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Molecular characterization of a factor VII deficient patient supports the importance of the second epidermal growth factor-like domain

GIOVANNA D'ANDREA
ANNA BOSSONE
MARIA ROSARIA LUPONE
FLORA PEYVANDI
GIOVANNA MAISTO
FABIO PERRICONE
ELVIRA GRANDONE
MAURIZIO MARGAGLIONE

A B S T R A C T

Background and Objectives. Although a large number of gene mutations have been characterized in patients with factor VII (FVII) deficiency, few naturally occurring mutations have been described in epidermal growth factor (EGF)-like domains. We investigated a 6-year old Italian girl who had low functional and antigenic FVII plasma levels.

Design and Methods. Plasma levels of FVII activity and antigen were evaluated in the propositus and her relatives. Mutation screening was performed by sequencing the FVII gene. The effect of the identified FVII mutations was investigated by protein expression in transfected cells.

Results The propositus was shown to be a compound heterozygote for a known (Arg110Cys) and a novel (Asp123Tyr) missense mutation both occurring in the second EGF-like domain. In transfected cells, expression of the Arg110Cys mutation reduced the amount of intracellular and secreted FVII protein (48% and 18%, respectively). Likewise, cells transfected with the Asp123Tyr mutation gave rise to low intracellular (40%) and extracellular (4%) FVII antigen levels. In conditioned media, FVII procoagulant activity was reduced accordingly (10% and <1%, respectively).

Interpretation and Conclusions Transient expression of the identified FVII mutations caused severely reduced but detectable FVII antigen and activity levels. The present findings suggest that the two naturally occurring missense mutations identified within the second EGF-like domain severely affect FVII protein handling, impairing the correct folding of FVII.

Key words: factor VII, gene, mutations, EGF-like domain, bleeding.

From the Unita' di Aterosclerosi e Trombosi, I.R.C.C.S. "Casa Sollievo della Sofferenza", S. Giovanni Rotondo (GDA, AB), Centro Regionale per le Emocoagulopatie, Azienda Ospedaliera "Santobono-Pausillipon" (MRL, GM, FP), "A. Bianchi Bonomi" Hemophilia and Thrombosis Center, Department of Internal Medicine, University of Milan (FP), Genetica Medica, Dipartimento di Scienze Biomediche, Università di Foggia, Italy (MM).

Correspondence:
Maurizio Margaglione, MD,
Cattedra di Genetica Medica,
Dipartimento di Scienze Biomediche, Università degli Studi di Foggia, viale Pinto, Foggia 71100, Italy.
E-mail: m.margaglione@unifg.it

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Human coagulation factor VII (FVII) deficiency is a rare, autosomal recessive trait first described in the early 1950s.¹ It produces severe deficiency in a homozygous state and a moderate deficiency, usually without clinical manifestations, in heterozygotes.² Subjects with FVII deficiency show a wide range of symptoms (epistaxis, menorrhagia, spontaneous and post-traumatic bleeding, etc.) that in some instances correlates poorly with the extent of the laboratory abnormalities.³ Since the first case of FVII deficiency described by Alexander *et al.*,¹ several patients have been described and a high number of different naturally occurring mutations have been reported⁴ (for further details see the factor VII database at: <http://europium.csc.mrc.ac.uk>). Most of these mutations are point mutations that severely affect FVII biosynthesis and give a concordant reduction of circulating levels

of both functional and immunological activity, but others allow for the presence in the bloodstream of an abnormal protein with impaired activity.^{2,5}

FVII is a vitamin K-dependent glycoprotein that, together with the tissue factor in the extrinsic pathway, triggers blood coagulation by activating factor IX and factor X.^{6,7} After binding to tissue factor, FVII activation occurs and the resultant two-chain active protein is composed of a light chain and a heavy one linked by a disulphide bond. FVII belongs to a family of homologous proteins that share a common domain organization, involving a N-terminal carboxylglutamic acid-rich (Gla) domain, 2 epidermal growth factor (EGF)-like domains (light chain) and a C-terminal serine protease domain (heavy chain).⁸ The serine protease domain contains the active site and is responsible for the activation of FX and FIX.⁹ On the other hand, the

Gla domain within the light chain confers the ability to bind to membrane containing negatively charged phospholipids. The first EGF-like domain shares, with the protease domain, the main sites of contact with tissue factor.^{10,11} The second EGF-like domain does not appear to play a major role in the ability of FVII to bind tissue factor or to catalyze the activation of other coagulation proteases. Although few naturally occurring mutations have been described in this domain,¹²⁻¹⁶ a similar prevalence of mutations in other domains has been reported.

Actually, 9 mutations out of 42 residues in the second EGF-like domain, as well as 10 at 7 out of 37 amino acids within the first EGF-like domain, have been described. This compares with 64 different mutations identified in the 254 residues of the catalytic domain (<http://europium.csc.mrc.ac.uk>).

We now report the molecular characterization of two naturally occurring mutations within the second EGF-like domain suggesting that these residues play an important role in the correct assembly of the FVII molecule.

Design and Methods

Informed consent was obtained from the proband and her relatives, after approval from the local Human Ethics Committee. The studies were carried out according to the Principles of the Declaration of Helsinki.

Materials

Reagents were of analytical grade or the best available commercial grade.

Blood collection and FVII measurements

Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2,000 g for 15 min to obtain platelet-poor plasma. Platelet-poor plasma was kept frozen (< -80°C) until analysis. FVII activity was measured in a one-stage clotting assay using Thromborel S (Behringwerke AG, Marburg, Germany) and a FVII-deficient plasma (International Laboratories, Milan, Italy). FVII antigen was measured by an enzyme immunoassay (EIA) (Asserachrom FVII:Ag, Diagnostica Stago, Asnières, France) and a normal plasma pool as standard. FVII activity and antigen were expressed as percentages of values measured in pooled normal plasma.

DNA analysis

Isolation of DNA and PCR analysis were performed according to standard procedures.¹⁷ All coding regions and intron/exon boundaries of the FVII gene were amplified using sense and antisense oligonucleotides designed on the basis of the known sequence of the

FVII gene locus (GenBank accession number J02933). Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Site-directed mutagenesis

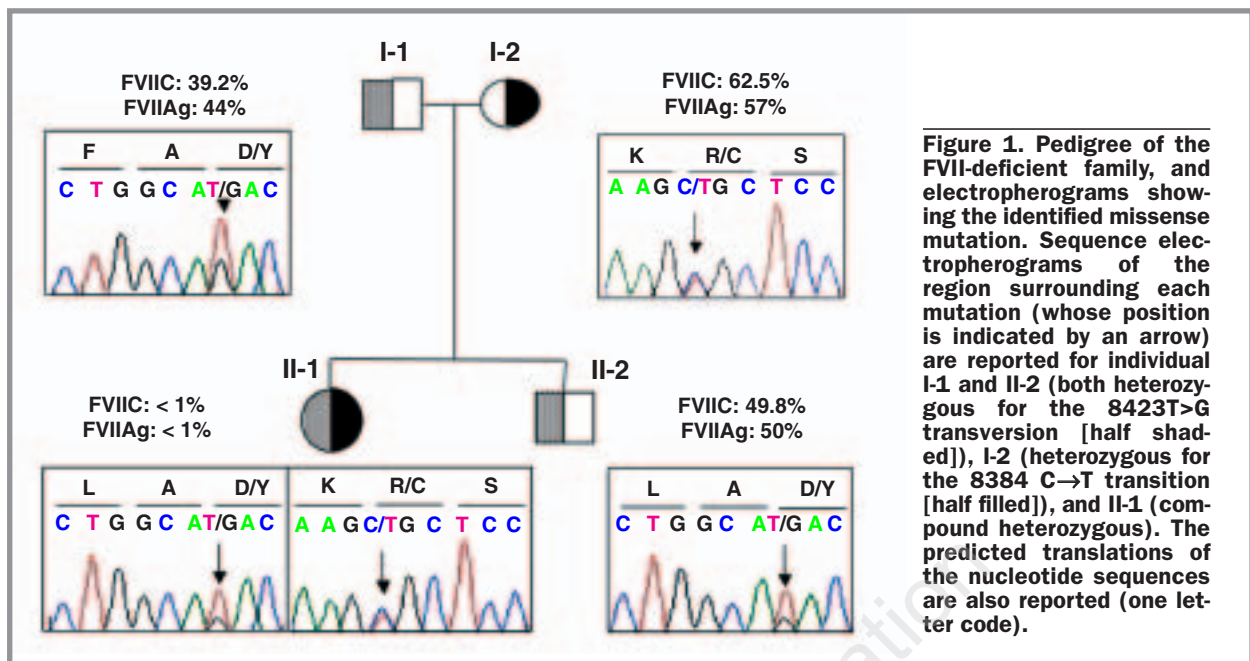
To study the effect of mutations identified, mutagenesis of the pED4 mammalian expression plasmid, containing the full-length FVII cDNA, was made by overlapping PCR. The mutagenic antisense primers (Life Technologies, Paisley, UK) 5'-GCACCGACAGGAG-CACTTGGTGCCCGT-3' (underlined letter indicates the mismatch) corresponding to nucleotide positions 8398-8372 (numbered according to GenBank accession number J02933) and 5'-GCACCGACAGGAGCTCTTGGTGCCCGT-3' were used to replace Arg110 with a Cys or a Ser codon, respectively.¹⁸ A 543bp construct was made using a sense primer (*comFw*), which extended from nucleotide 501 to 518 (5'-CAGCACTGCAGAGATTC-3'). The remaining 3' end of FVII cDNA was obtained employing the sense primer 5'-ACGGGCACCAAGTGCTGTCTCGGTGC-3' (Cys110) or the sense primer 5'-ACGGGCACCAAGAGTCCTGTCTCGGTGC-3' (Ser110) (nucleotide 8372-8398) and the antisense primer *comRev* 5'-TTGGCTTCTCACAGG-3' (nucleotide 11674-11692) to obtain a 939bp product. For the Asp123 to Tyr substitution, a 580bp construct was made using the sense primer *comFw* and a mutagenic antisense primer 5'-GGACACCCCGTATGCCAGCAGAGAGTA-3' (nucleotide 8434-8408). Then, the mutagenic sense primer 5'-TACTCTGCTGGCATACGGGGTGTCC-3' (nucleotide 8408-8434) and the antisense *comRev* were used to obtain a 903bp product. A second round of amplification, using the outermost primers *comFw* and *comRev*, was employed to obtain a 1,455bp product containing the entire FVII coding region. The resulting mutant plasmids were checked by sequencing. Plasmids used in transfection experiments were extracted by the EndoFree Plasmid Midi Kit (Qiagen, Hilden, Germany).

Cell cultures and transfections

COS-7 cells (SV40-transformed green monkey kidney cells) were cultured according to standard procedures.¹³ Transfection experiments were performed using the Lipofectamine 2000 reagent (Invitrogen) essentially as previously described.¹³

FVII measurements in conditioned media and cell lysates

FVII activity and antigen levels were measured 72 hours after transfection in conditioned media and cell lysates by a one-stage clotting assay and EIA. Standard curves were constructed with reference plasma diluted



1:100 to 1:6400 in Tris-buffered saline (50 mM Tris, 150 mM NaCl), pH 7.5. Conditioned media were collected in pre-chilled tubes containing a protease inhibitor (Complete; Roche, Basel, Switzerland), and stored at -80°C , after removal of cell debris by centrifugation. To obtain cell lysates, cells were washed three times with pre-chilled phosphate-buffered saline (PBS) and lysed for 1 hour on ice with lysis buffer containing $1\times$ PBS, 1% Triton X-100, and $1\times$ Complete. Cell lysates were centrifuged to remove cell debris.

Results

Case presentation

The patient was a 6-year old white girl whose parents were not consanguineous (Figure 1). Her family history was negative for a bleeding tendency. She had a moderate bleeding diathesis manifested by easy bruising, hematomas, and bleeding from the mouth. A diagnosis of FVII deficiency was made based on a normal activated partial thromboplastin time (aPTT), a very prolonged prothrombin time (PT), and undetectable FVII function ($>1\%$; normal range: 70–130%) as well as quantitative ($>1\%$; normal range: 50–150%) plasma levels. Both parents of the proband, as well as her brother, had low functional and quantitative levels of FVII (Figure 1).

Genetic characterization

Fragments covering the entire coding region of the FVII gene were amplified from the proband's genomic DNA. All the amplified segments proved to be identical to those obtained in controls except for that spanning

exon 5. Direct DNA sequencing of the PCR product from the patient showed a heterozygous C→T transition at cDNA position 8384 [numbered according to GenBank accession number J02933],¹⁸ leading to an Arg to Cys (CGC→TGC) substitution at amino acid position 110 (numbering omits the signal peptide) (Figure 1). The same mutation was found in the heterozygous state in her mother (I-2) who displayed reduced FVII levels. In addition, a single nucleotide heterozygous transversion (G→T) was identified at nucleotide 8423, leading to an Asp to Tyr (GAC→TAC) substitution at amino acid position 123. The same mutation was found in the heterozygous state in her father (I-1) and in her brother (II-2), who had reduced FVII levels (Figure 1). Screening for both the C→T and the G→T mutations failed to identify these molecular changes in 51 healthy subjects (102 chromosomes).

Transient expression of wild-type and mutant recombinant FVII alleles

To investigate the effect of the Arg110Cys and Asp123Tyr mutations on the secretion of the FVII antigen, recombinant mutated FVII alleles (Cys110 and Tyr123) were transiently and separately expressed in COS-7 cells, which do not express endogenous FVII. The 8384G→A nucleotide substitution was introduced into the pED4/FVII expression plasmid by site-directed mutagenesis to obtain the mutated constructs. Cells were transfected with either the wild-type or one of the mutant constructs. Serum-free media as well as cell extracts were analyzed for the presence of FVII antigen by EIA.

The concentration of FVII antigen in conditioned media of cells expressing the wild-type construct ranged

Table 1. Transient expression of wild-type and mutant FVII constructs.

	Wild-type	Cys110	Ser110	Tyr123
FVII antigen				
Conditioned media ng/mL (%)	410±125	74.0±5.7(18)	193.0±9.3(64)	17.5±0.7 (4)
Cell lysates ng/mL (%)	31.5±2.1	15.0±0.9 (49)	20.0±1.2 (47)	12.5±0.6 (39)
FVII activity Conditioned media %	100	9.7±0.3	39.2±2.6	<1

pED4/FVII (wild-type), pED4/FVII-Cys110, pED4/FVII-Asp123, or pED4/FVII-Ser110 were transiently transfected in COS-7 cells. Antigen levels of recombinant FVII were measured in both conditioned media and in the corresponding cell lysates by an EIA assay, 72 hours after transfection. Results represent means + standard deviation of three independent experiments, each performed in duplicate. The mean value of wild-type FVII is set as 100%.

from 321 ng/mL to 499 ng/mL. However, in conditioned media of cells transfected with pED4/FVII-Cys110 plasmid, an about 5-fold reduction in FVII antigen concentrations was recorded (74.0±5.7 ng/mL) (Table 1). Transfection with the pED4/FVII-Tyr123 plasmid gave rise to extracellular FVII antigen levels about 4% of the wild-type (17.5±0.7 ng/mL) (Table 1). In lysates of cells expressing the mutant alleles, FVII antigen levels were reduced to approximately 50 to 40% (15.0±0.9 ng/mL and 12.5±0.6 ng/mL, in cells transfected with the pED4/FVII-Cys110 or the pED4/FVII-Tyr123 plasmid, respectively) of those measured in cells expressing the wild-type allele (31.5±2.1 ng/mL) (Table 1). The importance of the Arg110 residue was further investigated by expressing a mutated plasmid containing a C-to-A transition (Arg110Ser) at cDNA position 8384. In conditioned media of cells transfected with the pED4/FVII-Ser110, FVII:Ag levels were reduced to 47% of the wild-type, whereas the levels in cell lysates were decreased to 64% (193.0±9.3 and 20.0±1.2 ng/mL, respectively). No FVII procoagulant activity was detectable in conditioned media of cells transfected with the pED4/FVII-Tyr123 plasmid (<1%), whereas the FVII activity of cells transfected with the pED4/FVII-Cys110 (9.7±0.3%) or the pED4/FVII-Ser110 (39.2±2.6%) plasmid was lower than antigen levels found.

Discussion

FVII is crucial for the correct maintenance of hemostasis, since patients presenting with a severe reduction of circulating levels suffer from a moderate to severe bleeding disorder.¹⁹ We have investigated the mechanisms responsible for FVII deficiency in an Italian patient presenting with a moderate bleeding diathesis and undetectable FVII levels (<1%). Sequencing of the entire coding sequence and intron/exon boundaries of the FVII gene revealed that the patient was a compound heterozygote for two missense mutations, Arg110Cys and Asp123Tyr. Both of them occurred within the second EGF-like domain and are highly conserved among different species (Figure 2). This domain is not involved in the direct contact between tissue factor and FVII but is necessary for optimal binding, merely imparting structure to the rest of the molecule.^{10,20} The EGF-like domains have been found to mediate protein-protein interactions. Experimental studies have shown that both the EGF-like and the serine protease domains are essential for the interaction between the tissue factor and FVII.²¹ The second EGF-like and the serine protease domains are known to interact with each other and a synthetic peptide corresponding to the N-terminal part of the second EGF-like domain exerts an antithrom-

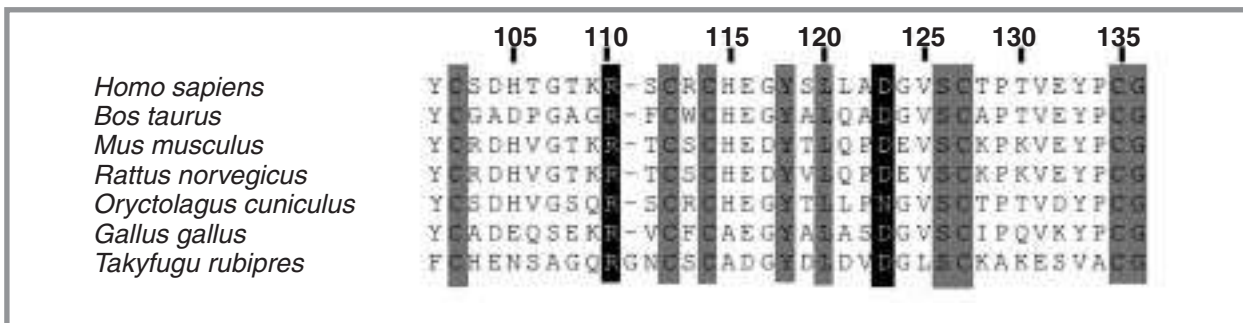


Figure 2. Conservation of Arg110 and Asp123 residues in the second EGF-like domain. Multiple alignment of FVII proteins from different species in the region containing the both Arg110Cys and Asp123Tyr mutations: human, bovine, mouse, rat, rabbit, chicken, and pufferfish FVII (accession numbers NP_062562, I46932, NP_034302, NP_690059, KFB07, AA033363 and AA033368, respectively). Identical residues are highlighted in gray, amino acid Arg110 and Asp123 in the human FVII molecule and the corresponding residues of the other FVII proteins are boxed in black. Numbering does not include the signal peptide and refers to the human sequence.

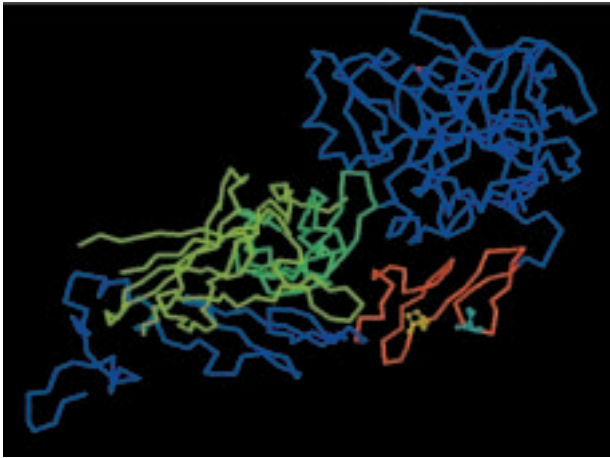


Figure 3. Structural analysis of the FVIIa (1DAN.PBD)¹⁰ using RasMol. The second EGF-like domain is indicated in red. The positions of Arg110 (yellow) and Asp123 (cyan) are indicated as a ball and stick.

botic effect disrupting the interaction between the two FVII domains.²² In keeping with this, FVII-deficient patients carrying a Glu100Arg missense mutation displayed very low to undetectable FVII antigen levels and activity.^{5,13,23} Actually, almost all natural mutations occurring within the second EGF-like domain have been identified between Cys91 and Cys102. To further unravel the role of mutations within this portion of the domain, we performed transient expression studies in COS-7 cells with cDNA encoding FVIIwt, FVIICys110, and FVIITyr123 and showed that the secretion of the two mutant proteins was severely impaired. In conditioned media, cells transfected with the FVIICys110 or FVIITyr123 mutant constructs showed much lower FVII antigen levels, about 5 and 25-fold, respectively, than concentrations measured in conditioned media from cells transfected with the wild-type allele. However, concentrations of FVII antigen in cell lysates of COS-7 cells transfected with the mutant constructs were reduced, by about 40 to 50% of the values found in control cells. Thus, these data are consistent with the possibility that the Arg110Cys and Asp123Tyr substitutions severely impair the processing/stability and the secretion of the FVII molecule causing intracellular degradation of the mutant protein by lysosomal proteolysis or by pre-Golgi or endoplasmic reticulum mechanism.

The Arg110Cys substitution has been recently found in a family with mild to moderate FVII deficiency.¹⁶ The proband was homozygous for the missense mutation and showed FVII activity level of 25% and FVII antigen level of 28% of the normal value, without severe bleeding episodes. These circulating values differ from those (<1%) found in the present patient. On the other hand,

the latter were in agreement with FVII concentrations found in conditioned media. It is likely that differences in other regions of the FVII gene locus, i.e. the promoter, may explain differences in plasma levels. The Arg110Cys mutation implies the substitution of an amino acid with a positively charged polar side chain, arginine, by a different amino acid containing an uncharged polar side chain, cysteine. The Arg 110 residue is located within one of the two hairpins that characterize the second EGF-like domain (Figure 3). Therefore, we speculated that the substitution might lead to an abnormal three-dimensional conformation of the FVII molecule, which would have a significant impact on the protein structure. To confirm this, we analyzed a FVII mutant containing a different amino acid with an uncharged polar side chain, Ser110. The Arg110Ser mutation leads to reduced levels of FVII in cell lysates (64%) and in conditioned media (FVII antigen levels: 47%; FVII activity: 39%). In addition to the different charge of the side chain, cysteine has a thiol group, which may form a disulphide bond with other cysteines through oxidation. The second EGF-like domain contains six cysteines that are arranged to form three intramolecular disulphide bonds, Cys91-Cys102, Cys98-Cys112, and Cys114-Cys127. We postulate that the formation of a new cysteine residue may interfere with the correct formation of disulphide bridges and further severely impair the structural integrity of the FVII molecule.

At variance with this, the Asp123Tyr missense mutation is a novel variant. The Asp123 residue is located in a turn of the second EGF-like domain (Figure 3) and the substitution with a tyrosine implies the presence of an amino acid containing an uncharged polar side chain instead of one with a negative charged polar side chain. The residue is conserved in other vitamin K-dependent coagulation proteins, being an aspartic acid in protein C (Asp129) and in protein Z (Asp121) and an asparagine in factor IX (Asn120), factor X (Asn121), and protein S (Asn155). In all these proteins, the homologous residue is found in a β turn and the substitution of Asn120 in the factor IX resulted in loss of circulating factor IX antigen,²⁴ further supporting a pivotal role of this residue in the correct folding of vitamin K-dependent coagulation proteins. As suggested for the Glu100Arg missense mutation,²³ mutation of FVII residues not directly involved in the interaction with the tissue factor, such as the Asp123Tyr substitution, may result in molecular variants of FVII unable to take part in the initiation of coagulation.

Transient expression of FVII mutations caused reduced but detectable FVII antigen and activity levels, whereas virtually undetectable FVII levels were measured *in vivo*. Since the patient was a compound heterozygote, the two abnormal FVII molecules are expect-

ed to have a joint effect *in vivo*, leading to intermediate FVII levels. The presence of unrecognized different common or rare variants within the FVII gene locus and/or related gene loci, which may modulate the FVII gene expression or protein handling, has to be taken into account. The assay employed to evaluate the effect of mutant FVII molecules may be not informative enough to fully appreciate the effect of missense mutations on protein secretion and folding. In keeping with this, folding and/or disulphide linkage disturbances in the second EGF-like domain are likely to affect protein stability severely and account for the absence of measurable FVII antigen and coagulant activity in the patient investigated.

In conclusion, we have identified two naturally occurring missense mutations within the second EGF-like

domain. These mutations severely affect FVII intracellular protein handling, probably profoundly impairing the correct folding of the native protein leading to FVII deficiency and further suggest the importance of the second EGF-like domain in the correct orientation of different FVII domains.

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