific activity. To corroborate these preliminary results and to measure the activity of the 402X variant using a more sensitive method, fluorogenic FXa generation assays were performed in patient's plasma. FXa generation activity measured for the homozygous 402X showed a 2.5-fold increase in activity when compared to a pooled normal plasma (PNP).

To study the contribution to the coagulation process of the 402X variant in a more complex system in which also pro- and anticoagulant forces are taken into account, thrombin generation assays were carried out. The difference between low antigen levels and activity were observed also in these experiments, in which the 402X in patient's plasma showed a thrombin generation activity 2-fold higher than that of equal amounts of PNP. In addition, with the exception of the 100% of PNP, the area under the curve indicating the endogenous thrombin potential (ETP) measured for 402X was found to be higher than that of equivalent or up to 5-fold greater PNP concentrations.

These findings pointed toward the presence of a protein with increased activity. Indeed, these experiments in patient's plasma permitted us to evaluate the specific activity of the 402X-FVII variant, which was found to be 2.5-fold higher than normal.

The asymptomatic phenotype of the patient homozygous for the 402X nonsense mutation should be due to the increased specific activity of the mutant protein, but this observation might be explained also by the presence of altered levels of other procoagulant factors as FIX and FX. For the purpose of excluding external contributions to the augmented activity observed for the 402X FVII, FIX and FX in patient's plasma were quantified by ELISA, but only physiological oscillations in antigen levels were found. Furthermore, an additional explanation for the augmented catalytic capacity could be given by the higher affinity of the 402X variant for a substrate different from FX, thus the higher capacity of activating FIX might be responsible for the discrepancy between the activity and the very low amounts of circulating protein. For this purpose, in order to avoid the contribution of

FVIIa/TF-mediated conversion of FIX to FIXa [30] in the FX activation pathway leading to the formation of thrombin, optimized fluorogenic FIIa generation assays were carried out in the presence of a commercially available anti-FIX antibody. The measurement of FIIa generation activity in the absence and in the presence of the anti-FIX antibody showed that the higher affinity for FIX rather than for FX was not responsible for the augmented activity previously observed in patient's plasma.

To further investigate this mutation and to corroborate our findings in patient's plasma, the recombinant 402X variant was expressed in transiently transfected BHK21 cells, known to correctly process FVII [31]. The r402X-FVII variant was secreted in conditioned medium at very low levels (below 1% of rWt-FVII), thus reflecting antigen levels observed for the natural 402X variant. The very low levels of the poorly secreted recombinant 402X variant did not permit us to reliably evaluate the residual activity. Thus, to better evaluate FVII protein and activity levels and to obtain a more reliable estimate of the specific activity, the r402X-FVII protein in conditioned medium was approximately 10-fold concentrated. By this way, antigen and activity assays were estimated by ELISA and FXa generation assays in FVII-deficient plasma. As for the R402X variant in plasma, the recombinant variant showed a specific activity higher than that of the rWt-FVII, confirming our findings in vivo and indicating that the increased activity might be due to intrinsic features of the truncated protein.

The interaction leading to the assembly of the FVIIa/TF/FX ternary complex have been extensively investigated, even if a clear picture of the complex molecular basis of these interactions has not been completely established. A great number of works have indicated the regions and aminoacids involved in protein-protein interactions between FVII, TF and FX. Protein structures as mainly the GLA domain and the two EGF-like domains, and partly the serine protease domain, have been clearly identified as the protein regions involved in the formation of the FVIIa/TF binary complex [32-38]. Based on available crystal structures for FVIIa complexed with TF (PDB code 1DAN, from [36]) a linear surface-exposed structure for the carboxy-terminus of FVII has been predicted. Being the stoichometric 1:1 FVIIa-TF interaction identified at the above mentioned protein domains, the surface exposed carboxyl-terminal region of FVII might be potentially involved in the interaction with the substrate FX in the FVIIa/TF/FX ternary complex during the FX activation pathway. Together with the involvement of GLA and EGF domains in protein-protein interactions observed for the FVIIa/TF complex, a role for exosites in determining

affinity and binding specificity to FX in the Xase complex have been proposed [39], as observed for other macromolecular complexes in which exosite-mediated interactions are involved [40,41]. Thus, a model for FX activation by the 402X-FVII variant in which the carboxyl-terminal region participate in exosite-like interactions might be hypothesized. Noticeably, a FVII with a single aminoacid change conferring an augmented capacity to activate FX has been reported [42] and, in addition, also a natural FIX variant (FIX Padua) showing a specific activity higher than that of the normal FIX protein has been described [43]. Interestingly, a wide alanine scanning study conducted on FVII residues thought to be involved in FX interaction, indicated that some alanine-substituted residues showed a diminished or enhanced rate of FXa formation depending on the residue involved [36]. In particular, the residue L400 in the heavy chain of FVII showed a high rate of conversion of FX to FXa and a low dissociation constant, and thus a high affinity, for phospholipid reconstituted TF was observed. This finding theoretically points toward a not essential participation of FVII terminal residues in FX activation but suggests that a little or wider modification of this region could be account for variations in the capacity of activating FX by FVII in the FVIIa/TF complex. Intriguingly, this aminoacid in the linear FVII primary protein sequence is located only one residue before the L401, which is the last surface-exposed aminoacid in the 402X FVII natural mutant. This observation might suggest a possible involvement of this residue in exosite-like interactions during the activation of FX, thus in the conversion of the zymogen form of FX to the active protease FXa, therefore being responsible for the observed 2.5-fold increase in specific activity observed for the 402X mutant.

Although extremely intriguing, the very low expression levels of the 402X-FVII variant did not enable us to proceed with a more precise biochemical characterization of the molecule and in particular its activation, activity toward substrates FIX and FX or its interaction with its cofactor, Tissue Factor.

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PART II

The carboxyl-terminal region of coagulation factor VII as a candidate epitope for an inhibitory antibody developed in a FVII-deficient patient

This study was aimed at characterizing the feature of an inhibitory anti-FVII antibody developed in a FVII deficient patient upon infusion with exogenous FVII. Since the patient is homozygous for the A294V-11125delC, leading to significant alteration of the FVII carboxyl-terminal region, we explored the hypothesis that this region was one of the major epitopes recognized by the antibody.

The study involved either experiments in patient's plasma or with recombinant FVII variants in which the carboxyl-terminal region was progressively deleted by premature stop codons.

3.4 The A294V-11125delC mutation

The previously characterized A294V-11125delC mutation in coagulation FVII is associated to very low residual levels and variable phenotypes ranging from asymptomatic to severe forms of FVII deficiency, due to reduced antigen and activity both in vitro and in vivo.

The prolonged treatment of the patient under study with plasma-derived FVII or NovoSeven rFVIIa induced the development of an anti-FVII inhibitory antibody. This FVII variant is characterized by a missense mutation in the catalytic domain of FVII and by a cytosine deletion at position 11125 responsible for a frameshift at the carboxyl-terminal codon 404. This frame shift predicts the formation of a 28 residues longer carboxy-terminus due to a stop codon which localization was predicted to be downstream from the natural TAG triplette (Fig. 3.4.1). Thus, in the patient's plasma homozygous for the A294V-11125delC mutation a normal mature FVII molecule is absent and consequently the treatment with rFVIIa should expose to a recombinant factor different, mainly at the carboxyl-terminal region, from that of the patient, thus indicating this region as a potential candidate epitope for the inhibitory antibody developed in the patient's plasma.

Analysis of two independent collections of patient's plasma for the anti-FVII antibody content provided an inhibitor titre of 12.3 and 7.2 Bethesda Units ml^{-1} (BU ml^{-1}). As

definition, in hemophilia, frequently associated to development of inhibitory antibodies, 1 BU/ml represents the amount of inhibitor that neutralizes 0.5 U/ml of coagulation factor.

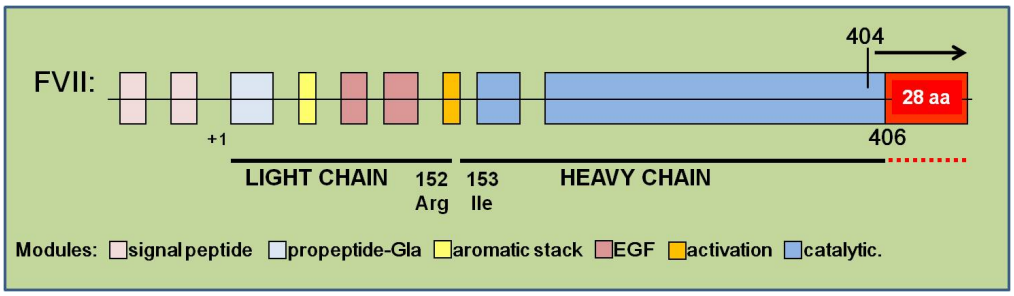

Figure 3.4.1. Schematic representation of theA294V-11125delC FVII variant.
At the end of the normal full-length FVII (ending with the 406 residue) the frame shift caused by the cytosine deletion is predicted to add a 28-aminoacid tail (red rectangle) to the terminal tract of FVII starting from the residue 404 (black arrow). Domain organization of the protein is indicated as differently colored modules. Light and heavy chains are indicated as black lines; Arg152 and Arg153 in the activation peptide are also shown. The +1 position in the amino-terminus of the protein indicates the first aminoacid of FVII protein after the signal peptide removal.

3.5 Binding of inhibitory antibody to rFVIIa and zymogen FVII

Activity assays aimed at determining the inhibition of rFVIIa and FVII from pooled normal plasma (PNP) were performed. Incubations at 2 and 4 hours of either rFVIIa or PNP dilutions with patient's plasma provided the evidence of a dose-dependent inhibition of both rFVIIa and PNP, confirming the presence of an anti-FVII antibody that recognized and inhibited FVII (data now shown).

This finding pointed toward the presence in patient's plasma of a molecule capable to interact with FVII causing its inhibition and thus its incapability to activate its physiological substrate FX, even if it is not clear whether the molecule recognized by the inhibitory antibody was represented by the activated or zymogen form of FVII or both.

First, we investigated by western blotting the capacity of anti-FVII antibody developed in patient's plasma to recognize rFVIIa both in reducing and non-reducing conditions. Moreover, considering the high homology of FVII to other coagulation factor such as FX, plasma-derived FXa was used as negative control to test the specificity of antibody for FVII (Fig. 3.5.1).

Western blotting analysis showed that the anti-FVII antibody was capable to recognize in a concentration-dependent manner the non-reduced double-chain form of rFVIIa (Fig. 3.5.1, lane 2 and 4), where a disulphide bridge links FVII heavy and light chains, while the heavy

chain derived from reduced rFVIIa either at 10 or 20 ng of concentration was not recognized (Fig. 3.5.1, lane 1 and 3). Furthermore, as plasma-derived FXa was not recognized by the antibody (Fig. 3.5.1, lane 5), this result provided the evidence of a specific interaction with rFVIIa even in the presence of the activated form of a highly homologous factor. This finding potentially indicated the involvement of antibody in the inhibition of the activated form of FVII and not of other coagulation factors albeit in a complex network such as coagulation.

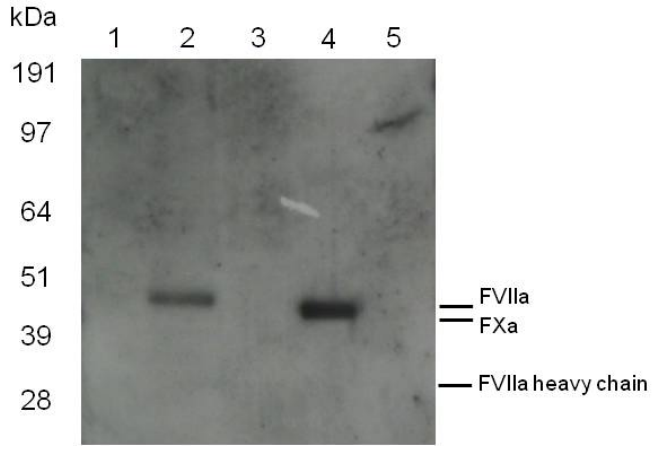

Figure 3.5.1. Western blotting for detection of anti-FVII inhibitory antibody against rFVIIa. Lane 1: reduced rFVIIa (10 ng); lane 2: rFVIIa (10 ng); lane 3: reduced rFVIIa (20 ng); lane 4: rFVIIa (20 ng); lane 5: plasma-derived FXa (20 ng) used as negative control. On the left are indicated molecular weights (kDa) and on the right the corresponding migrations of FXa (46 kDa), FVIIa (50 kDa) and FVIIa heavy chain (30 kDa) are referred.

Results from western blotting experiments helped to shed light on the ability of plasma anti-FVII inhibitory antibody to discriminate the rFVIIa molecule from a highly homologous activated factor and to preferentially recognize the non-reduced protein. To analyze the response of anti-FVII antibody to different concentrations of rFVIIa, an ELISA-based assay with coated rFVIIa was performed. These experiments showed for the antibody-rFVIIa interaction a dose-dependent response to different concentrations of either rFVIIa or anti-FVII antibody (Fig. 3.5.2). Concentrations of coated rFVIIa between 1 and 8 ng/ml indicated that only curves for 1 and 8 ng/ml showed differences in the rate of binding, while slight differences for 2 and 4 ng/ml were observed. In addition, none of the tested concentrations were saturated by an anti-FVII antibody titre ranging from 0,005 to 0,04 BU/ml (Fig. 3.5.2, a). On the contrary, after having increased the concentrations of both rFVIIa and anti-FVII antibody a better dose-response was observed (Fig. 3.5.2, b). In

this case, antibody concentrations between 0,005 and 0,08 BU/ml were able to bind in a concentration-dependent manner a coated rFVIIa at 25 and 50 ng/ml. Moreover, in these conditions significant differences between the concentrations of rFVIIa were observed and a binding trend near to saturation was reached.

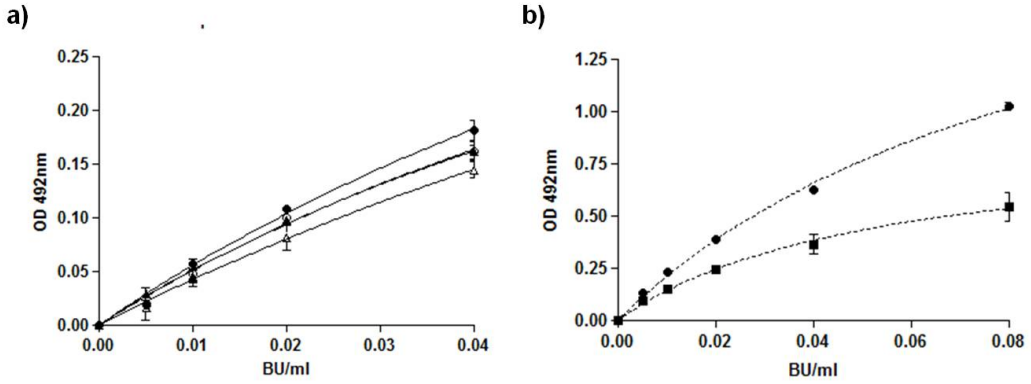

Figure 3.5.2. Binding of the anti-FVII antibody in plasma to rFVIIa. Coated rFVIIa was used to measure the interaction between rFVIIa and anti-FVII antibody by ELISA assays. a) filled circles: 8 ng/ml; empty circles: 4 ng/ml; filled triangles: 2 ng/ml; empty triangles: 1 ng/ml. b) filled circles: 50 ng/ml; filled squares: 25 ng/ml. In the x-axis is reported the concentration of plasma anti-FVII antibody expressed as Bethesda Units (BU)/ml and in the Y-axis is indicated the rate of binding expressed as OD.

Previous experiments indicated the specific recognition and the dose-dependent interaction between rFVIIa and plasma anti-FVII antibody, but there was the possibility that the antibody might recognize the zymogen form of FVII.

To investigate the capability of the plasma anti-FVII antibody to identify zymogen FVII and to determine whether rFVIIa and FVII are differently recognized by the antibody, ELISA-based assays with either rFVIIa or plasma-derived zymogen FVII were performed (Fig. 3.5.3). As previously observed for low concentrations of rFVIIa, also in this case 2 and 4 ng/ml of both rFVIIa and zymogen FVII did not show a dose-dependent recognition by the antibody and, as for rFVIIa alone, a saturation condition was not reached in a range of anti-FVII antibody from 0,005 to 0,04 BU/ml (Fig. 3.5.3, a). Results observed for low concentrations of rFVIIa e FVII, identical to that of rFVIIa alone, led us to explore conditions in which higher concentrations of coated proteins were tested. Interestingly, experiments with amounts of coated proteins increased to achieve a concentration of 100 ng/ml for both rFVIIa and zymogen FVII showed a similar binding capacity of anti-FVII antibody to either rFVIIa or FVII (Fig. 3.5.3, b). Noticeably, for points relative to low concentrations of anti-FVII antibody (under 0,04 BU/ml) also the 100 ng/ml concentration

assayed showed identical binding capacity of the antibody to both rFVIIa and zymogen FVII, as previously observed for 2 and 4 ng/ml of coated proteins.

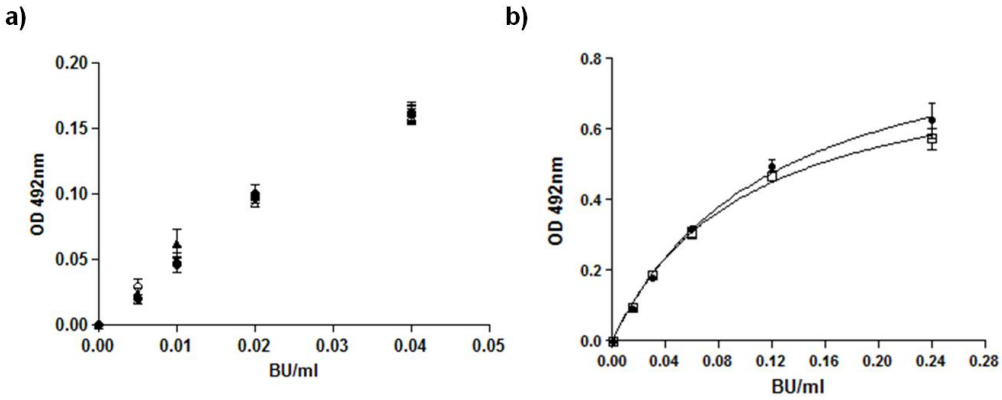

Figure 3.5.3. Binding of plasma anti-FVII antibody to rFVIIa and plasma-derived zymogen FVII. Coated rFVIIa and FVII were used to measure the interaction between rFVIIa and FVII with anti-FVII antibody by ELISA assays. a) filled circles: 4 ng/ml rFVIIa; empty circles: 2 ng/ml rFVIIa; filled triangles: 4 ng/m FVII; empty triangles: 2 ng/ml FVII. b) filled circles: 100 ng/m rFVIIa; empty squares: 100 ng/ml FVII. In the x-axis is reported the concentration of plasma anti-FVII antibody expressed as Bethesda Units (BU)/ml and in the Y-axis is indicated the rate of binding expressed as OD.

Moreover, since a similar recognition features for both rFVIIa and zymogen FVII was observed, quantification of the anti-FVII IgG in patient's plasma by ELISA assays with both rFVIIa and plasma-derived zymogen FVII was assessed.

Results from experiments on the 12.3 BU/ml plasma sample conducted with coated rFVIIa indicated an anti-FVII IgG content of 412.8±27.6 ng/ml, while experiments with coated zymogen FVII indicated a concentration of 403.3±30.5 ng/ml. Altogether these results revealed a similar recognition of rFVIIa and plasma-derived FVII by the anti-FVII inhibitory antibody, and showed a mean plasma concentration of 408.0±6.7 ng/ml. For the plasma sample with a 7.2 BU/ml antibody titre the concentration of anti-FVII IgG was 216.5±73.9 ng/ml (Table 3.5.1).

	12.3 BU/ml	**7.2 BU/ml**
Anti-FVII IgG (ng/ml)	408.0±6.7	216.5±73.9

Table. 3.5.1. Concentration of two independent collections of patient's plasma having an inhibitory antibody titre of 12.3 and 7.2 BU/ml. Concentration is expressed as mean ± standard deviation.

3.6 Expression of recombinant proteins

Data obtained from western blotting and ELISA-based assays shed light about the interaction of both rFVIIa and plasma-derived zymogen FVII with the anti-FVII inhibitory antibody found in the patient homozygote for the A294V-11125delC mutation.

To investigate the involvement of FVII carboxyl-terminal region as the candidate epitope for the plasma anti-FVII antibody a deletion scanning of this terminal region was performed. For this issue a panel of progressively truncated mutants was created and evaluated both at secretion and activity levels.

By site-directed mutagenesis with specific primers carrying termination codons, the wild-type carboxy-terminus, composed of 406 aminoacids in the mature protein, was shortened in order to obtain the recombinant variants r406X, r405X, r404X, r403X and ultimately, as control, the r402X variant, being the last a mutated protein known to be secreted at very low levels (see Part I). A representation of truncated proteins produced by mutagenesis is depicted on Fig. 3.6.1.

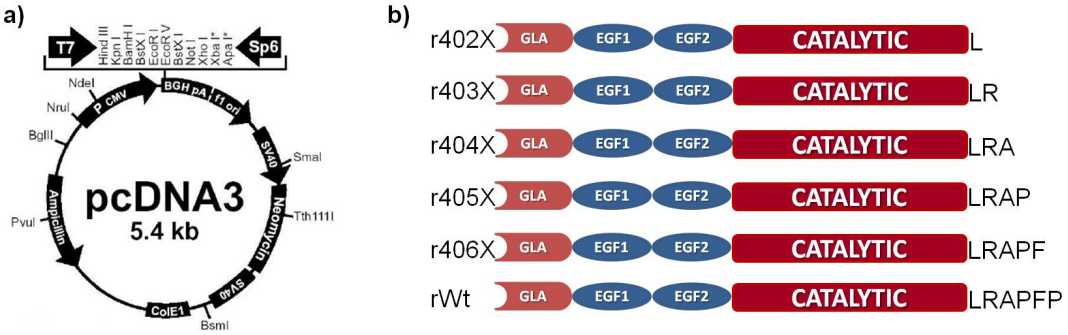

Figure 3.6.1. Recombinant variants produced by progressive deletion of FVII carboxy-terminus. For each mutant the natural stop codon TAG has not been removed or changed. Recombinant Wt, 406X, 405X, 404X, 403X and 402X variants are 406, 405, 404, 403, 402 and 401 aminoacid-long, respectively. a) pcDNA3 plasmid in which FVII cDNA was cloned; b) panel of progressively deleted variants produced.

Eukaryotic BHK cells were transiently transfected to evaluate the secretion of these variants in conditioned medium. After a 72-hour after transfection, conditioned media were collected and the FVII content was quantified by ELISA, which showed an inverse relationship between secretion levels and the extent of deletion in the carboxyl-terminal region (Fig. 3.6.2, a). In other words, the shortening of the carboxyl-terminal region of FVII revealed a detrimental effect on secretion. Protein levels in media were 57.8±5.1%, 16.6±4.5%, and 11.9±3,7% for r406X-FVII, r405X-FVII, r404X-FVII, respectively, while

r403X-FVII and r402X-FVII variants were found to be expressed at very low levels (less than 1% of rWt). This result indicated that the carboxyl-terminal region of FVII is importantly involved in the biosynthesis/secretion process of the protein.

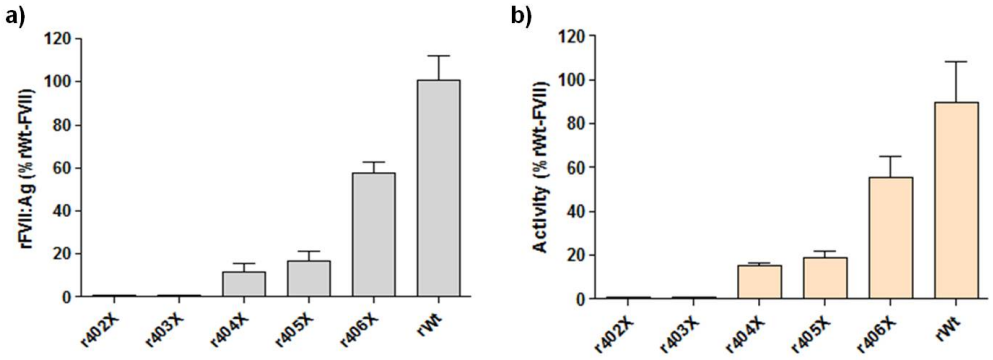

Figure 3.6.2. Expression levels (a) and activity (b) of FVII variants in conditioned media from transiently transfected BHK cells. Antigen and activity levels are reported as mean percentage ± standard deviation obtained from three independent experiments.

To determine the residual activity of these mutants in conditioned media, and thus the effects of deletions in the carboxy-terminus, a fluorogenic FXa generation assay in FVII-deficient plasma was performed. As observed for expression levels of these variants in transient transfections, FVII residual activity measured in FXa generation was in inverse relationship with the extent of the deletion (Fig. 3.6.2, b) The activity levels observed in FXa generation assays for the well-detectable variants r406X-FVII, r405X-FVII and r404X-FVII were 55.3±10.1%, 18.9±2.8% and 15.2±1,1%, respectively, while the activity observed for r403X and r402X was under 1% of rWt and thus barely detectable. The same results were observed in FXa generation assays carried out in the reconstituted system (data not shown).

3.7 Specific activity of recombinant variants

The specific activity of the best-secreted recombinant variants was evaluated in FXa generation assays conducted both in the reconstituted system and in FVII-deficient plasma (Fig. 3.7.1, a and b). Activity was measured only for those variants which showed well-detectable protein levels without the need of concentration protocols. For r403X activity measured after concentration of conditioned media showed a specific activity similar to that of the rWt protein, while for concentrated r402X-FVII a 2.5-fold increase in specific activity was observed (see Part I).

Data from assays carried out in the reconstituted system showed for the rWt-FVII a specific activity of 16054.4±4279.8 Rfu/min/nM FVII, while for r406X-FVII, r405X-FVII

and r404X-FVII the specific activities observed were 15676.1±3547.6 Rfu/min/nM FVII, 15369.6±2803.1 Rfu/min/nM FVII and 15464.1±2901.6 Rfu/min/nM FVII, respectively (Fig. 3.7.1, a). Considering the FVII-deficient plasma system the specific activity observed for rWt was 1043.4±138.7 Rfu/min/nM FVII, and for r406X-FVII, r405X-FVII and r404X-FVII the specific activity measured was 983.8±181.8 Rfu/min/nM FVII, 953.0±212.5 Rfu/min/nM FVII and 967.8±264.3 Rfu/min/nM FVII, respectively (Fig. 3.7.1, b).

Our results from activity assays indicated that these truncated FVII variants have a specific activity similar to that of the recombinant wild-type FVII in both reconstituted and FVII-deficient plasma systems (Fig. 3.7.1, c).

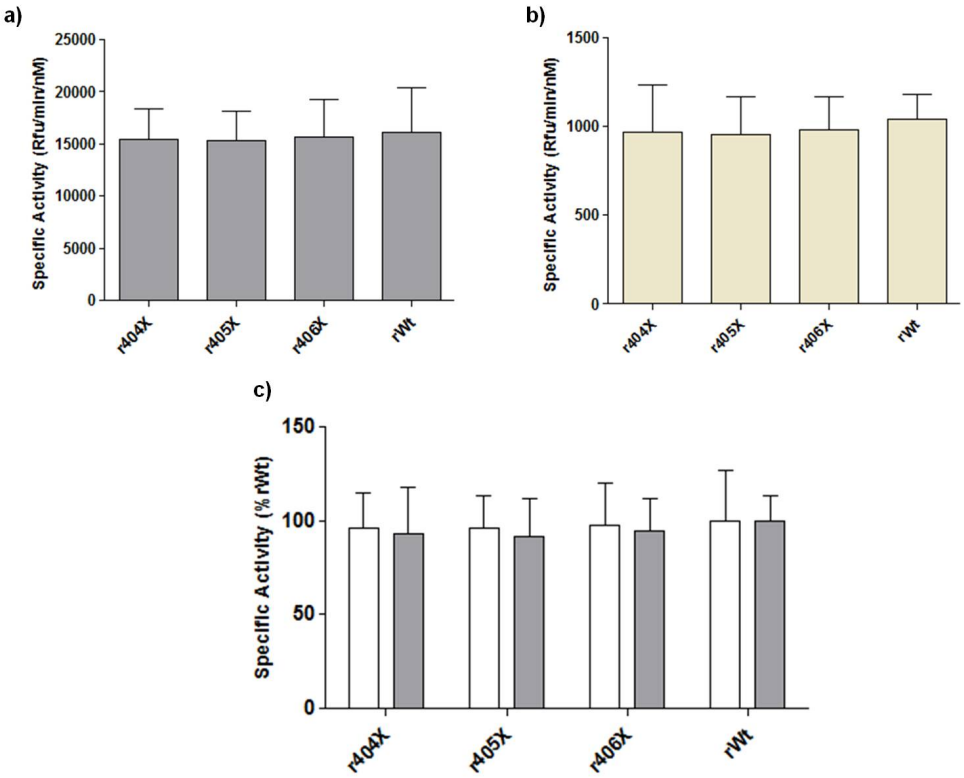

Figure 3.7.1. Specific activity of recombinant FVII variants r404X, r405X, r406X compared with rWt.
a) Specific activity in FXa generation assays in reconstituted system. b) Specific activity in FXa generation assay in FVII-deficient plasma. In a) and b) specific activity is expressed a mean ± standard deviation of Relative Fluorescence Units (Rfu)/min/nM FVII. c) Comparison between specific activities from FXa generation in reconstituted system (white) and in FVII-deficient plasma (grey); specific activity is expressed as mean percentage ± standard deviation. At least three independent experiments were performed for each activity assay.

3.8 Activity assays with anti-FVII inhibitory antibody

Interaction studies conducted on both rFVIIa and plasma-derived zymogen FVII indicated that the binding of anti-FVII antibody to these proteins was very similar. For this reason,

once obtained the recombinant FVII variants r406X-FVII, r405X-FVII and r404X-FVII, we investigated the activity of these progressively truncated proteins in the presence of the antibody.

Activity assays were performed in order to determine the potential involvement of the carboxyl-terminal region as a candidate epitope for the anti-FVII antibody. Recombinant wild-type FVII, as rFVIIa and plasma-derived zymogen FVII, differs from the r406X-FVII, r405X-FVII and r404X-FVII for 1, 2 and 3 aminoacids, respectively.

Residual activity of rWt-FVII and recombinant truncated variants was assessed in Bethesda-based FXa generation assays carried out in FVII-deficient plasma either in the absence or in the presence of patient's plasma containing the anti-FVII inhibitory antibody. The specific activity was calculated and expressed as percentage of residual activity considering for each variant the specific activity in the absence of antibody as 100%.

Activity assays conducted on r406X-FVII and rWt-FVII (normalized for the antigen of the r406X variant) showed at 0.16, 0.08 and 0.01 BU/ml a similar rate of inhibition by the anti-FVII antibody (Fig. 3.8.1) even if at antibody concentrations of 0.04 and 0.02 BU/ml a slightly less inhibition of rWt was appreciated.

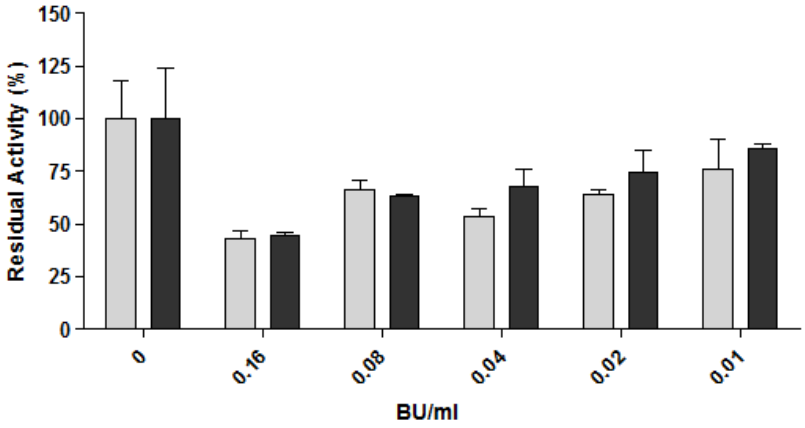

Figure 3.8.1. Residual activity of recombinant FVII variants r406X and rWt from conditioned media in Bethesda-based FXa generation assays carried out in the FVII-deficient plasma system. Recombinant r406X-FVII (grey) and rWt (black) normalized for antigen were incubated at 37°C for 2 hours in FVII-deficient plasma with or without serial dilutions (0.16, 0.08, 0.04, 0.02 and 0.01 BU/ml) of plasma anti-FVII inhibitory antibody. Residual activity is expressed on the y-axis as mean percentage ± standard deviation of specific activity. Activity of each protein in the presence of the inhibitory antibody was calculated and normalized considering the activity in the absence of the antibody as 100%.

This result indicates that the shortening of only one aminoacid in the carboxyl-terminal region of FVII had no effects on inhibition rate exerted by the anti-FVII antibody.

Data obtained from activity measured in experiments carried out on r405X-FVII and rWt-FVII (normalized for the antigen of the r405X-FVII variant) indicated that r405X exhibited an inhibition rate different from that of the rWt-FVII (Fig. 3.8.2). Indeed, at antibody concentrations ranging from 0.04 to 0.16 BU/ml the activity measured for the rWt protein was more affected by the antibody when compared to that of the r405X-FVII variant, whereas at lower concentrations (0.01 and 0.02 BU/ml) a similar effect on residual activity was observed. This observation could be accounted for the little difference (2 aminoacids) between rWt and r405X-FVII carboxyl-terminal regions and represented a first hint to the involvement of the carboxy-terminus of FVII as a potential epitope for the plasma anti-FVII antibody.

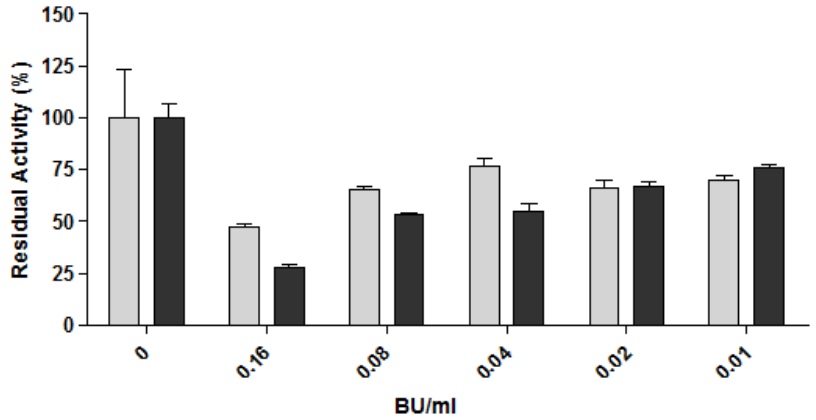

Figure 3.8.2. Residual activity of recombinant FVII variants r405X and rWt from conditioned media in Bethesda-based FXa generation assays carried out in the FVII-deficient plasma system. Recombinant r405X-FVII (grey) and rWt (black) normalized for antigen were incubated at 37°C for 2 hours in FVII-deficient plasma with or without serial dilutions (0.16, 0.08, 0.04, 0.02 and 0.01 BU/ml) of plasma anti-FVII inhibitory antibody. Residual activity is expressed on the y-axis as mean percentage ± standard deviation of specific activity. Activity of each protein in the presence of the inhibitory antibody was calculated and normalized considering the activity in the absence of the antibody as 100%.

Experiments conducted on r404X-FVII and rWt-FVII (normalized for the antigen of the r404X variant) showed a significant difference in the inhibition rate of the two proteins (Fig. 3.8.3). Differently from r405X-FVII for the r404X-FVII truncated variant a residual activity higher than that of the rWt was appreciated at every tested concentrations (from 0.01 to 0.16 BU/ml) of anti-FVII antibody. Especially at 0.16 BU/ml the r404X variants exhibited the most significant difference in residual activity, as also observed for the r405X-fvii protein.

This result pointed really towards a consistent involvement of the carboxyl-terminal region of FVII as a candidate site contributing to the formation of the epitope recognized by the antibody found in the patient's plasma homozygous for the A294V-11125delC mutation.

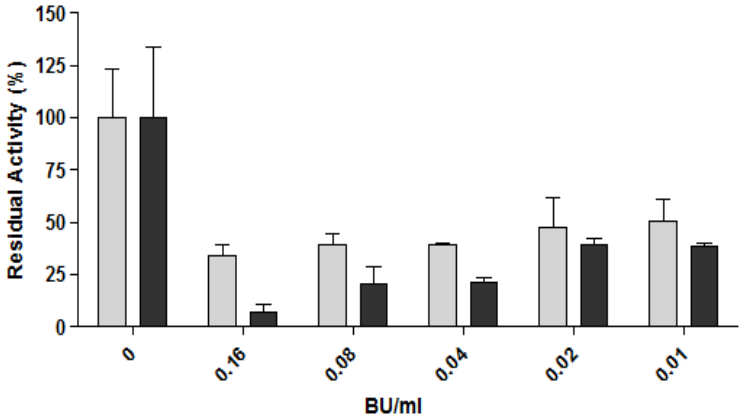

Figure 3.8.3. Residual activity of recombinant FVII variants r404X and rWt from conditioned media in Bethesda-based FXa generation assays carried out in the FVII-deficient plasma system. Recombinant r404X-FVII (grey) and rWt (black) normalized for antigen were incubated at 37°C for 2 hours in FVII-deficient plasma with or without serial dilutions (0.16, 0.08, 0.04, 0.02 and 0.01 BU/ml) of plasma anti-FVII inhibitory antibody. Residual activity is expressed on the y-axis as mean percentage ± standard deviation of specific activity. Activity of each protein in the presence of inhibitory antibody was calculated and normalized considering the activity in the absence of antibody as 100%.

Our findings pointed toward an implication of the carboxy-terminus of FVII as main epitope for the anti-FVII antibody.

Significant differences between residual activity of r405X-FVII and r404X-FVII, but not for r406X-FVII, and rWt-FVII protein were appreciated, indicating that the shortening of the carboxyl-terminal region of FVII led to a rate of inhibition that is inversely proportional to the extent of the deletion, thus implying that the shortest but well-detectable variant was less recognized by the antibody. Moreover, we observed a progressive shift in the antibody concentration able to inhibit the recombinant variants tested. Therefore, activity observed in independent experiments in which residual function of each truncated variant was compared to that of the rWt-FVII at different concentrations of anti-FVII antibody might suffer from the differences in protein concentration used in each assay.

For this reason, and to corroborate these results, recombinant variants and wild-type FVII were normalized for the antigen of the least secreted truncated protein (r404X) and were

assayed in the presence of the antibody concentrations (0.16 and 0.08 BU/ml) at which the most significant differences were observed (Fig. 3.8.4).

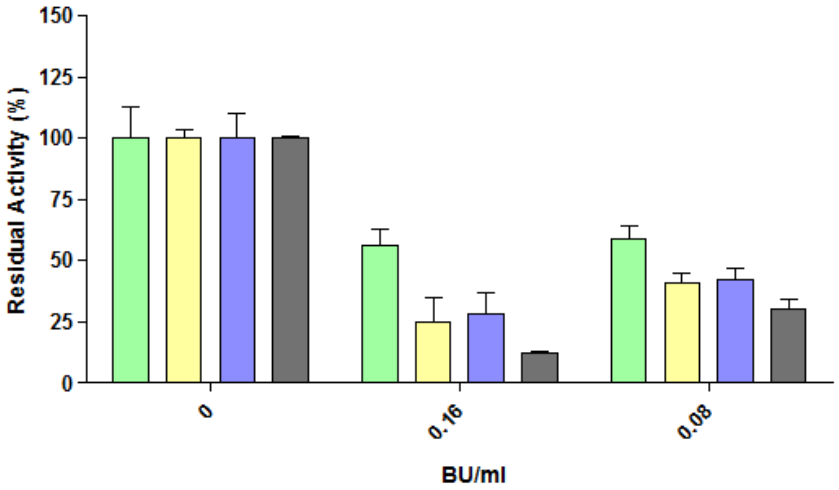

Figure 3.8.4. Residual activity of recombinant FVII variants r404X, r405X, r406X and rWt from conditioned media in Bethesda-based FXa generation assays carried out in the FVII-deficient plasma system. Recombinant r404X (green), r405X (yellow), r406X (blue) and rWt (black) FVII were incubated at 37°C for 2 hours in FVII-deficient plasma with or without plasma anti-FVII inhibitory antibody. r405X, r406X and rWt proteins were normalized for the antigen of the least-secret r404X variant. Antibody was diluted to 0,16 BU/ml and 0,08 BU/ml. Activity of each protein in the presence of the inhibitory antibody was calculated and normalized considering the activity in the absence of the antibody as 100%. Residual activity is expressed on the y-axis as mean percentage ± standard deviation of specific activity.

Interestingly, rWt protein exhibited a rate of inhibition higher than that observed for the r406X-FVII, r405X-FVII and r404X-FVII variants at each anti-FVII antibody concentration. Moreover, the residual activity measured in these experiments for r404X-FVII and rWt-FVII showed the same inverse relationship between carboxyl-terminal length and the extent of inhibition as previously observed (Fig. 3.8.3). Noticeably, the r404X-FVII variant at the antibody concentration of 0.16 BU/ml displayed a residual function very similar to that measured at 0.08 BU/ml, as observed even in previous experiments (Fig. 3.8.3). Evidence of a significant difference in residual activity was observed mainly for the highest antibody concentration (0.16 BU/ml), even if at 0.08 BU/ml a greater activity principally for r404X-FVII, which exhibited a 2-fold increase in respect of the wild-type FVII, was appreciated (Table 3.8.1). In these experiments also r406X-FVII and r405X-FVII were found to be less inhibited than the rWt-FVII, mainly at 0.16 BU/ml (Table 3.8.1), displaying a 2-fold higher activity in respect of the wild-type. This result observed for r405X-FVII and r406X-FVII suggested for the antibody a high

interaction affinity to the wild-type protein, thus reflecting a low affinity for r406X or r405X and so the need of higher concentrations of these proteins to achieve a greater or complete inhibition (Fig. 3.8.1 and 3.8.2).

	0 BU/ml	**0.16 BU/ml**	**0.08 BU/ml**
r404X-FVII	100 ± 12.4	56.5 ± 6.7	58.9 ± 5.4
r405X-FVII	100 ± 3.1	25.1 ± 9.9	41.0 ± 4.1
r406X-FVII	100 ± 10.3	28.1 ± 8.9	41.9 ± 5.2
RWt-FVII	100 ± 0.7	12.4 ± 0.8	30.3 ± 3.8

Table. 3.8.1. Residual activity of recombinant FVII variants r404X, r405X, r406X and rWt measured in Bethesda-based FXa generation assays with or without two dilutions (0.16 and 0.08 BU/ml) of plasma anti-FVII inhibitory antibody. Recombinant 405X-FVII, r406X-FVII and rWt-FVII proteins were normalized for the antigen of the least-secreted r404X-FVII variant. Residual activity is expressed as mean percentage ± standard deviation of specific activity. Activity of each protein in the presence of the inhibitory antibody was calculated and normalized considering the activity in the absence of the antibody as 100%.

As a final consideration, the A294V-11125delC protein present in patient's plasma did not confer any addition to the activity assayed in each experiment conducted on conditioned media, as demonstrated by the very low activity (<1%) showed by the patient's plasma itself used as negative control in these experiments.

3.9 Competition assays with mimicking peptides

Our findings provided the experimental evidence for the carboxyl-terminal region of FVII as a candidate epitope for the anti-FVII developed in the patient's plasma bearing the A294V-11125delC mutation in the homozygous condition.

If the FVII carboxyl-terminal region represents the epitope for the anti-FVII antibody, short peptides mimicking its end should displace the antibody and compete it away, thus vanishing inhibition. To test this hypothesis, the two synthetic peptides RSEPRPGVLLRAPFGG-OH (#1) and RSEPRPGVLLRAPF-OH (#2) were synthesized and initially exploited in binding assays.

ELISA-based assays were optimized to study the competition between the two synthetic peptides and rFVIIa in the binding to the anti-FVII antibody (Fig. 3.9.1). Coated rFVIIa was incubated with plasma containing the antibody either in the absence or in the presence

of the two different synthetic peptides. Prior to incubation with rFVIIa, plasma containing anti-FVII antibody was incubated for 1 hour at 37°C with either peptide #1 or #2. Binding of antibody to coated rFVIIa in the absence of peptides was considered as 100%, while the potential effect of peptides in avoiding binding of the antibody to rFVIIa was evaluated as a decrease in measured OD, and thus in a decrease in percentage of binding to rFVIIa.

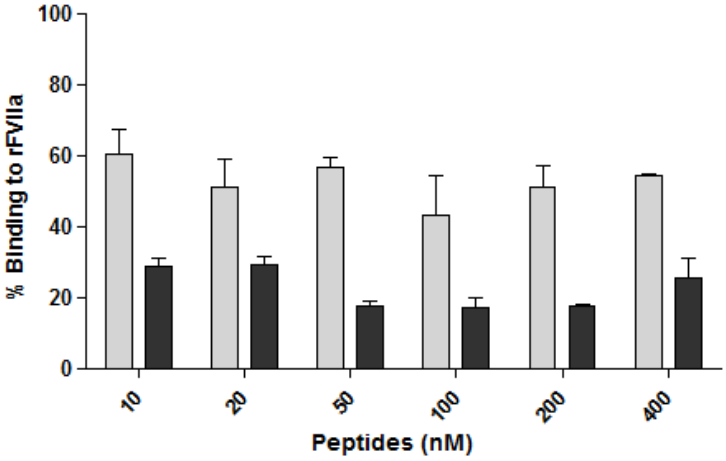

Figure 3.9.1. Interaction of anti-FVII plasma antibody with rFVIIa in the presence of synthetic peptides. Coated rFVIIa (2 nM) was incubated with the anti-FVII antibody (0.035 BU/ml) in the presence of the two synthetic #1 (grey) and #2 (black) peptides at concentrations ranging from 10 to 400 nM. The interaction is expressed as percentage ± standard deviation of binding to rFVIIa considering the binding to rFVIIa without peptides as 100%.

Results obtained in these binding assays revealed a dose-dependent effect of peptides at concentrations ranging from 10 to 400 nM, in particular for the peptide #2 (RSEPRPGVLLRAPF-OH). Indeed, while for peptide #1 the observed decrease in binding of anti-FVII antibody to rFVIIa was around 50% up to 43.3±11.2%, for peptide #2 the higher decrease in binding capacity was observed between 50 and 200 nM with 17.9±1.2%, 17.3±2.5% and 17.7±0.2% of binding to rFVIIa, respectively.

Assays aimed at assessing the functional effect of these peptides are currently in progress.

DISCUSSION

Among the coagulation disorders haemophilia is the most common. Haemophilia A and haemophilia B are X-linked recessive inherited bleeding disorders characterized by FVII and FIX deficiencies, respectively. As for other coagulation disorders, based on the activity of the coagulation factor detectable in the patient's plasma, haemophilia may be classified as mild (>5%), moderate (1–5%) or severe (<1%).

The treatment of these disorders is represented by the infusion of the deficient/missing factor, but the exposure to the exogenous proteins can trigger the development of inhibitory antibodies, thus reversing the benefits brought by the replacement therapy. Indeed, the formation of alloantibodies against factor FVIII or factor IX represents the most severe complication of replacement therapy in patients with haemophilia. Inhibitors occur at a frequency of 25% in severe haemophilia A and around 5% in haemophilia B [1,2]. More in general in approximately 10% of all patients with haemophilia the development of an antibody in response to the therapy with the deficient factor has been reported [3]. As a definition, an inhibitor antibody is a polyclonal high affinity immunoglobulin that neutralizes the procoagulant activity of a specific coagulation factor. The population of inhibitory antibodies found in patients with haemophilia A and B after exposure to exogenous FVIII and FIX consists of polyclonal IgG antibodies, being the IgG4 subtype the most abundant, even if all IgG subclasses have been observed [4,5]. The inhibitory titre is measured and expressed in Bethesda units (BU), 1 BU being defined as the concentration of antibody able to inhibit 0.5 U/ml of coagulation factor. In other words, 1 BU is the inverse of the IgG concentration, referred as the inverse of the plasma dilution inhibiting 50% of the activity in normal plasma [6]. Depending on the amount of inhibitory antibody detected in plasma, inhibitor levels are classified as high or low titre when these are upon or below 5 BU, respectively [7].

The development of inhibitory antibodies has been observed as dependent on the type of mutation by which either FVIII or FIX were involved. In general, in severe haemophilia A and B those gene alterations that result in the absence or severe truncation of the FVIII/FIX proteins are associated with the highest risk for inhibitor formation, while mutations associated with the presence of a gene product even at low amounts confer a low risk for inhibitor production [8]. For example, haemophilia B patients with nonsense or frame shift mutations have a risk of approximately 20%, whereas for those having complete deletions

or rearrangements of the FIX gene the risk of inhibitor development may arise up to approximately 50% [9]. Contrarily, missense mutations represent the main mutation type in mild to moderate haemophilia, with an inhibitor prevalence of 5%. This low incidence is due to the presence in these patients of some endogenous, although non-functional, protein that is sufficient to induce immune tolerance, even if different missense mutations in FVIII and FIX reflect different risk for inhibitor formation, indicating that the inhibitor prevalence in missense mutations is also dependent on the position in which the alteration is localized [10].

Moreover, inhibitory antibodies are characterized by several features that may have implications for the immune process by which they occur. Indeed, the FVIII antibodies are mainly directed towards the A2, A3 and C2 domains, thereby interfering with the function of the factor Xase complex, the binding of FVIII to von Willebrand factor, and the binding of FVIII to phospholipid membranes [11,12]. Noticeably, FVIII antibodies with catalytic activity [13,14], thus exerting their inhibitory effect by a proteolytic cleavage mechanism, and antibodies developed in acquired haemophilia A preventing inactivation of FVIII by APC [12] have also been reported. The FIX epitopes are localized to the amino-terminal γ-carboxyglutamic acid (GLA) region and the serine protease domain [15]. In some patients antibodies directed against both areas have been found, but antibodies against the EGF-like domains have never been. The inhibitory effects of these anti-FIX antibodies include inhibition of the FVIII-dependent FX activation, the GLA domain binding to phospholipids and the binding of the cofactor FVIII [16].

Overall, in addition to an inhibitor prevalence for haemophilia A, more patients with haemophilia B than with haemophilia A have been found to be CRM^+ on the basis of detectable FIX antigen. Since inhibitory antibodies in haemophilia B have been found to develop more frequently in CRM^- than CRM^+ individuals, it has been postulated that patients with detectable FIX polypeptide (CRM^+) might develop tolerance to the 'self' protein which then is extended to the FIX infused in replacement therapy [17]. However, the larger fraction of haemophilia B patients with detectable FIX antigen on the basis of molecular genetics may not provide the sole explanation for the lower incidence of FIX inhibitors. Given that acquired deficiency of FIX is much less common than FVIII, it is thought that FIX may be a less immunogenic protein than FVIII. Thus, the high conservation of amino acid sequence among vitamin K-dependent coagulation factors

(factors II, VII, IX, X, protein C and S) could be a reason for decreased FIX immunogenicity [17].

Genetic risk factors are known to be of importance in the development of inhibitors, whereas the impact of non-genetic factors is less clear. Accumulating evidence indicates that other genetic factors (e.g., major histocompatibility complex alleles and other immune-modulatory genes) and factors associated with treatment (e.g., type of FVIII concentrate, route of administration, and age of first exposure) may also influence the risk of inhibitor development [16].

The lower prevalence of potentially null mutations in FIX (and particularly in FVII) may partially explain the lower incidence of alloantibodies in haemophilia B, as observed for FVII deficiency. In haemophilia B genetic conditions predisposing to antibody formation are mainly represented by nonsense changes followed by large deletions, which are very rare or absent in FVII deficiency [18].

Given that, for the above mentioned reasons, alloantibodies against FVII are very rare.

In our study, we focused the attention to one of the almost unique case of severe FVII deficiency associated with the presence of an anti-FVII inhibitory antibody. Two other cases of inhibitory antibodies directed against FVII have been reported (19,20].

The patient was characterized by being homozygous for the A294V-11125delC mutation that, beside the aminoacid substitution at position 294, causes a frameshift and predicts the synthesis of an elongated FVII, bearing a 28 aminoacid-long tail in the carboxyl-terminal region downstream from the 404 residue (Fig. D1).

The A294V-11125delC mutation has been previously described as a mutation associated to very low residual levels both in vivo and in vitro and to a clinical phenotype ranging from asymptomatic to severe forms of FVII deficiency [21,22]. The distribution of the A294V-11125delC mutation has been primarily localized in the central Europe as the geographic area, but a high incidence has been mainly observed in patients from the Eastern Europe [23-27]. In these studies, patients have been found to be homozygous, heterozygous or double heterozygous for the mutation, and even in the homozygotes a great variability of the clinical phenotypes has been observed.

As for the double mutation A294V-11125delC, also the A294V has been found in several patients with variable phenotypes [28-30] and its biochemical characterization revealed that this mutation affects the interaction of the mutant protein with activators, TF and substrates [31].

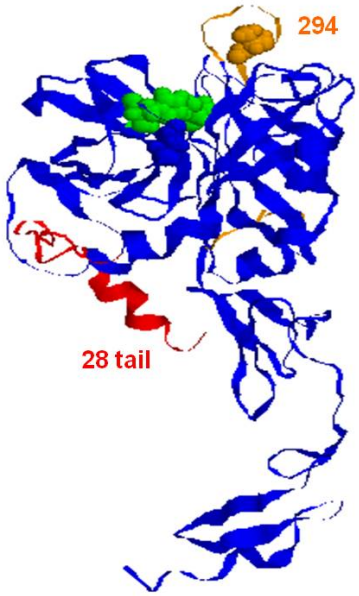

Figure D1. Tridimensional model of the A294V-11125delC FVII variant.
The normal full-length 406 aminoacid-long FVII molecule (blue) is elongated at the carboxyl-terminal region due to a frameshift caused by the deletion of a cytosine that finally results in a terminal tail of 28 aminoacids (red). The A294V mutation is indicated in orange.

The patient understudy, and homozygous for the A294V-11125delC mutation, developed an anti-FVII inhibitory antibody after the treatment with plasma-derived FVII and rFVIIa. Analysis of two independent collections of patient's plasma for the anti-FVII antibody content provided an inhibitor titre of 12.3 and 7.2 Bethesda Units ml^{-1} (BU ml^{-1}).

Based on the molecular defect, the patient's plasma is characterized by the exclusive presence of traces of a carboxyl-terminal-elongated FVII, which suggests the carboxyl-terminal region as a candidate epitope for the inhibitory antibodies.

Experiments aimed to study the binding of this antibody to rFVIIa or zymogen FVII showed that the antibody was able to recognize both the FVII and FVIIa forms. Moreover, western blot showed that the antibody was able to discriminate between reduced and non-reduced rFVIIa, the last being the recognized form. In addition, the antibody was found to be unable to interact with a highly homologous factor as FXa, indicating the involvement of antibody in the specific interaction of the sole rFVIIa and not with other coagulation factors albeit in a complex network such as coagulation. ELISA-based assays helped us to observe a rate of binding to rFVIIa very similar to that observed for plasma-derived zymogen FVII. Since after activation of FVII the surface-exposed carboxyl-terminal is less rearranged than other internal regions [32], this finding further support the hypothesis that

this FVII region is involved in the formation of the epitope recognized by the inhibitory antibody.

Inspection of the primary FVII structure shows that the reading frame shift starts from position 404, thus meaning that rFVIIa, FVII and the A294V-11125delC protein share a common sequence until the residue 403, whereas the downstream sequence is different, or completely absent, between the A294V-11125delC variant and both rFVIIa and zymogen FVII.

This observation led the rationale for the deletion scanning approach of wt-FVII as the appropriate model to explore the FVII carboxyl-terminal region as one of the main epitopes of the anti-FVII antibody. For this purpose, the progressively truncated FVII recombinant variants r406X-FVII, r405X-FVII, r404X-FVII, r403X-FVII and r402X-FVII, differing from the full-length FVII in the order of 1, 2, 3 and 4 aminoacids respectively, have been produced.

Results from transiently transfected cells revealed an inverse relationship between the extent of the deletion and the amount of recombinant proteins in conditioned medium, thus indicating the importance of the carboxyl-terminal region for the biosynthesis/secretion of the FVII protein, as previously shown for FIX and PC [33-35]. The same inverse relationship was also observed when the FXa generation activity of these truncated variants was assayed in both the reconstituted system and in FVII-deficient plasma. In these experiments, the r406X-FVII, r405X-FVII and r404X-FVII were the best-secreted variants, being the r404X-FVII the least expressed but well-detectable protein, while the r403X-FVII and r402X-FVII were barely detectable both at the antigen and the activity levels. However, the r402X-FVII protein was known to be expressed at very low levels both in vitro and in vivo (see Part I). Noticeably, considering only the well-detectable r406X-FVII, r405X-FVII and r404X-FVII variants, data from secretion and FXa generation activity showed for these truncated proteins a specific activity similar to that of the wild-type FVII in both the reconstituted and FVII-deficient plasma systems.

Results obtained on these recombinant truncated proteins offered a good model to study the inhibition by the anti-FVII antibody. Intriguingly, experiments with serial dilutions of the plasma anti-FVII antibody showed a different rate of inhibition among the truncated variants. Independent experiments with r406X-FVII, r405X-FVII and r404X-FVII compared with rWt-FVII indicated that the r406X-FVII was the most inhibited protein, almost as the wild-type, the r404X-FVII the least inhibited, while the r405X-FVII showed

an intermediate rate of inhibition. Given that in each independent set of experiments the rWt-FVII was normalized to reach the antigen of the least secreted protein assayed (r406X-FVII, r405X-FVII or r404X-FVII), these observations were probably suffering from the different concentrations of the assayed proteins. Interestingly, when each truncated protein was normalized to the antigen of the least-expressed r404X-FVII variant and assayed using the two inhibitor concentrations shown to be the most effective in our previous experiments, our results indicated that each truncated protein was less recognized, and thus less inhibited, by the antibody, while the rWt-FVII activity was the most affected. This observation was consistent with the view that depicts the FVII carboxyl-terminal region as a candidate epitope for the antibody, since in these experiments the inhibition exerted by the anti-FVII antibody was escaped in a manner dependent on carboxyl-terminal region extension of the truncated variants. In addition, as previously observed, the r404X-FVII variant showed the lower rate of inhibition, and thus the higher residual activity, among the truncated variants at both concentrations of anti-FVII antibody. Indeed, the r404X protein exhibited a residual activity from 2- to 4.5-fold higher than that of the wild-type protein. Moreover, the discrepancy between the results observed in independent experiments carried out on each variant compared with the rWt-FVII and experiments with the entire set of truncated proteins, indicated that the binding of the antibody to proteins having a shortened carboxy-terminus occurred with an affinity lower than that exerted against the wild-type full-length protein. Indeed, when assayed at the lower concentrations reflecting the r404X-FVII antigen level, the r406X-FVII and r405X-FVII variants were found to be less inhibited than an equal amount of rWt-FVII, suggesting a for the antibody a high interaction affinity for the rWt-FVII, thus in parallel reflecting a low affinity for r406X-FVII or r405X-FVII and so the need of higher concentrations of these proteins to achieve a greater or complete inhibition.

Importantly, our findings indicated that the r404X truncated variant was the least inhibited by the anti-FVII antibody in patient's plasma, reinforcing the hypothesis of an involvement of the FVII carboxyl-terminal region in the formation of the epitope recognized by the inhibitory antibody, being the r404X the sole truncated protein having a sequence that is the most similar to that of the A294V-11125delC.

The presence of the inhibitory antibody seriously affects coagulation factors against which has been developed, by compromising different interaction mechanism as binding to phospholipids, cofactor, substrates, activators and, in the case of FVIII, molecular carriers

as vWF. The use of little synthetic peptides has been exploited not only for the determination of the main epitopes in molecular targets (as FVIII or FIX) recognized by antibodies, but represents a valuable tool for avoiding the antibody-target recognition and interaction. These peptides are synthesized based on specific linear sequences found in the target molecules and their capability to act as decoy systems and restore the coagulant activity in a dose-dependent manner has been demonstrated both in vitro and in vivo [36,37]. For this reason ELISA-based assays in the presence of the inhibitory antibody and with different concentrations of two synthetic peptides mimicking the terminal tract of the FVII carboxyl-terminal region were performed. Results obtained with these peptides showed a dose-dependent effect of peptides on the interaction between rFVIIa and antibody, indicating that, at least in part, this effect might be widely extended in vivo to contrast the antibody action. In addition, even in the absence of data indicating a restore in functional activity, our findings suggest these synthetic peptides as a helpful tool to prevent the rFVIIa-antibody interaction and further shed light on the participation of the FVII carboxyl-terminal region in the formation of the epitope for the anti-FVII antibody found in the patient's plasma.

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PART III

Study of the role of the carboxyl-terminal region of coagulation factor X

In this study we investigated the role of carboxyl-terminal region of coagulation FX on its biology by the in vitro expression of sequentially truncated recombinant variants. Furthermore, given the high homology among coagulation serine proteases, and particularly between FX and FVII, we created two FX and FVII recombinant variants with swapped terminal regions.

3.10 Secretion levels of deleted FX proteins

Deletion studies on the carboxyl-terminal region of FVII demonstrated the existence of an inverse relationship between secretion efficiency and the extent of the deletion, thus suggesting an essential role for this region in biosynthesis/secretion of the mature protein rather than for the specific activity (see Part II). On the other hand, the 402X-FVII variant, albeit poorly secreted, exhibited an increased 2.5-fold specific activity (see Part I), which indicate a not yet explained functional role.

Alignment of primary sequences of highly homologous factor VII (FVII), factor IX (FIX), protein C (PC) and FX provides the evidence for significant differences in the carboxyl-terminal region of these proteins, being the FX carboxy-terminus the most extended.

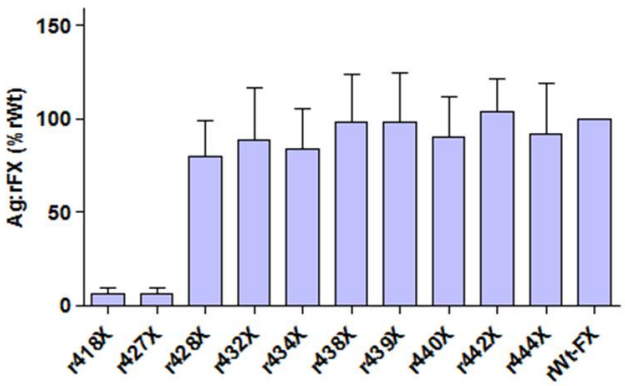
To investigate the contribution of the FX carboxyl-terminal region in protein secretion and activity a deletion scanning approach similar to that performed on FVII was carried out (Fig. 3.10.1).

Figure 3.10.1. Deletion scanning in exon 8 of FX cDNA cloned in the pCMV4 plasmid. Nucleotide and primary sequence of both FX cDNA and protein are shown. Nucleotide change causing premature stop codons are indicated in red under the wild-type triplettes. The natural stop codon TGA is located at codon 449.

Eukaryotic HEK293 cells were transiently transfected with pCMV4 plasmids bearing premature stop codons at different positions in the cloned FX cDNA sequence. To evaluate the secretion of these recombinant variants, conditioned media were collected 72-hour upon transfection and the secreted recombinant FX concentration was quantified by ELISA.

Interestingly, recombinant proteins having a carboxyl-terminal region ranging from 443 (r444X-FX) to 427 (r428X-FX) aminoacids showed a secretion level similar to that of the rWt-FX (Fig. 3.10.2, a). The mean concentration of rWt-FX evaluated was 2346.2±274.6 ng/ml. Noticeably, decrease in the secretion level of recombinant FX truncated variants was not significantly appreciated for deletions up to 21 aminoacids, while the r427X-FX levels showed a dramatic decrease, with levels of 6.1±3.7% of that of wt-FX. This observation was confirmed by the low levels of the shortest r418X-FX molecule (6.7±3.2%).

a)



b)

	Ag:rFX (%rWt)
rWt-FX	100 ± 0.113
r444X	92.2 ± 26.7
r442X	103.9 ± 17.9
r440X	90.7 ± 20.9
r439X	98.5 ± 26.7
r438X	98.7 ± 25.3
r434X	84.2 ± 21.1
r432X	88.9 ± 27.7
r428X	79.7 ± 19.8
r427X	6.1 ± 3.7
r418X	6.7 ± 3.2

Figure 3.10.2. Expression levels of recombinant FX variants in conditioned media from transiently transfected HEK293 cells, represented as a column graph (a) and a table reporting concentration of each recombinant variant (b). Antigen levels are expressed as mean percentage ± standard deviation obtained from at least five independent experiments.

These findings suggest a not essential role for the carboxyl-terminal region of factor X in biosynthesis and secretion thus pointing toward a possible implication in function.

3.11 Activity of deleted FX variants in a plasma system

To mimic the physiological conditions in which FX interacts with its natural activators (FVIIa and FIXa), cofactor (FVa) and substrate (prothrombin) we optimized thrombin generation assays in a plasma system. Recombinant FX in conditioned medium was added with 1/10 dilution of FX-deficient plasma and Innovin, a source of TF, Ca^{2+} and

phospholipids, was used to form the FVIIa/TF activation complex. Activity of FX variants was measured by monitoring fluorescence emission after the addition of a synthetic peptidyl fluorogenic substrate specific for thrombin. Specific activities observed in FX-deficient plasma are referred as the ability of recombinant FX proteins to convert prothrombin in its active form thrombin. Using this approach, the thrombin generation rates of r428X-FX, r432X-FX, r434X-FX and r438X-FX variants were found to be around 25% of rWt-FX, being these activities 23.8±0.8%, 26.4±12.0%, 20.6±2.4% and 27.0±11.9%, respectively. With the exception of r444X-FX, for which a thrombin generation activity of 34.2±3.6% was observed, the least-deleted proteins showed the highest specific activity. Indeed, r442X-FX and r440X-FX showed 48.9±15.9% and 65.1±9.6% of activity in generating thrombin (Fig. 3.11.1). In these experiments the measured specific activity for the wild-type FX was 22.1±3.5 Rfu/min/nM FX.

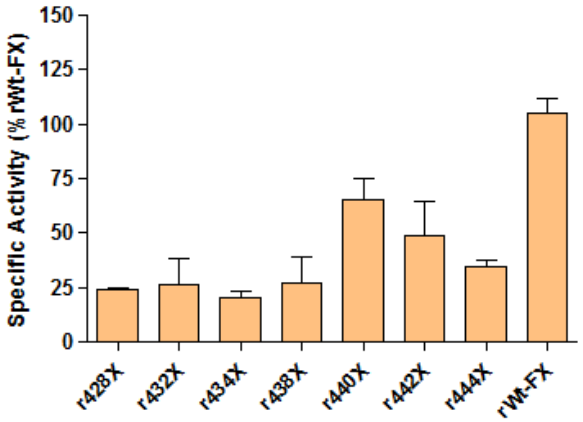


Figure 3.11.1. Specific activity of recombinant FX truncated variants in optimized FIIa generation assays in the FX-deficient plasma system. Serial dilutions of rWt-FX were used as standard curve. Specific activity is expressed as mean percentage ± standard deviation considering the specific activity of wild-type FX as 100%.

These results revealed that, in a plasma system, the activity of the recombinant truncated FX variants was significantly reduced.

3.12 Amidolytic activity of deleted FX variants

In the attempt to dissect the functional impact of deletions on FX function, we also assessed the amidolytic activity of variants by exploiting the Russell's viper venom (RVV) protease as non-physiological FX activator and a specific peptydyl fluorogenic substrate.

In these studies we tested the longest (r444X-FX) and the shortest but well-detectable (r428X-FX) truncated variants and other two mutants of intermediate length (r440X-FX and 438X-FX).

These functional assays revealed that the r444X-FX, r440X-FX and r428X-FX have a specific activity respectively of 87.2±5.5%, 92.8±9.9 and 107.7±15.2% of that of Wt-FX, which corresponded to 32.7±3.3 Rfu/min/nM FX. The r438X-FX variant, on the contrary, displayed a specific activity of 47.4±17.3% of Wt-FX (Fig. 3.12.1).

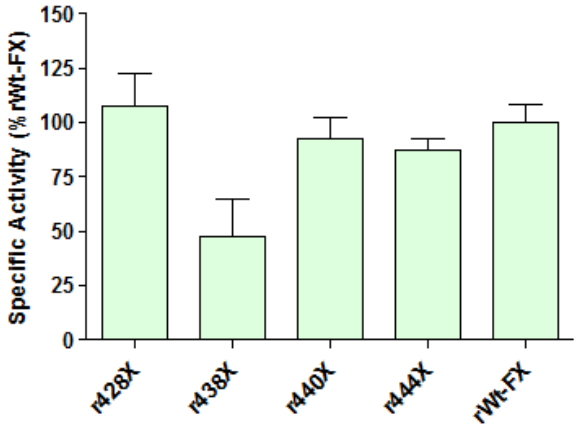


Figure 3.12.1. Specific activity of recombinant FX truncated variants in amidolytic assays after activation by RVV. Recombinant proteins in conditioned media were incubated for 15' at 37°C in the presence of the non-physiological activator RVV from Russell's viper venom. Serial dilutions of rWt-FX was used as a standard curve. Specific activity is expressed as mean percentage ± standard deviation considering the specific activity of wild-type FX as 100%.

These results suggested that deletion of terminal aminoacids up to residue 427 have a little impact on catalytic activity of FX, with the sole exception of r438X-FX, for which the lowest amidolytic activity was observed.

To better interpret these data, the amidolytic activity of the r438X-FX variant was tested at varying RVV concentrations. The r438X-FX and rWt-FX proteins were normalized to reach a concentration of 500 ng/ml, corresponding to 8.5 nM, and incubated at 37°C for 15 minutes with different RVV concentrations. RVV was diluted to 0.34, 0.17, 0.085, 0.042 and 0.021 nM, corresponding to 1/25, 1/50, 1/100, 1/200 and 1/400 dilution in respect of the FX concentration. Amidolytic activity was measured towards the FXa fluorogenic substrate and expressed as Relative Fluorescent Units (Rfu) and then the activity data observed were elaborated and expressed as Relative Fluorescent Units (Rfu) per second (Rfu/sec) as a function of RVV concentration (nM) (Fig. 3.12.2).

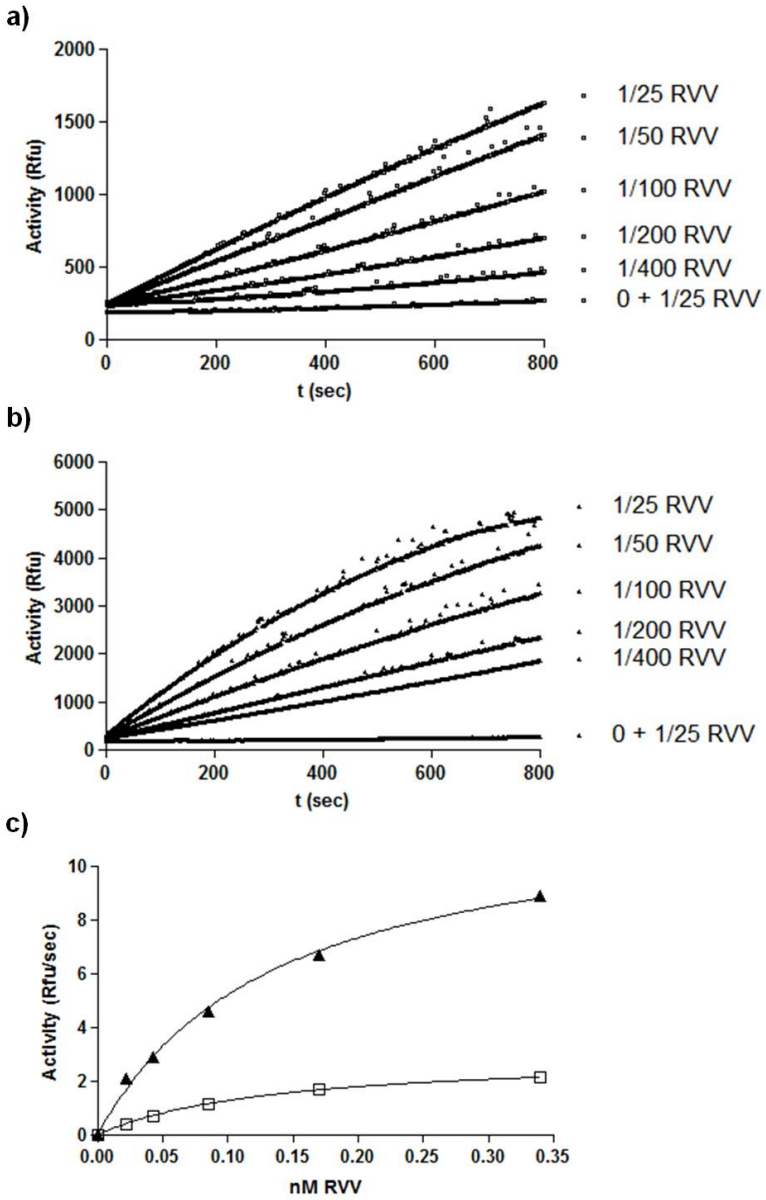


Figure 3.12.2. Amidolytic activity of r438X and rWt-FX towards FXa fluorogenic substrate after activation by RVV at different concentrations. Recombinant proteins were normalized to reach a concentration of 8.5 nM FX and then incubated at 37°C for 15 minutes with 1/25, 1/50, 1/100, 1/200 and 1/400 serial dilutions of activator, corresponding to 0.34, 0.17, 0.085, 0.042 and 0.021 nM RVV, respectively. a) Activity of rWt-FX. b) activity of r438X-FX. c) Activation of r438X-FX and rWt-FX at serial dilutions of RVV. Activity is expressed as Relative Fluorescence Units (Rfu) as a function of time (a,b) and as Rfu per second (Rfu/sec) as a function of RVV concentration (c). The negative control in each assay was represented by the highest RVV concentration added to medium collected from cells transfected with the empty plasmid.

In these experimental conditions, we observed that the activity of the r438X-FX variant was lower than that of the rWt-FX (Fig. 3.12.2, a,b,c). Even at the higher concentration of activator (0.34 nM RVV) the r438X-FX amidolytic activity was below 2000 Rfu, while the

rWt-FX at this RVV concentration reached a near-plateau activity with a Rfu value around 5000. In addition, at 0.34 nM RVV the r438X-FX protein showed the same activity observed for rWt-FX at the lowest concentration of activator (0.021 nM).

Although further investigation is needed to support any conclusion, these preliminary data suggest that the FX carboxyl-terminal region is somehow involved in the FX activation process and the mechanisms leading to the achievement of the functionally competent FXa form.

3.13 The proline 439 in the conserved alanine-proline site

Results obtained from transient expression of deleted variants showed that progressive removal of terminal aminoacids did not affect secretion.

The inspection of the aminoacid sequence of the carboxy-terminus among coagulation vitamin K-dependent serine proteases FVII, FX and PC revealed the presence of a conserved proline at position 404, 439 and 419, respectively (Fig. 3.13.1). Based on the biochemical properties of proline residues and their involvement in defining protein structure, this conserved proline could introduce structural constraints and thus influence processes such as biosynthesis or function.

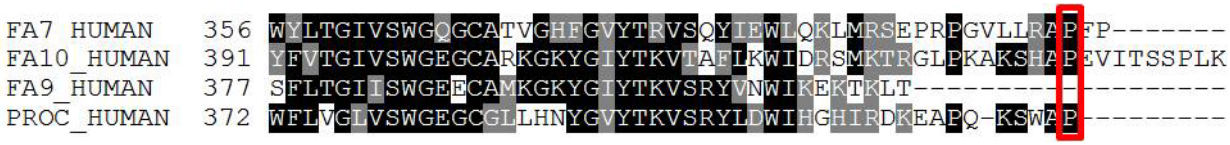


Figure 3.13.1. Primary sequence alignment of human FVII, FX, FIX and PC. The conserved proline residue is indicated by the red box.

To investigate the contribution of this conserved proline residue to the structure of FX carboxy-terminus, we substituted the proline 439 of FX with an alanine in order to determine the effects on secretion and activity of the resulting mutated protein rP439A-FX. In addition, also the truncated r439X-FX variant, lacking the sequence downstream from the proline 439, was produced.

Media from transiently transfected HEK293 cells were collected and both antigen and activity were evaluated (Fig. 3.13.2). For the r439A-FX variant, as for r439X-FX, neither significant differences in secretion nor in amydolytic activity levels were appreciated.

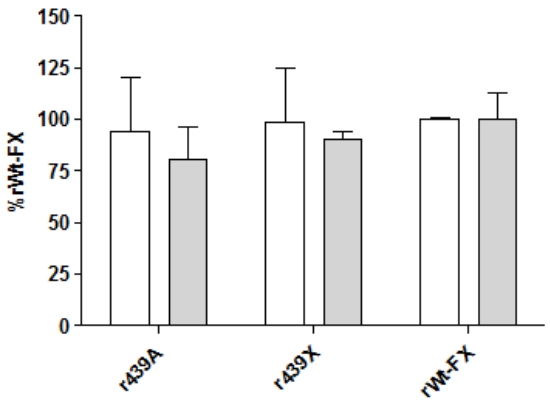


Figure 3.13.2 Antigen levels (white columns) and specific activity (grey columns) of r439A, r439X and rWt-FX in conditioned media. Specific activity was measured in amidolytic activity assays after activation by RVV. Antigen and activity levels are expressed as mean percentage ± standard deviation of wild-type FX from at least three independent experiments.

Proline 439 in FIX is not present and in PC represents the last aminoacid, while FVII and FX bear other aminoacids downstream from this residue. For this reason, due to the great difference in the terminal sequence among these serine proteases, and in particular between FX and FVII, we also investigated whether a factor-specific role by the carboxyl-terminal region of FX and FVII was exerted.

To evaluate the effect mediated by exchanging the FX and FVII carboxy-termini a swapping of terminal tracts downstream from the conserved AP site was performed. Site-directed mutagenesis with primers bearing the sequence to be inserted downstream from the AP site was exploited to create the three mutated plasmids pFX-FVIIter (Fig. 3.13.3), pFVII-FXterEVITS and pFVII-FXterEVITSSPLK (Fig.X.Y). Creation of the pFX-FVIIter plasmid by insertion of the nine terminal nucleotides of FVII was directly performed on the wt-FX cDNA sequence cloned in the pCMV4 expression vector.

As regards the swap of the FX terminal tract in FVII a two-step mutagenesis was needed in order to insert the entire 30 nucleotide-long FX terminal. A first mutagenesis on wt-FVII sequence cloned in the pcDNA3 plasmid was performed to insert 15 of the 30 nucleotides of the FX terminal, leading to the creation of the intermediate pFVII-FXterEVITS plasmid (Fig. 3.13.4, a). Then, a second mutagenesis in which the pFVII-FXterEVITS plasmid was used as the template allowed us to obtain the pFVII-FXterEVITSSPLK (Fig. 3.13.4, b) bearing the entire FX terminal sequence.

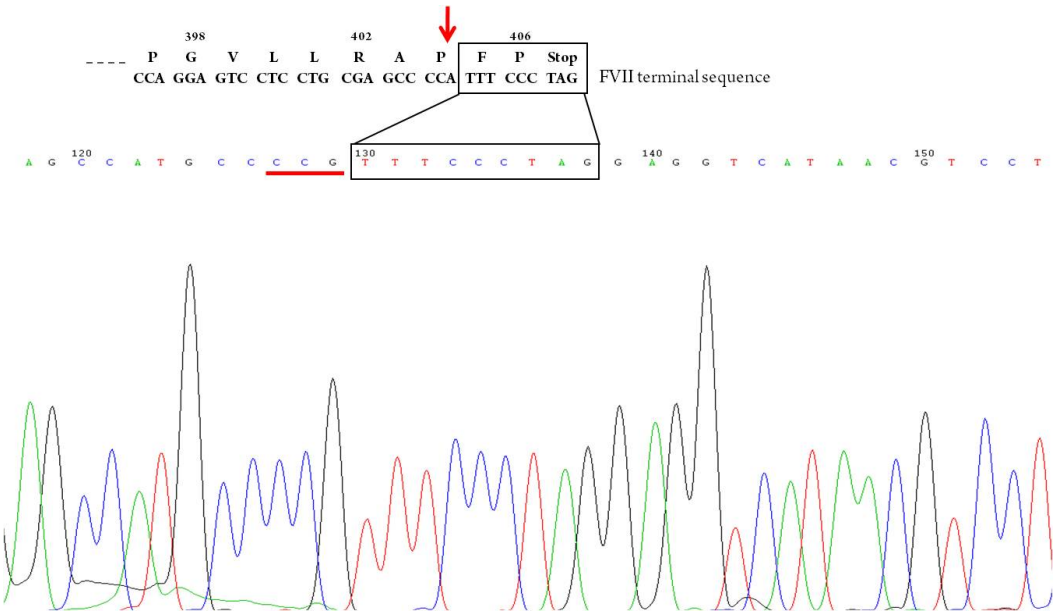
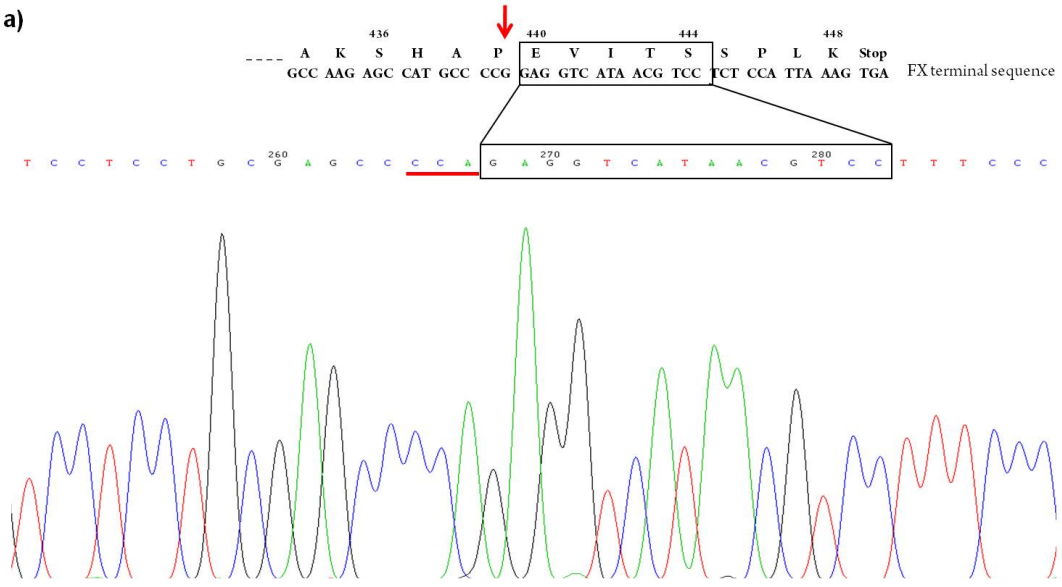


Figure 3.13.3. Nucleotide sequence of FX terminal tract in the pFX-FVIIter plasmid bearing the insertion of the FVII terminal nucleotides downstream from the conserved AP site. The FVII terminal sequence is depicted above the chromatogram and the inserted FVII nucleotides are outlined in black. Conserved proline of the AP site in the FVII sequence (red arrow) and the triplette downstream from which in FX the insertion starts (red line) are also shown.



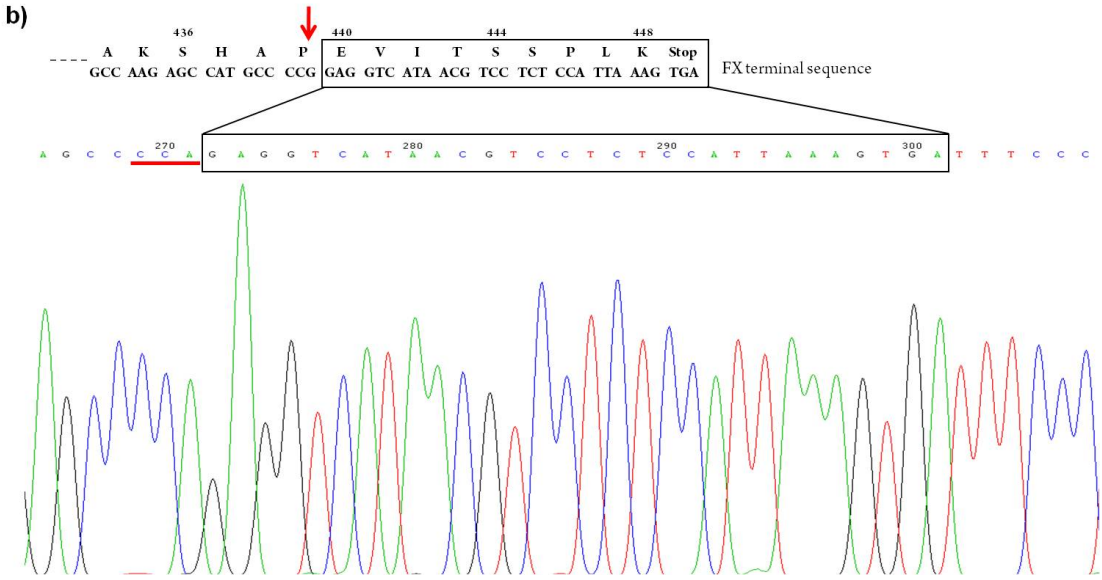


Figure 3.13.4. Nucleotide sequence of FVII terminal tract in the pFVII-FXterEVITS (a) and pFVII-FXterEVITSSPLK (b) plasmids bearing the insertion of the FX terminal tract downstream from the conserved AP site. The FX terminal sequence is depicted above each chromatogram and the inserted FX nucleotides are outlined in black. Conserved proline of the AP site in the FX sequence (red arrows) and the triplette downstream of which in FVII the insertion starts (red lines) are shown.

To test whether protein expression is affected by the exchange between FX and FVII terminal sequences, HEK293 (for FX) and BHK cells (for FVII) were transiently transfected with the three plasmids. After 72 hours media were collected and tested for antigen and activity levels. ELISA assays on conditioned media from cells transfected with the pFX-FVIIter expression vector produced FX secretion levels of 6.3±0.2% of that of wild-type FX, for which a value of 2470.4±200.8 ng/ml was measured (Fig. 3.13.5, a). When media collected from cells transfected with either the pFVII-FXterEVITS or pFVII-FXterEVITSSPLK plasmids were evaluated, the antigen levels were 25.4±6.7% and 2.3±0.6%, respectively (Fig. 3.13.5, b). The expression of rWt-FVII was 276.4±32.2 ng/ml.

Although protein levels were low or very low for both rFX-FVIIter and rFVII-FXter, amidolytic and FXa generation activity, respectively, were assessed. For rFX-FVIIter the amidolytic activity after RVV activation was 5.6±0.5% of rWt-FX, while in FXa generation assays the activity observed for the rFVII-FXter variant in respect of rWt-FVII was 0.7±0.1%.

These results obtained for both FX and FVII indicated that the sequence swapping of the terminal tracts downstream from the conserved AP site dramatically affected the

biosynthesis of the resulting proteins. The same result was observed for activity of these recombinant variants. Noticeably, in the case of FVII, both expression and activity were affected in a way proportional to the extent of the two-step inserted sequence. Intriguingly, even if for FX a not essential role for the carboxyl-terminal region in biosynthesis was observed, the replacement of the carboxyl-terminal tract seemed to be more detrimental than progressive deletion of terminal residues.

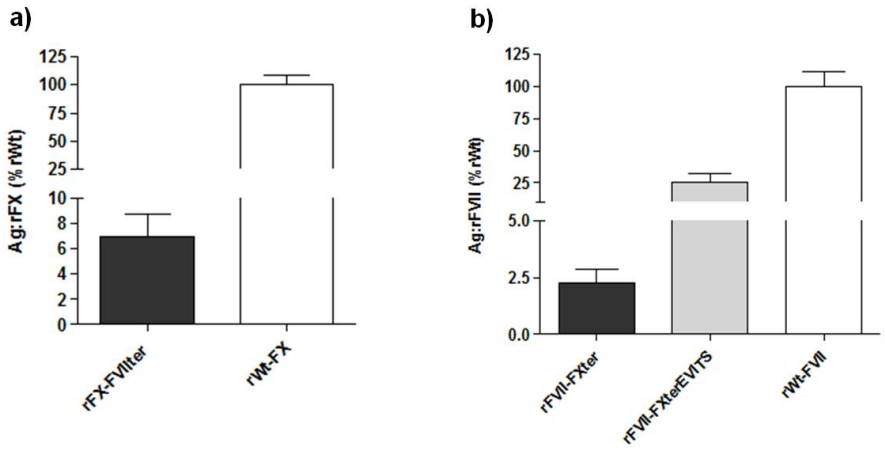


Figure 3.13.5. Protein levels from swapped FX and FVII plasmids transiently transfected in HEK293 cells. a) rFX-FVIIter from transfection with the pFX-FVIIter plasmid. b) rFVII-FXterEVITS and rFVII-FXter from transient transfections with pFVII-FXterEVITS and pFVII-FXterEVITSSPLK, respectively. Antigen levels are expressed as mean percentage ± standard deviation of wild-type FX (a) or FVII (b) form three independent transfections.

Together these results pointed toward a model in which the carboxyl-terminal region of these two coagulation serine proteases might have a factor-specific role both in biosynthesis/secretion and activity/activation, mainly for FX,

DISCUSSION

Vitamin K-dependent serine proteases of coagulation share a common structure organization both at gene and protein levels. In particular Factor X (FX) is highly homologous to procoagulant proteins such as Factor VII (FVII), Factor IX (FIX) and the anticoagulant Protein C (PC). Noticeably, despite of the high sequence homology, the terminal tract of the carboxyl-terminal region remarkably differs either in terms of extension or in aminoacid composition (Fig. D2). Indeed, in the terminal tract of the carboxy-terminus of these coagulation factors are located the most variable residues, being only few conserved. The gene and protein organization shared by these members of the serine protease family is due to the origin from a common ancestor protein characterized by the general structure Gla-EGF1-EGF2-SP [1,2].

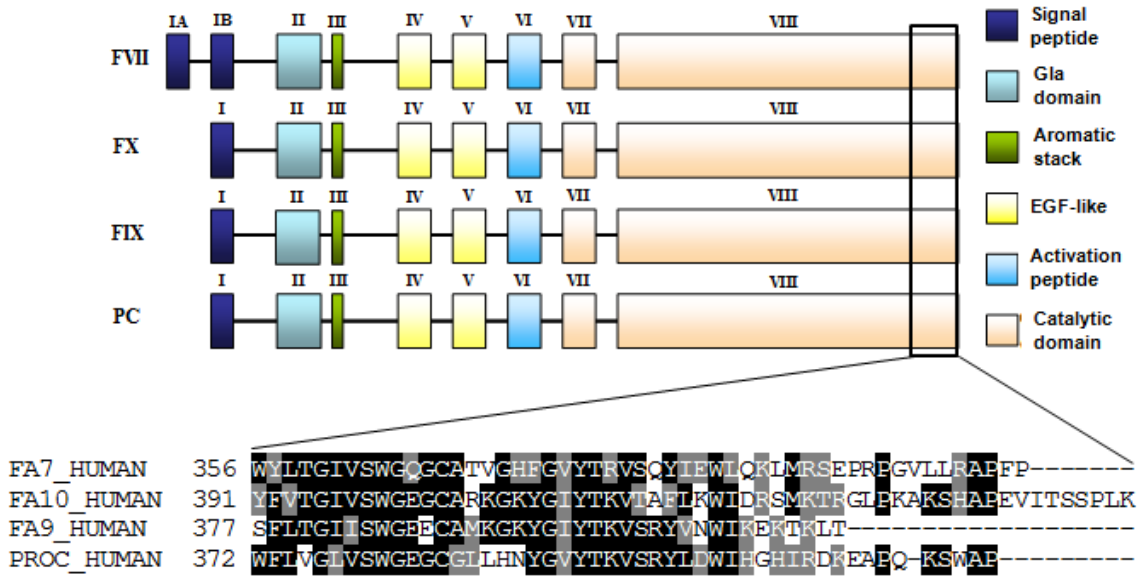


Figure D2. Homology regions in the gene and protein structure of coagulation serine proteases FVII, FX, FIX and PC. Roman numbers above each protein domain indicates the corresponding coding exon.

The alignment of primary sequences of these serine proteases showed the FX carboxyl-terminal region as the most extended, followed by FVII, PC and ultimately FIX. Interestingly, this carboxyl-terminal region was indicated as an essential sequence for the expression of FIX, PC and FVII, as demonstrated by experiments that showed a detrimental effect mainly on protein secretion due to substitutions or deletions of some carboxyl-terminal residues [3,4, and Part II].

We investigated the role of the carboxyl-terminal region of FX in secretion and activity through a deletion scanning of terminal residues similar to that performed on FVII (see Part II). Results obtained from experiments on FVII secretion and activity gave results according to the observation indicating that alteration of the carboxy-terminus affects protein secretion.

A set of recombinant FX variants (Fig. D3) was transiently expressed in HEK293 cells and then conditioned media were tested for secreted antigen levels. Interestingly, a secretion level similar to that of the full-length wild-type FX was shown for recombinant proteins with a carboxyl-terminal region in which a sequence spanning 21 aminoacids was deleted. Thus, this observation pointed towards a non-essential participation of the FX carboxyl-terminal region in the secretion process, differently from that observed for other coagulation factors. A dramatic decrease in the amount of secreted protein was observed for the r427X protein, lacking for 22 residues, and also the most deleted mutant r418X was barely detectable.

391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPKAKSHAPEVITSSPLK rWt (448 aa)
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPKAKSHAPEVIT r444X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPKAKSHAPEV r442X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPKAKSHAP r440X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPKAKSH r438X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGLPK r434X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMKTRGL r432X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSMK r428X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAFLKWIDRSM r427X
391-YFVTGIVSWGEGCARKGKYGIYTKVTAF r418X

Figure D3. Recombinant FX variants obtained by deletion scanning of the carboxyl-terminal domain.

Given that secretion was not affected by the deletion of terminal residues, an effect on the activity would be expected. Activation of rFX variants by the non-physiological RVV (Russell's viper venom) protease was assessed in order to determine the amidolytic activity of a selected set of recombinant variants, and among them only the r438X protein showed a decrease in the specific activity towards the synthetic FXa fluorogenic substrate, while the most deleted but well-detectable r428X-FX protein showed a specific activity similar to that of the rWt-FX.

This result might be explained by an effect due to position, since removed residues reside in a region distant from the catalytic site, thus only a minor effect on the activity of the catalytic centre would be expected. As the r438X-FX was the sole variant with a decreased

specific activity, an activation defect was hypothesized, and experiments with different concentrations of the activator pointed toward a defect in the activation process. Noticeably, FX sequences involved in the activation process by RVV have been identified by assaying synthetic peptides for the inhibition of FX activation (Chattopadhyay 1989). Among the three peptides that exerted the highest rate of inhibition, the peptide spanning the FX sequence from residue 417 to 431was found to prevent FX activation in a dose-dependent manner, even if it showed the least potency (below 50% of inhibition), and thus a participation of the sequence between residues 417 and 431 in the activation of FX by RVV has been proposed. Thus, a role for residues residing downstream from the 431 in the activation by RVV would be speculated.

The activation of zymogen FX consists of a first proteolytic cleavage between Arg194 and Ile195 in the N-terminal region of the heavy chain of FX leading to the α form of activated FX (FXaα, [5,6]), and, in the presence of Ca^{2+} and phospholipids, a second autoproteolytic cleavage between Arg429 and Gly430 at the carboxy-terminus of the heavy chain giving rise to FXaβ [7]. No difference in function has been observed between the α and β forms of the protease [8]. The r428X variant lacks only two residues in respect of the full-length FXaβ, thus the normal activity of this protein could be due to a FXaβ-like structure. In addition, since the sequence 417-431 have been involved in the activation of FX by RVV, the activity of the r428X-FX protein, even if it lacks four of the residues localized in this sequence, was not affected maybe because this variant retains the major determinants for activation by RVV.

Since the conversion from FXaα to FXaβ needs the presence of phospholipids and RVV is unable to activate FX in an environment in which phospholipids are present [9], the potential contribution of the β form to the activity of the truncated variants was not yet evaluated. In addition, to investigate the activity and activation of these mutants in a more complex system as the plasma, in which also protein-protein and exosite-mediated interactions occur, an optimized FIIa generation assay was performed. These experiments showed that in the generation of thrombin all the mutants were affected in a different extent by deletions, resulting in a specific activity ranging from 25 to 65% in respect of the rWt-FX.

Inhibition studies have proved the participation of the peptide having the 417-431 sequence not only in the activation of FX by RVV but also in the activation mediated by FVIIa [10]. In addition, important FX sequences involved in the prothrombinase complex have been

identified in two different works, by the indication of the 417-431 peptide as one of the most effective in the inhibition of FXa-mediated formation of thrombin [11] and the sequence 415-429 an important site of interaction between FXa and prothrombin in the prothrombinase complex [12].

In these experiments FXa activation was triggered by the addition of Innovin, a source of TF, Ca^{2+} and phospholipids, and the activity was measured by adding a specific FIIa fluorogenic substrate. As for the activation by RVV, also in the case of activation mediated by the FVIIa/TF complex, the deletion of carboxy-terminal residues might affect the amount of the thrombin generated in the assay because: i) the FVIIa/TF complex fails to fully activate the FX variants due to a decrease in the interaction rate; ii) the carboxyl-terminal region lacks residues involved in exosite-mediated interactions, known to be important for thrombin formation [13-15]. With the exception of the FXaβ-like r428X-FX, an additional mechanism responsible for the decreased activity observed for the FX truncated variants might be the failed conversion to the β form. Since the sequence recognized by the FXa itself during the autoproteolysis for the conversion from the α to the β form of FXa is the same as the rWt-FX, even if this process might suffer from the different length and aminoacid composition of the carboxy-terminus between the truncated variants and the wild-type.

The most deleted r428X-FX variant, after activation by RVV, showed an amidolytic activity similar to that of the rWt-FX, whereas in FIIa generation the activity was highly affected. Based on the importance of the sequence 417-431, the decreased activity of the r428X-FX variant might be explained by the lack of four aminoacids being, at least in part, a major determinant for the activation mediated by the TF/FVIIa complex or for the interaction between FXa and prothrombin in the prothrombinase complex.

The alignment of FX and FVII primary sequences show the presence in the carboxyl-terminal tract of few highly conserved residues, referred as P432, A438 and P439 for FX and P397, A403 and P404 for FVII. This conserved “AP-site” represents the point from which the two sequences differ significantly in aminoacid composition and particularly in extension. These alanine and proline residues found in FX and FVII are also conserved in PC (P413, A418 and P419), but in FIX are absent.

The conservation of this short sequence among FX and FVII led us to investigate the effects of different modifications by deleting the FX proline 439 and downstream aminoacids, by changing this proline to an alanine and by the swapping of the downstream

sequence between FX and FVII (Fig. D4). As for the other truncated mutants, the deletion of the P439 residue did not affect secretion, and the same result was observed for the rP439A-FX variant. In addition, either for the r439X or the rP439A protein only little differences in the amidolytic activity were observed. This result further confirmed our previous findings illustrating a non essential role for the carboxyl-terminal region in biosynthesis and secretion of FX.

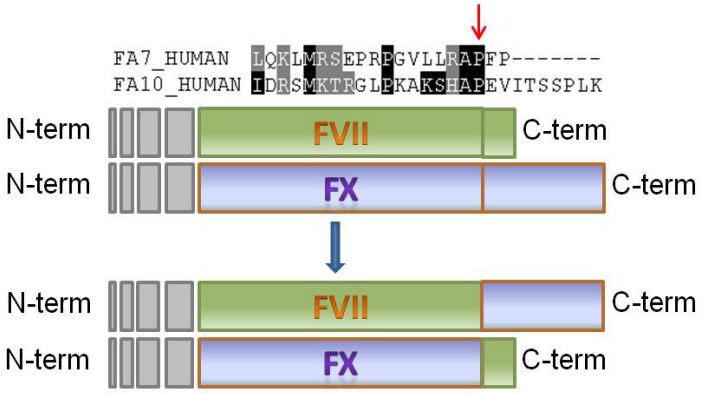


Fig. D4. Sequence swapping between FX and FVII carboxy-terminal regions. Terminal residues in FX and FVII carboxyl-terminal regions are exchanged downstream from a proline located in a conserved "AP-site" (red arrow).

To study and understand the role and involvement of the different domains of the homologous factor FX, FIX and PC, the replacement of sequences has been extensively approached, and an identification of a factor-specific role of each interchanged domain has been reported [16-20]. The substitution of the terminal tract of FX with that of FVII and vice versa indicated a dramatic decrease, near to barely detectable levels, of both secretion and activity. Interestingly, the replacement of the FVII terminal tract with that of FX downstream to the "AP-site" required a two-step insertion due to the high extension of the FX terminal sequence (9 aminoacids, 30 nucleotides stop codon included). Noticeably both expression and activity of the two forms of FVII were affected in a manner proportional to the extent of the two-step inserted sequence. These results indicated that the replacement of the terminal tracts downstream from the conserved AP site dramatically affected the biosynthesis of the resulting chimeric proteins.

Together these findings pointed toward a model in which the carboxyl-terminal region of these serine proteases might have a factor-specific role, as previously observed in experiments of protein regions replacement. The importance and the factor-specific role of the carboxyl-terminal region has been reported also for other coagulation factors, even if not homologous to FX, FVII, FIX and PC, as TFPI, in which the carboxy-terminus confers

the maximal anticoagulant activity [21] and has a direct role in the inhibition of FXa [22], and in antithrombin III, in which the carboxy-terminus is involved in interactions with both FX and thrombin [23].

The different susceptibility to deletion or substitution of terminal residues observed among FX, FVII, FIX and PC might be explained by the molecular evolution of these highly homologous serine proteases. Genes for FIX and PC are located on separate chromosomes, while the genes encoding for FVII and FX are arranged in a head-to-tail manner on the same chromosome. The great difference in susceptibility mainly observed between the closely related FX and FVII might be due to an evolutionary mechanism in which the elongation of the carboxyl-terminal region potentially had a determinant impact. A similar picture has been reported for the evolution of the three genes coding the three chains of fibrinogen, for which a carboxyl-terminal-extended variant has been observed [24].

Interestingly, the low influence exerted by deletions of the FX carboxyl-terminal region suggests for these terminal residues an evolutionary mechanism of tolerance to alterations, also indicated by the removal of carboxyl-terminal residues downstream from Arg429 after FX activation. Noticeably, differently from coagulation factors as FIX, FVII and PC, in FX only very rare natural nonsense mutations have been found, carried in the double heterozygous state in both cases [25,26]. It is known that C→T transitions account for the majority (46%) of all nonsense mutations found in the human genome, being the CGA (coding for arginine) to TGA one of the most frequent change giving rise to nonsense mutations (21%, [27]). The high proportion of C-T transitions that result in a TGA stop codon (24% of all nonsense mutations) is explicable in terms of the methylation-mediated deamination of 5-methylcytosine (5mC) within the CpG dinucleotides, known to be hot-spot sites for mutation [28,29]. Intriguingly, the analysis of nucleotide and protein sequences shows that FX lacks arginine residues coded by the CGA codon in the carboxyl-terminal region and, more extensively, in the whole coding sequence. Thus, the codon most frequently responsible for nonsense changes is totally absent. This observation might reflect, at least in part, the very low frequency of nonsense mutations found in FX. Considering only the arginine residues coded by the CGA codons in FVII, FIX and PC, all the CGA triplettes coding for arginine in FVII, FIX and PC were found to be associated with nonsense codons due to C→T transitions [30-43] (Table D2).

Protein	Number of CGA codons	Nucleotide change	Codon	Position	CpG	References
FVII	2	C→T	TGA	152	yes	[30,31]
		C→T	TGA	402	yes	[32,33, present work]
FIX	6	C→T	TGA	29	yes	[34]
		C→T	TGA	116	yes	[35]
		C→T	TGA	248	yes	[36]
		C→T	TGA	252	yes	[37]
		C→T	TGA	333	yes	[38]
		C→T	TGA	338	yes	[39]
PC	2	C→T	TGA	157	yes	[40,41]
		C→T	TGA	306	yes	[42,43]

Table D2. CGA codons and C→T transitions in FVII, FIX and PC.

Together, data from deletion scanning and region swapping experiments indicated that the carboxyl-terminal region of FX is not essential for biosynthesis and secretion, but might have a functional role either in the activation mechanism or in exosite-mediated interactions.

Further investigations are needed to corroborate our preliminary results and to shed more light on the functional role of the carboxyl-terminal region either in the activity or in the activation of FX and/or in the interactions in which exosites are involved. The perspectives for this study include purification of truncated FX variants as the shortest but well-detectable r428X-FX protein and the r427X-FX to better investigate the effective contribution of the carboxyl-terminal to the interactions in the prothrombinase complex, being this protein the one for which a dramatic decrease in secretion levels was observed.

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CONCLUSIONS

Factor VII (FVII), Factor X (FX), Factor IX (FIX) and protein C (PC), which belongs to the family of coagulation serine proteases, share high gene and protein homology, both in structure and sequence. This is explained by their evolution from a common ancestor that drove the narrow specialization of these proteins which, by a fine interplay, ensure the tight regulation of a complex coagulation network.

In spite of the several similarities, the carboxyl-terminal region of these proteins displays remarkable differences, both in extension and aminoacid composition. Previous works showed the fundamental role of the carboxyl-terminal region for the biosynthesis and secretion of FIX and PC.

In these studies, by taking advantage of both natural occurring mutations and in-vitro expression of recombinant protein variants, we investigated the role of this region for biosynthesis/secretion and/or activity of FVII and FX, so far virtually unexplored.

The investigation of the natural nonsense mutations R402X found in a homozygous FVII deficient patient enabled us to identify a truncated FVII variant that, albeit poorly secreted, possesses an increased specific activity. On the other hand, through the expression of progressively truncated FVII variants we demonstrated that its carboxy-terminus is essential for an efficient secretion.

The studies in plasma from a patient bearing a FVII variant with an altered and extended carboxy-terminal region who developed inhibitory anti-FVII antibodies enabled us to suggest this region as a main epitope.

Expression of a panel of progressively truncated FX variants at the carboxy-terminus enabled us to suggest a role for the activity of this serine protease rather than for biosynthesis.

Altogether these results provide experimental evidence for the importance of the carboxyl-terminal region of coagulation serine proteases and contribute to elucidate the relationship between protein structure and function. The knowledge of these molecular mechanisms would help understanding the processes leading to the divergence and evolution of coagulation serine proteases.

To strengthen our results and to support our conclusions we have planned other investigations that include the purification of selected FX truncated variants and their biochemical characterization, combinatorial mutagenesis at position 401 of FVII and identification of IgG subtype responsible for the immunological complication in the FVII deficient patient.

LIST OF PUBLICATIONS

Thromb Res. 2009 Apr;123(6):914-8

Characterization of the intracellular signalling capacity of natural FXa mutants with reduced pro-coagulant activity

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INTRODUCTION: Factor X (FX) is a serine-protease playing a crucial role in the blood coagulation pathway and triggering intracellular signalling in a variety of cells via protease-activated receptors (PARs). By exploiting naturally occurring variants (V342A and G381D, catalytic domain; E19A, gamma-carboxyglutamic acid (GLA)-rich domain), we investigated the relationship between the pro-coagulant activity and the signal transduction capacity of FX.

MATERIALS AND METHODS: Recombinant FX (rFX) variants were expressed in Human Embryonic Kidney cells and purified by immunoaffinity chromatography. Activated rFX (rFXa) variants were characterized for pro-coagulant, amidolytic and thrombin generation activity. rFXa signalling was assessed through evaluation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation in C2C12 myoblasts.

RESULTS AND CONCLUSIONS: rFX variants showed reduced (rFX-342A, 29%; rFX-19A, 12%) or not detectable (rFX-381D) amidolytic activity. Thrombin generation activity in a plasma system was also decreased either upon activation by Russell's viper venom (rFX-342A, 38%; rFX-19A, 7%; rFX-381D, not detectable) or by the extrinsic pathway (rFX-342A, 36%; rFX-19A, rFX-381D, not detectable). The rFXa-381D mutant displayed little or no enzymatic activity, and did not induce any appreciable signal transduction capacity. The rFXa-342A mutant induced a dose-dependent signalling with a 50% reduced signalling capacity. At the highest concentration (174 nM), signalling progressed with a time course similar to that of rFXa-wt. Zymogen rFX-19A showed defective and incomplete activation resulting in strongly reduced enzymatic activity and signalling. Taken together our data are consistent with a close correlation between pro-coagulant activity and intracellular signalling capacity.

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Chronic sleep deprivation markedly reduces coagulation factor VII expression

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Chronic sleep loss, a common feature of human life in industrialized countries, is associated to cardiovascular disorders. Variations in functional parameters of coagulation might contribute to explain this relationship. By exploiting the mouse model and a specifically designed protocol, we demonstrated that seven days of partial sleep deprivation significantly decreases (-30.5%) the thrombin generation potential in plasma evaluated upon extrinsic (TF/FVIIa pathway) but not intrinsic activation of coagulation. This variation was consistent with a decrease (-49.8%) in the plasma activity levels of factor VII (FVII), the crucial physiologicalal trigger of coagulation, which was even more pronounced at the liver mRNA level (-85.7%). The recovery in normal sleep conditions for three days completely restored thrombin generation and FVII activity in plasma. For the first time, we demonstrate that chronic sleep deprivation on its own reduces, in a reversible manner, the FVII expression levels, thus influencing the TF/FVIIa activation pathway efficiency.

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Regular Article

Characterization of the intracellular signalling capacity of natural FXa mutants with reduced pro-coagulant activity

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ABSTRACT

Introduction: Factor X (FX) is a serine-protease playing a crucial role in the blood coagulation pathway and triggering intracellular signalling in a variety of cells via protease-activated receptors (PARs). By exploiting naturally occurring variants (V342A and G381D, catalytic domain; E19A, γ-carboxyglutamic acid (GLA)-rich domain), we investigated the relationship between the pro-coagulant activity and the signal transduction capacity of FX.

Materials and methods: Recombinant FX (rFX) variants were expressed in Human Embryonic Kidney cells and purified by immunoaffinity chromatography. Activated rFX (rFXa) variants were characterized for pro-coagulant, amidolytic and thrombin generation activity. rFXa signalling was assessed through evaluation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation in C2C12 myoblasts.

Results and conclusions: rFX variants showed reduced (rFX-342A, 29%; rFX-19A, 12%) or not detectable (rFX-381D) amidolytic activity. Thrombin generation activity in a plasma system was also decreased either upon activation by Russell's viper venom (rFX-342A, 38%; rFX-19A, 7%; rFX-381D, not detectable) or by the extrinsic pathway (rFX-342A, 36%; rFX-19A, rFX-381D, not detectable). The rFXa-381D mutant displayed little or no enzymatic activity, and did not induce any appreciable signal transduction capacity. The rFXa-342A mutant induced a dose-dependent signalling with a 50% reduced signalling capacity. At the highest concentration (174 nM), signalling progressed with a time course similar to that of rFXa-wt. Zymogen rFX-19A showed defective and incomplete activation resulting in strongly reduced enzymatic activity and signalling. Taken together our data are consistent with a close correlation between pro-coagulant activity and intracellular signalling capacity.

Human coagulation factor X (FX) is a plasma glycoprotein that plays a crucial role in the coagulation cascade. It can be activated (FXa) by factor VIIa/tissue factor (FVIIa/TF) in the initiation phase, and by factor IXa/factor VIIIa (FIXa/FVIIIa) in the subsequent propagation phase. In the prothrombinase complex, FXa activates prothrombin into thrombin in the presence of factor Va (FVa), phospholipids and Ca^{2+} leading to fibrin deposition and clot formation [1,2].

FXa is also able to induce intracellular signalling via protease-activated receptors (PARs) [3,4], G-protein-coupled receptors that are activated by proteolytic cleavage [5–7]. Four PARs have been so far characterized [8]. Thrombin is able to activate PAR -1, -3 and -4 [9,10], but not PAR-2, which can be activated by multiple trypsin-like proteases including the upstream coagulant proteases FVIIa and FXa [11,12]. In addition to PAR-2, FXa signalling can be mediated by PAR-1 depending on the cell type [13,14].

In particular, PAR-2 is the main endogenous FXa receptor in endothelial cells [15,16] and vascular smooth muscle cells [17] suggesting that this receptor might mediate vascular FXa signalling. By contrast, PAR-1 appears to be the major determinant of FXa signalling in HeLa cells [18].

Through these mechanisms FXa triggers a variety of cellular responses, including release of cytokines [19], expression of

Abbreviations: ERK1/2, extracellular signal-regulated kinase 1 and 2; FX, factor X; FXa, activated factor X; FVIIa, activated factor VII; FIXa, activated factor IX; FVIIIa, activated factor VIII; FVa, activated factor V; GLA, γ-carboxyglutamic acid-reach domain; HEK 293, Human Embryonic Kidney cells; MAPK, mitogen-activated protein kinases; PAR, protease-activated receptors; P-ERK1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; rFX, recombinant FX; RVV, Russell's viper venom; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TF, tissue factor; t-ERK1/2, total extracellular signal-regulated kinase 1 and 2.

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adhesion molecules [20] and TF [13]. It has been reported that FXa enhances proliferation of fibroblasts [21] and stimulates smooth muscle cells mitosis [22,23]. Very recently, we have shown that FXa signalling is responsible for fibroblast migration and differentiation into myofibroblasts, thus facilitating wound healing [24,25].

The cellular effects of FXa, upon PAR activation, are mediated by the mitogen-activated protein kinases (MAPK), i.e. extracellular signal-regulated kinase 1 and 2 (ERK1/2), which are central cell regulators being involved in protein synthesis, cell proliferation and differentiation [26].

Recently we have identified a number of FX mutants in patients with mild to severe FX deficiency, one of the rarest inherited haemorrhagic conditions [27]. Among them, two variants of the catalytic domain (FX-342A, FX-381D) and one of the GLA domain (FX-19A) were chosen as models to investigate the correlation between the enzymatic activity and the signal transduction capacity of FXa.

Materials and methods

Cell culture

Murine myoblasts C2C12 (CRL-1772) were obtained from the ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 4 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (Gibco, Gaithersbur, MD) and maintained at 37 °C in a 5% CO_2 atmosphere.

Expression and purification of rFX variants

Expression vectors for recombinant FX (rFX) variants were created by site-directed mutagenesis of the human FX cDNA cloned into the pCMV4 vector through the site-directed Quick-change mutagenesis kit (Stratagene, la Jolla, CA, USA). As a control we also expressed the active site variant FX-379A. To create stably expressing cells, Human Embryonic Kidney cells (HEK 293), known to properly carry out the extensive post-translational modifications of FX, were co-transfected with pCMV4-FX and pCDNA3, providing the neomycin resistance gene, as previously described [28]. Upon selection with G-418, the highly expressing clones were expanded in a cell factory system. The rFX proteins in conditioned media were purified by ion-exchange followed by immunoaffinity chromatography as previously described [29]. FX concentration was determined by measuring OD at 280 nm (extinction coefficient 1.16 mL mg-1 cm-1) and protein purity was assessed by staining with Coomassie brilliant blue after 12% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE).

Activation and activity of rFX variants

Purified rFX was activated by Russell's viper venom (RVV, Haematologic Technologies Inc, Essex Junction, VT), a potent non-physiological FX activator [30], at 1:200 molar ratio (RVV/FX), for 15 min at 37 °C in the presence of 8 mM $CaCl_2$. Activation of rFX variants by RVV was estimated through Western blotting analysis [31].

The amidolytic activity of rFXa was assayed toward the FXa fluorogenic substrate (Spectrofluor™ Xa, CH_3SO_2-D-CHA-Gly-Arg-AMC.AcOH; American Diagnostica, Greenwich, CT).

Thrombin generation activity was carried out in diluted (1:20) FX-depleted plasma supplemented with either RVV-activated rFX (see above) or 10 nM zymogen rFX variants. In the latter condition, coagulation was triggered via the extrinsic pathway by using Innovin (Dade-Behring, Marburg, D) as source of TF, phospholipids and calcium [31]. Thrombin generation was evaluated by adding 300 µM thrombin fluorogenic substrate (Benzoil-Phe-Val-Arg-AMC; MP Biomedicals, Costa Mesa, CA). Fluorescence (360 nm excitation, 465 nm emission) was monitored over time at 37 °C in a continuous model on a SpectraFluor Plus microplate reader (Tecan, Salzburg, A) and analysed with the software Magellan.

The initial rate of thrombin generation was used to calculate the relative activity of rFX/FXa variants. Serial dilutions of rFX-wt or rFXa-wt were exploited to create standard curves and to extrapolate relative activity (% of wt) of variants.

Cell stimulation

C2C12 cells were grown at 70% confluence in 12-well plates, washed three times with phosphate buffered saline (PBS) and serum-starved for 4 hours prior to stimulation. rFXa variants were then added to the medium as indicated in figure legends. FCS (20%), RVV and zymogen FX were used as independent control assays. Upon removal of medium, cells were rapidly lysed in 100 mM Tris/HCl, 4% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue, pH 6.8, and scraped. Cell lysates were stored at −20 °C until use.

Western blot analysis of ERK1/2 phosphorylation

Cell lysates were incubated 5 min at 95 °C, and 20 µl samples were run on 10% SDS-PAGE. Proteins were transferred onto an immobilon-P PVDF membrane (Millipore, Billerica, MA), which was blocked with TRIS-buffered saline supplemented with 0.1% Tween-20 (TBS-T) and 3% low-fat milk powder. Membranes were then incubated overnight at 4 °C with primary antibodies directed to phosphorylated ERK1/2 and total ERK1/2 (Cell Signalling Technology, Beverly, MA) in TBS-T. The detection was carried out with a horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation, Glostrup, DK).

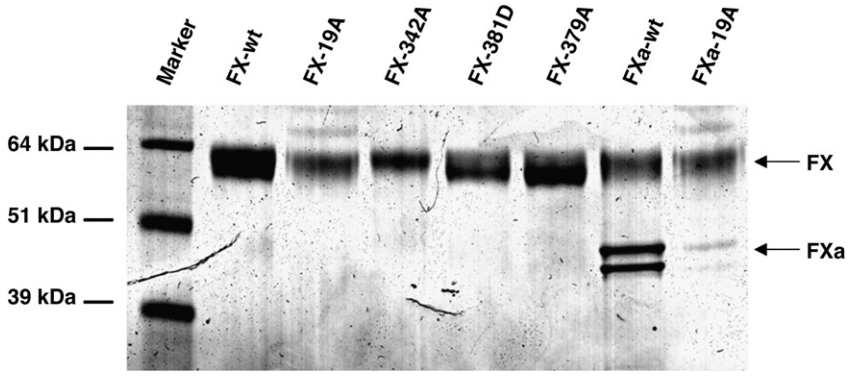

Fig. 1. SDS-PAGE analysis of purified FX molecules. Purified rFX molecules were separated by 12% SDS-PAGE and visualized by Coomassie staining. Bands of 59 kDa represent the zymogen form of each FX molecule. Bands of 49 kDa and 45 kDa represent the RVV activated forms, α and β respectively, of rFXa-Wt and rFXa-19A.

Images were acquired with a cooled CCD camera (Laser 3000, Luminescent image analyser, FUJIFILM, Düsseldorf, D) after incubation of membrane with the Lumilight Plus ECL substrate (Roche, Basel, CH). Chemiluminescence intensities were quantified by laser densitometry using AIDA analyzer software. Fold-induction (mean ± S.E.M.) of phosphorylated ERK1/2 was normalized for the amount of total ERK1/2.

Results

Activity of purified recombinant FX mutants towards synthetic substrate and prothrombin

Selected natural amino acid substitutions (V342A, G381D, E19A) that do not impair FX biosynthesis or secretion, as indicated by protein levels in media from transiently transfected cells, were characterized for their effect on the FX activity toward synthetic substrates or prothrombin. For this purpose, each variant was stably expressed and purified. Evaluation through SDS-PAGE confirmed the presence of highly purified products that migrated as a single band with a molecular mass of 59 kDa (Fig. 1).

Activation by RVV showed a similar electrophoretic pattern for rFX-wt, rFX-342A, rFX-381D and rFX-379A (as control). Differently, rFX-19A showed reduced activation (5-10% of rFX-wt), as evaluated by densitometric analysis of the FXa-α form (49 kDa) (Fig. 1). This finding was unexpected since our previous studies in plasma from the FX-19A homozygous patients and with the recombinant protein in conditioned medium from transiently transfected cells suggested a normal activation of the rFX-19A by RVV [31]. Whereas the presence of other proteins in our purified preparation (Fig. 1) is unlikely, the very different experimental conditions, and particularly the purification steps, might contribute to explain the discrepancy between our previous and present data.

The activity of rFX variants, also obtained by extrinsic activation in plasma, is summarized in Table 1.

The RVV-activated rFX-342A variant showed a moderately reduced activity toward the peptidyl substrate (28.6 ± 1.9% of rFXa-wt) and a roughly proportional thrombin generation activity in plasma (38 ± 8.1%), which was confirmed after extrinsic activation (36 ± 0.4%).

The rFX-19A variant showed appreciable thrombin generation (6.8 ± 2.3%) and amidolytic (11.8 ± 0.3%) activities after RVV activation. It showed undetectable enzymatic activity toward prothrombin after extrinsic activation.

The G381D substitution markedly impaired amidolytic and thrombin generation activity. In addition, we produced mutant of the active site rFX-379A (substitution of the catalytic serine) as negative control that, as expected, did not show any enzymatic activity.

Table 1
Pro-coagulant, amidolytic and thrombin generation activities of rFX mutants.

FX mutant	FX:C	Amidolytic activity after RVV activation	Thrombin generation activity in FX depleted plasma after RVV activation	Thrombin generation activity in FX depleted plasma after extrinsic activation
FX-342A	73[b]	28.6 ± 1.9	38 ± 8.1	36 ± 0.4
FX-19A	<1 (17)[a]	11.8 ± 0.3	6.8 ± 2.3	n.d.
FX-381D	<1[a]	n.d.	n.d.	n.d.
FX-379A	n.d.	n.d.	n.d.	n.d.

FX coagulant activity (FX:C, as percentage of pooled normal plasma) was measured in plasma of homozygous ([a]) or heterozygous ([b]) patients by prothrombin time based assays after extrinsic or RVV activation (in brackets). The amidolytic and thrombin generation activities of rFXa mutants are expressed as percentage of the rFXa-wt, and reported as mean ± standard deviation from three independent experiments. n.d., not detectable [28,31,35].

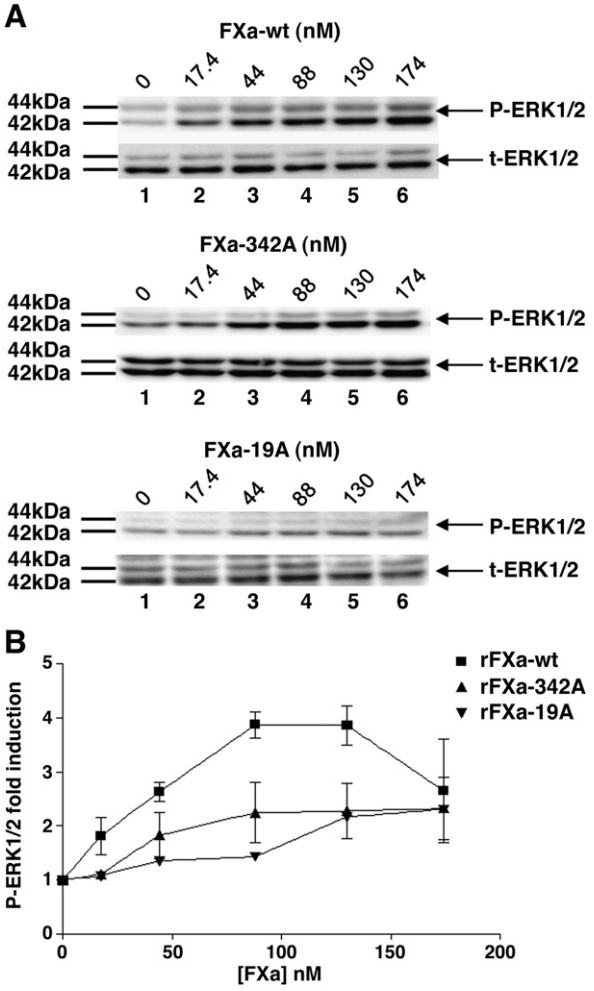

Fig. 2. Dose-dependent induction of ERK1/2 phosphorylation in C2C12 cells by rFXa variants. (A) Western blot analysis of ERK1/2 phosphorylation induced in C2C12 cells by 0 to 174 nM of rFXa (lanes 1 to 6) for 30 minutes. Total ERK1/2 (t-ERK1/2) was used as loading control. (B) Fold-induction (mean ± S.E.M.) of phosphorylated ERK1/2 (P-ERK1/2) by increasing concentrations of rFXa variants, upon normalization for the amount of total ERK1/2. Induction was calculated by densitometric analysis of bands normalized to t-ERK1/2, in three independent experiments.

ERK1/2 phosphorylation induced by FXa variants in C2C12 cells

The signalling properties of the recombinant natural FX variants were evaluated in C2C12 cells in which we have recently demonstrated that FXa signals via PAR-2 [24].

We first examined the dose-responsiveness of FXa-induced signal transduction by incubating serum-starved cells with concentrations of either rFXa-wt or mutant rFXa (from 0 to 174 nM) similar to those already exploited to assess PAR signalling [11,25]. As shown in Fig. 2, stimulation of C2C12 cells with a concentration as low as 17.4 nM (lane 2) of rFXa-wt for 30 minutes was sufficient to trigger ERK1/2 phosphorylation, as compared to cells stimulated with PBS (lane 1). The rFXa-342A and rFXa-19A variants induced a dose-dependent ERK1/2 phosphorylation, but an appreciable signal was only detectable at 44 nM and above 100 nM FXa, respectively. Maximal phosphorylated ERK1/2 levels induced by these variants were reduced to about 50% as compared to rFXa-wt (Fig. 2). As expected, the active site mutant rFXa-379A did not induce ERK1/2 phosphorylation even at the highest concentration (174 nM for 30 minutes, data not shown).

To determine the time dependency of rFXa-mediated ERK1/2 phosphorylation we stimulated C2C12 cells with 174 nM of each variant (Fig. 3). For rFXa-wt, phosphorylation of ERK1/2 was

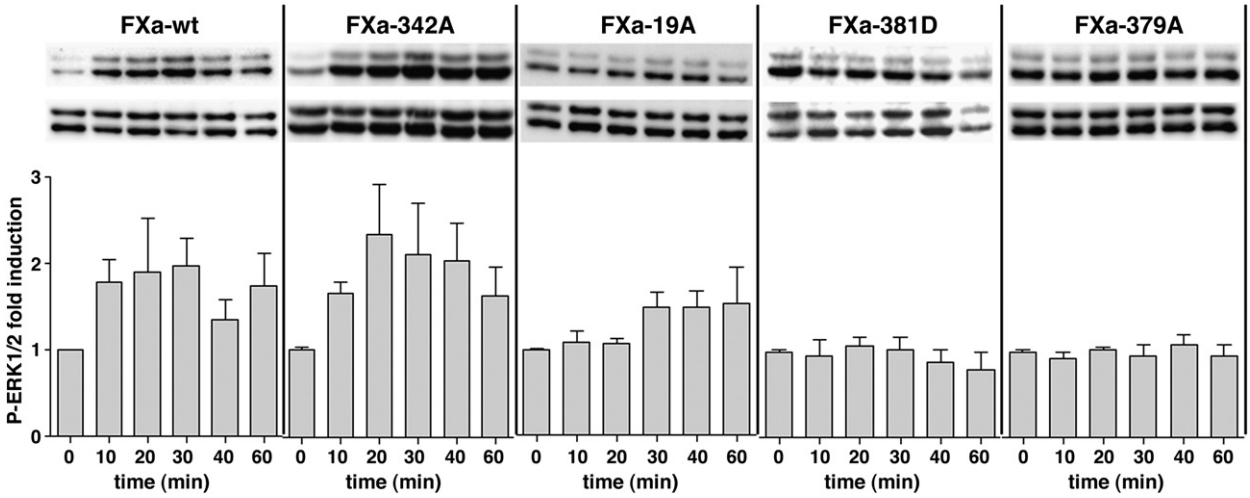

Fig. 3. Time-dependent induction of ERK1/2 phosphorylation in C2C12 cells by rFXa variants. Upper. Western blot analysis of ERK1/2 phosphorylation for 0 to 60 minutes induced by 174 nM rFXa. Total ERK1/2 (t-ERK1/2) was used as loading control. Bands of 42 and 44 kDa, corresponding to phosphorylated ERK1/2 (P-ERK1/2) and t-ERK1/2, are shown. Lower. Fold induction (mean ± S.E.M.) of P-ERK1/2 at the different time points. Calculations were made by densitometric analysis of bands, upon normalization for the amount of t-ERK1/2, in three independent experiments.

detectable at 10 minutes, peaked at 30 minutes and was still present after 60 minutes stimulation. The fold induction of phosphorylated ERK1/2 by rFXa-wt was comparable to that obtained by plasma-derived FXa at the same concentration (174 nM) (data not shown).

The efficiency and time-dependence of rFXa-342A-induced activation of ERK1/2 was comparable to that of the rFXa-wt. In contrast, the rFXa-19A variant showed a reduced signalling capacity and a delayed response, with an appreciable phosphorylation of ERK1/2 detectable only after 30 minutes of stimulation. The rFXa-381D mutant was not able to induce ERK1/2 phosphorylation, thus behaving like the inactive rFXa-379A mutant. Zymogen rFX-wt, even at the highest concentration and longest incubation time tested (174 nM for 60 minutes), did not show any appreciable signalling activity (not shown).

Discussion

Although FXa-mediated signalling has been demonstrated in different cellular models [15,21,23–25], and a number of FX mutants has been described in FX deficiency [27], the relationship between FXa pro-coagulant activity and the signal transduction capacity has not been investigated yet. The comparison between activity in the coagulation pathway and cellular effects has successfully been pursued for a few thrombin [32] or activated protein C [33] recombinant variants.

Among naturally occurring FX mutations only those compatible with normal biosynthesis and secretion, representing a minor fraction, represent appropriate tools to address this issue.

The molecular characterization of several FX deficient patients in our laboratory provided us with three dysfunctional natural FX variants characterized by amino acid substitutions that, through different molecular mechanisms, are responsible for mild or severe reduction of pro-coagulant activity. These naturally-occurring FX variants were exploited in the current study which was focused on the relationship between FX coagulant and cell signalling activity.

As cellular model to evaluate the signalling properties of FXa variants we selected myoblasts, key determinants of vascular pathology in which FXa-induced intracellular signalling via PAR-2 has been demonstrated [24].

Incubation of these cells with the virtually inactive rFXa-381D, found to be associated with severe hemorrhagic symptoms and a barely detectable coagulant activity (<1%) [28], indicated that an appreciable albeit some pro-coagulant activity is required for signalling in myoblasts. In fact, the signal transduction efficiency mediated by this variant was indistinguishable from that of the rFXa-379A mutant, which lacks a functional catalytic triad. This finding is consistent with previous data with active-site inhibitors of FXa (i.e. ZK-807834), which demonstrated the direct dependence of FXa signalling in vascular wall cells from its proteolytic function [34].

Compared to rFXa-wt, two other investigated variants exhibited apparently reduced cellular signalling: one (rFX-19A) with a mutation affecting the GLA domain and one (rFX-342A) with a catalytic domain mutation.

The rFXa-342A mutant, found in the heterozygous condition in a patient with mild FX deficiency [35], showed a comparable reduction in pro-coagulant and signal transduction activity, as indicated by dose dependency experiments. The rFX-19A mutant, found to be associated in the homozygous condition with a moderate reduction of FX antigen and markedly reduced FX coagulant activity upon extrinsic activation [31], revealed a low thrombin generation activity and a reduced signalling capacity. Although the evaluation of the signalling properties of this mutant is complicated by the impaired RVV-mediated rFX-19A activation, the resulting limited amount of rFXa-19A appears to account for the observed ERK1/2 phosphorylation and pro-coagulant activity.

This observation suggests that the activated rFX-19A variant triggered PAR-2 intracellular signalling with an appreciable efficiency. We infer that the alteration of the FXa GLA domain produced by the E19A substitution, not affecting the catalytic activity, is compatible both with a residual coagulant activity and PAR-2 activation.

Taken together, our data obtained with naturally occurring FX variants suggest that the signalling capacity of rFXa in myoblasts is roughly proportional to its pro-coagulant activity. Extending this approach to other dysfunctional FX variants showing inconsistencies between pro-coagulant and signalling activities might allow highlighting exosite-dependent interactions mediating these processes.

Conflict of interest statement

The authors declare no conflict of interest.

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Chronic sleep deprivation markedly reduces coagulation factor VII expression

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ABSTRACT

Chronic sleep loss, a common feature of human life in industrialized countries, is associated to cardiovascular disorders. Variations in functional parameters of coagulation might contribute to explain this relationship. By exploiting the mouse model and a specifically designed protocol, we demonstrated that seven days of partial sleep deprivation significantly decreases (-30.5%) the thrombin generation potential in plasma evaluated upon extrinsic (TF/FVIIa pathway) but not intrinsic activation of coagulation. This variation was consistent with a decrease (-49.8%) in the plasma activity levels of factor VII (FVII), the crucial physiologicalal trigger of coagulation, which was even more pronounced at the liver mRNA level (-85.7%). The recovery in normal sleep conditions for three days completely restored thrombin generation and FVII activity in plasma.

For the first time, we demonstrate that chronic sleep deprivation on its own reduces, in a reversible manner, the FVII expression levels, thus influencing the TF/FVIIa activation pathway efficiency.

Key words: Hodgkin, transplantation, refractory.

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Introduction

Chronic sleep debt affects millions of people in more developed countries and it is emerging as a co-factor in the development of metabolic and endocrine dysfunctions, as well as of cardiovascular and cerebrovascular pathologies.[1-5] One of the well-characterized causes of chronic partial sleep deprivation (PSD), sleep apnea, has been clearly associated with cardiovascular disease.[6-8]

Among many factors, variations in the plasma levels of key proteins able to shift the hemostatic balance might contribute to the association between sleep deprivation and cardiovascular disorders. A number of studies have reported the association between levels of procoagulant (soluble Tissue Factor, fibrinogen, von Willebrand factor) and anti-fibrinolytic (Plasminogen activator inhibitor-1) molecules and sleep apnea.[9-14] On the other hand, little information is available on the coagulant impact of sleep deprivation on its own.[15]

In this paper we exploited the mouse model and a well established protocol to induce partial sleep deprivation[16] to demonstrate that it strongly affects, in a reversible manner, the expression levels of factor VII (FVII), the serine-protease triggering the coagulation process.[17]

Design and Methods

Animal colony, maintenance and housing conditions

Experiments were performed with C57BL/6J mice (n=44; Jackson Laboratory, Bar Harbor, ME, USA) kept in a 12h light : 12h dark cycle (LD 12:12; lights on at 07:00). It is conventional to divide the 24-hour LD cycle into 24 one-hour Zeitgeber time (ZT) units and indicate the time of lights on as ZT0 and the time of lights off as ZT12. Mice had free access to food and water. The housing and sleep recording environments were sound attenuated and temperature controlled (21°C). The sleep deprivation wheels are 9.0 inch diameter stainless steel rotating wheels (Nalgene, Pittsburgh, PA, USA). Each one is fixed between a solid steel plate on one side and a clear piece of plastic on the other. The wheel cages are designed to allow simultaneous wheel rotation and free access to food and water. Wheel speed was maintained at 1.0 or 1.7 revolutions per minute (r.p.m.).

Experimental design

Mice (n=18) underwent 20h of sleep deprivation using a slowly rotating wheel (1.0 r.p.m. x 20h/day = 1,200 total wheel revolutions/day) after which they were transferred to their home cage for a 4-hour sleep opportunity in the first part of the light phase from ZT0 to ZT4. Mice were subdivided in 3 groups (n=6 each group) and

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subjected to three days of sleep deprivation, seven days of sleep deprivation or seven days of sleep deprivation followed by a recovery in normal conditions for three days. Non-sleep deprived mice (n=18; 6 for each sleep deprived group) were placed in a non-rotating wheel for 20h/day and in a home cage for 4h/day for experimental control over the recording environments.

A second group of mice (n=8) was exploited to investigate the effect of forced activity on coagulation. A group (n=4) was subjected for seven days to forced activity (1.7 r.p.m.) for 12h/day from ZT12 to ZT24. This daily amount of activity was equivalent to the amount of activity performed by mice subjected to partial sleep deprivation (1,224 wheel revolutions/day). Mice were subsequently transferred to their home cage for a 12-hour sleep opportunity during the light phase (from ZT0 to ZT12). The control mice (n=4) were housed for seven days in a non-rotating wheel for 12h/day and in a home cage for 12h/day.

The last day of treatment, at ZT0, all experimental and control mice were anesthetized with isoflurine, subjected to retro-orbital bleeding to isolate plasma, and then sacrificed to isolate livers, as previously described.[18]

Mice subjected to sleep deprivation showed a modest reduction in weight (about 2-3% after three days and about 5-6% after seven days of PSD). The mice regained most of the weight loss after three days of recovery. All treatments were conducted under the guidelines established by the Institutional Animal Care and Use Committee of the Morehouse School of Medicine.

Functional assays in plasma

Thrombin generation assays, optimized to evaluate the efficiency of the extrinsic or the intrinsic activation coagulation pathways, as well as the FVII activity assays were conducted in mouse plasma as previously described.[18-21] Student's t-test was used to determine significant differences ($P<0.05$).

q-PCR

DNase-treated total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and used to perform cDNA synthesis (iScriptTM cDNA synthesis kit, Biorad, Milan, Italy). cDNA was PCR-amplified with a Chromo4 real-time PCR Detection System using iQTM SYBR Green Supermix (Biorad, Milan, Italy). Primers for mouse FVII mRNA quantifications were 5'-GACTTTGACGGTCGGAACTGTG-3' and 5'-GCGGCTGCTGGAGTTTCTTT-3'. Mouse GAPDH (5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACA-CATTGGGGGTAGGAACA-3') was used as housekeeping gene. The comparable amplification efficiencies of FVII and GAPDH transcripts prompted us to exploit the comparative threshold cycle method (CT), as previously described.[18] Each CT value used for these calculations is the mean of three replicates of the same reaction. One-way ANOVA was used to determine significant differences ($P<0.05$) and the Dunnett's *post hoc* test was applied to compare experimental groups with the control group.

Results and Discussion

We have previously shown that the circadian clock plays an important role in the modulation of the coagula-

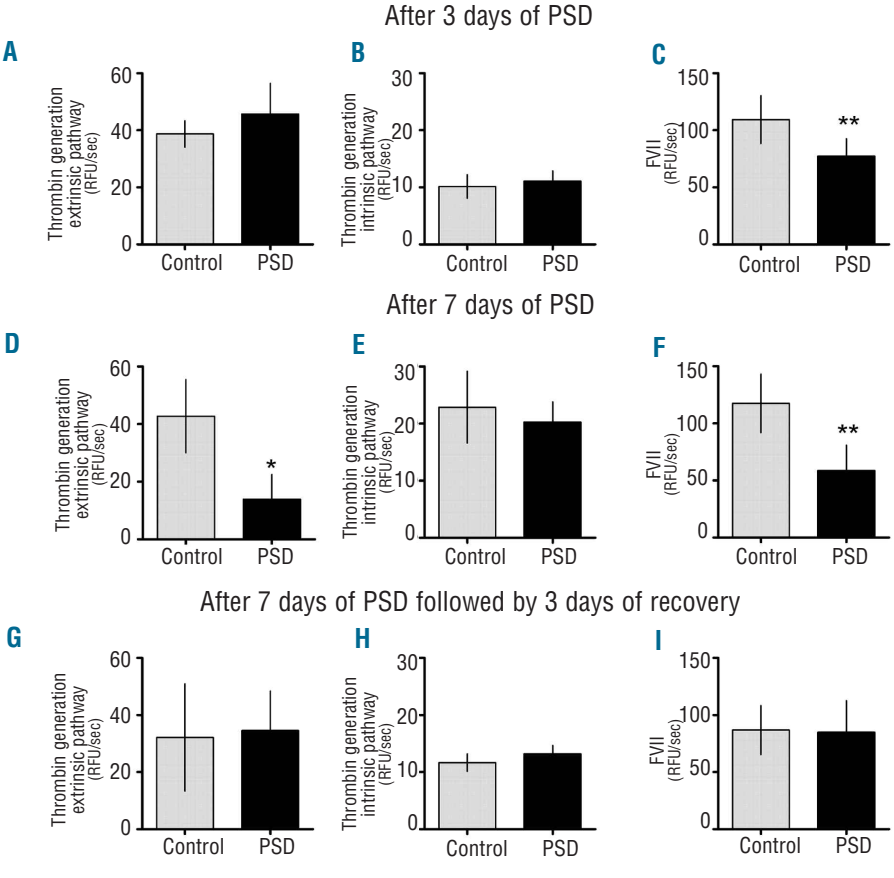

Figure 1. Effects of PSD on thrombin generation activity levels upon extrinsic (A,D,G) or intrinsic (B,E,H) activation, and on FVII activity (C,F,I) levels. For thrombin generation assays, the coagulation in diluted mouse plasma (1:40) was triggered via the extrinsic or the intrinsic pathway by adding an excess of Innovin™ (Dade Behring, Deerfield, IL) as a source of tissue factor, calcium, and phospholipids, or aPTT reagent (Actin-FS, Dade Behring, Germany) as a source of cephalin and negatively charged surfaces, respectively. The fluorogenic substrate (200 μM) for thrombin (Benzoil-Phe-Val-Arg-AMC; ICN Biomedicals, Costa Mesa, CA, USA) was then added and relative fluorescence units (RFU) monitored over time. FVII activity was evaluated by measuring the generation of activated factor X (FXa) in mouse plasma diluted 1:40 in human FVII depleted plasma (Dade Behring). Upon triggering of coagulation with an excess of Innovin, the fluorogenic substrate (200 μM) for FXa (MeSO2-D-CHA-Gly-Arg-AMCAcOH; American Diagnostica, USA) was added and relative fluorescence units monitored over time. In each assay the activity was evaluated as the initial rate expressed as RFU per second. Values represent the mean ± SEM of 6 samples per group (*$P<0.01$; **$P<0.001$).

tion cascade efficiency, mainly by modulating the TF/FVIIa pathway.[18-21] Since sleep and wake cycles and the circadian system closely interact with each other, we investigated whether partial sleep deprivation has any effect on the thrombin generation activity in plasma, a parameter defining the coagulation cascade efficiency.[22]

To this purpose, we exploited the mouse model and a recently developed PSD protocol.[16] This is a very effective method to induce partial sleep deprivation in rodents and although micro-sleep events may occur, animals lose about 60% of their normal daily sleep during the PSD protocol (KN Paul, unpublished data, 2009).

In mice subjected to three days of partial sleep deprivation, we did not observe a statistically significant variation of thrombin generation activity levels in plasma, neither upon extrinsic nor intrinsic activation of coagulation (Figure 1A and B; $P>0.1$ and $P>0.4$, respectively). When the PSD period was prolonged to seven days, we detected a significant reduction (-30.5 %; $P<0.01$) of thrombin generation activity levels upon extrinsic activation (Figure 1D). Conversely, upon intrinsic activation, these levels were comparable to those of control mice (Figure 1E).

To corroborate these findings we monitored the thrombin generation activity upon extrinsic trigger in the PSD mice after three days of undisturbed sleep and found that it returned to levels that were undistinguishable from those of controls (Figure 1G-I; $P>0.1$).

These data indicate that partial sleep deprivation significantly affects, in a reversible manner, the efficiency of the extrinsic activation pathway, characterized by the activity of the TF/FVIIa complex. Since, in our functional assay, TF is added in excess to mouse plasma, the variation of FVII activity levels likely explains the observed phenomenon. Other features of FVII support our mechanistic hypothesis: 1) FVII has a very short half-life (2-4h) which allows quick level fluctuations over time; and 2) FVII gene expression is directly controlled by the circadian clock machinery[18] which is strongly interlaced with the sleep/wake mechanisms.

In accordance with this hypothesis, the activity levels of FVII in plasma from mice subjected to three and seven days of partial sleep deprivation were reduced by 29.2% (Figure 1C; $P<0.001$) and 49.8% (Figure 1F; $P<0.001$) when compared to controls, respectively. Furthermore, three days of undisturbed sleep was able to completely restore FVII activity levels (Figure 1I; $P>0.1$) in line with the short half-life of this factor.

To corroborate this finding and to provide insights into the regulation at the transcriptional level, we investigated FVII gene expression in liver mRNA previously isolated from PSD mice (Figure 2). We observed a clear relationship between PSD conditions and a reduction in FVII mRNA levels in the liver, particularly after seven days (-85.7%;

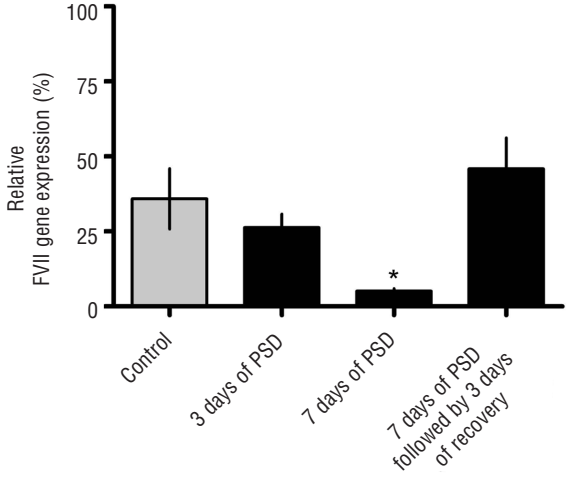

Figure 2. Liver FVII mRNA expression in PSD mice. Values were normalized with respect to the maximum value (100%) measured for each condition. The mean ± S.E.M. is shown. Dunnett's *post hoc* test was applied. No statistical differences in FVII mRNA expression were found among control groups ($P>0.50$), indeed the data for the 3 control groups of mice (N=18) were pooled. (*$P<0.01$).

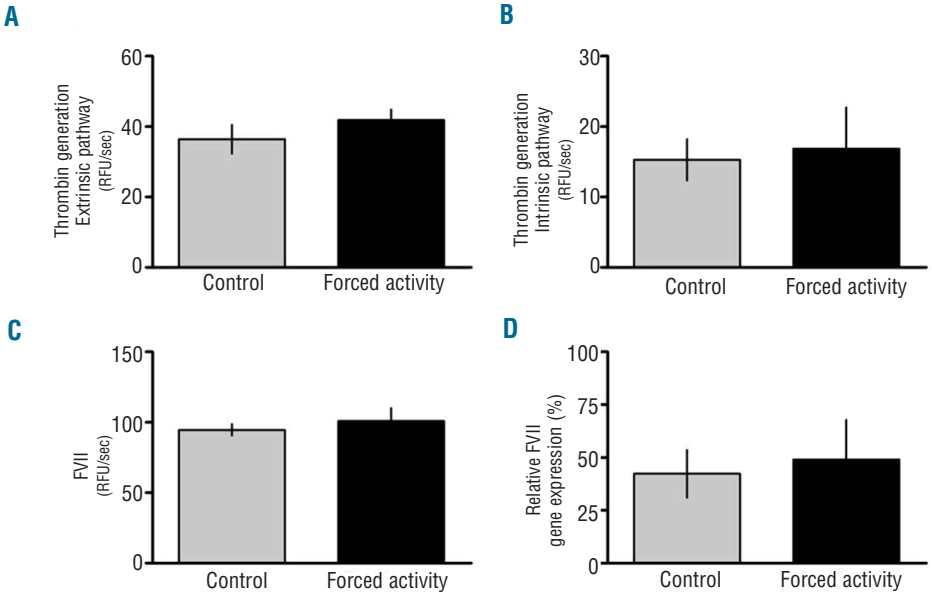

Figure 3. Effects of forced activity on thrombin generation activity levels upon extrinsic (A) or intrinsic (B) activation, on FVII activity levels (C) and on liver FVII mRNA expressions (D). Values represent the mean ± SEM of 4 samples per group. The coagulation parameters in the control group were not statistically different from those measured in the control mice in the PSD study ($P>0.1$ in all cases).

$P<0.01$). As observed for FVII activity, the normal FVII mRNA expression was restored by three days of undisturbed sleep.

One might argue that the variations reported here are due to increased levels of physical activity rather than lack of sleep. We, therefore, investigated the effect of forced activity on thrombin generation and FVII activity levels. The forced activity (12h/day from ZT12 to ZT24 for seven days) did not result in a significant change in any of the parameters under investigation (Figure 3 A-D; $P>0.1$ in all cases) thus supporting our main findings on the effects of partial sleep deprivation.

Very recently, Liu *et al.*[15] suggested that a one-day sleep deprivation results in a slight shortening of coagulation times (PT, APTT) in 10 healthy humans. Other studies, mainly focused on sleep apnea patients, reported a positive association between daily sleep disturbance/reduction and levels of specific prothrombotic factors (i.e. soluble Tissue Factor, von Willebrand factor, fibrinogen)[9-10,12-14,23] thus supporting a relationship between sleep deprivation and cardiovascular risk. Our data do not fit this hypothesis and would instead support a counteracting mechanism. We could speculate that the reduction of FVII levels may combine with the hemostatic balance through compensatory mechanisms operating in the coagulation/hemostasis pathways. A similar mechanism has been suggested by the parallel temporal oscillations in levels of FVII and of its direct inhibitor tissue factor pathway inhibitor.[19] Further studies aimed at evaluating the impact of sleep deprivation on plasma levels of a wide panel of coagulation and fibrinolytic factors are needed to address the physiopathology of this complex pathway.

For the first time, through the use of a well controlled animal model and a specifically designed experimental protocol, we demonstrated that chronic sleep deprivation on its own strongly affects and reduces, in a reversible fashion, the expression levels of FVII, thus influencing the TF/FVIIa activation pathway efficiency. These data further highlight the complexity of the modulation of the clotting cascade.

Authorship and Disclosures

MP, CB, FB, KNP and GT designed research; KB, SC, EF, NC, AB and JCE performed research; MP, CB, KNP, KB, SC, EF, NC, AB and GT analyzed and interpreted data; MP, CB, KNP, JCE, FB and GT wrote the paper.

The authors declare no conflict of interest.

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