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A New Mutation (Arg251Trp) in the Ca^{2+} Binding Site of Factor X Protease Domain Appears to be Responsible for the Defect in the Extrinsic Pathway Activation of Factor X Padua

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Summary: Factor X Padua, first described a few years ago, is characterized by a defect only in the extrinsic system. In this present paper, the molecular basis for this peculiar defect is investigated. Polymerase chain reaction amplification and direct sequencing of the entire FX coding sequence and of exon-intron junctions detected in the proposita a C-to-T translocation in exon 8 of nucleotide 875 at the homozygous level. This resulted in the substitution of tryptophan for arginine 251. A niece of the proposita was shown to be heterozygous for the abnormality. Molecular modeling suggested that the mutation does not alter significantly folding and stability of the protein but may be involved in the Ca^{2+} binding site.
Key Words: FX defects—Factor X mutations—FX variants.

Factor X (FX) is a vitamin K–dependent protein that participates in the middle phase of blood coagulation (1). After the specific activation by the complex tissue factor/activated factor VII (TF/FVIIa) in the initiation phase, process is amplified in the propagation step where FX interacts with the complex activated factor VIII/factor IX (FVIII) (2). Activated FX (FXa) converts prothrombin to thrombin in the presence of activated factor V (FVa), phospholipids, and Ca^{2+}. FX deficiency is a rare congenital hemorrhagic condition inherited as an autosomal recessive disorder. Clinical presentation, in our experience, usually correlates fairly well with the laboratory data. Genotyping and molecular analysis represent a valuable method to correlate the structure of FX to its functions. In this paper, we characterized the genetic basis of the previously reported FX Padua, a peculiar FX abnormality with a defect mainly in the activation via the extrinsic pathway (3).

PATIENTS, MATERIALS, AND METHODS

The proposita was a 56-year-old female who was familiar to us (3). She had been sent to us for evaluation after the disclosure of a prolonged prothrombin time. Bleeding history was unremarkable. Routine laboratory investigations and specific factor X assays have been performed as previously reported (3–5). SDS-PAGE was performed according to Laemmli (6). Genomic DNA was prepared from leukocytes by standard procedures. Amplification of exons I through VIII and respective splice junctions of the factor X gene were performed using oligonucleotide primers kindly supplied by Dr. H. James (Tyler, TX) according to previously described methods (7). Conformation sensitive gel electrophoresis (CSGE) for FX screening mutation was performed as recently described from our group (8). Sequencing was performed on an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City,

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CA) using ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems). Forward and reverse primers were the same as those used for polymerase chain reaction (PCR) amplifications. The Sequencing Analysis 3.3 computer program (Applied Biosystems) analyzed sequencing data. Results were compared with factor X sequence as reported in the GenBank database (GenBank accession number AF503510).

The crystallized model of wild-type human FX has been used for comparing our mutant model (PDB: 1HCG). The structure analysis was performed in interactive graphics using the software Swiss-pdbViewer as described by others (9). Energy minimization was performed by GROMOS96 implementation of Swiss-pdbViewer.

RESULTS AND DISCUSSION

As previously extensively described (3), laboratory characterization of the family members confirmed the prolongation of prothrombin time (PT) together with a normal partial thromboplastin time (PTT). FX antigen level was normal whereas we found a reduction of factor X activity level (30 u/dL) when tested by the extrinsic pathway. The result didn't change when we used thromboplastins from different sources. A chromogenic assay yielded also moderately decreased levels. On the contrary, the factor X result was always normal when tested with intrinsic system or the RVV-Cephalin method (3). All other clotting factors were with normal limits. A similar pattern was observed in the proband's father,

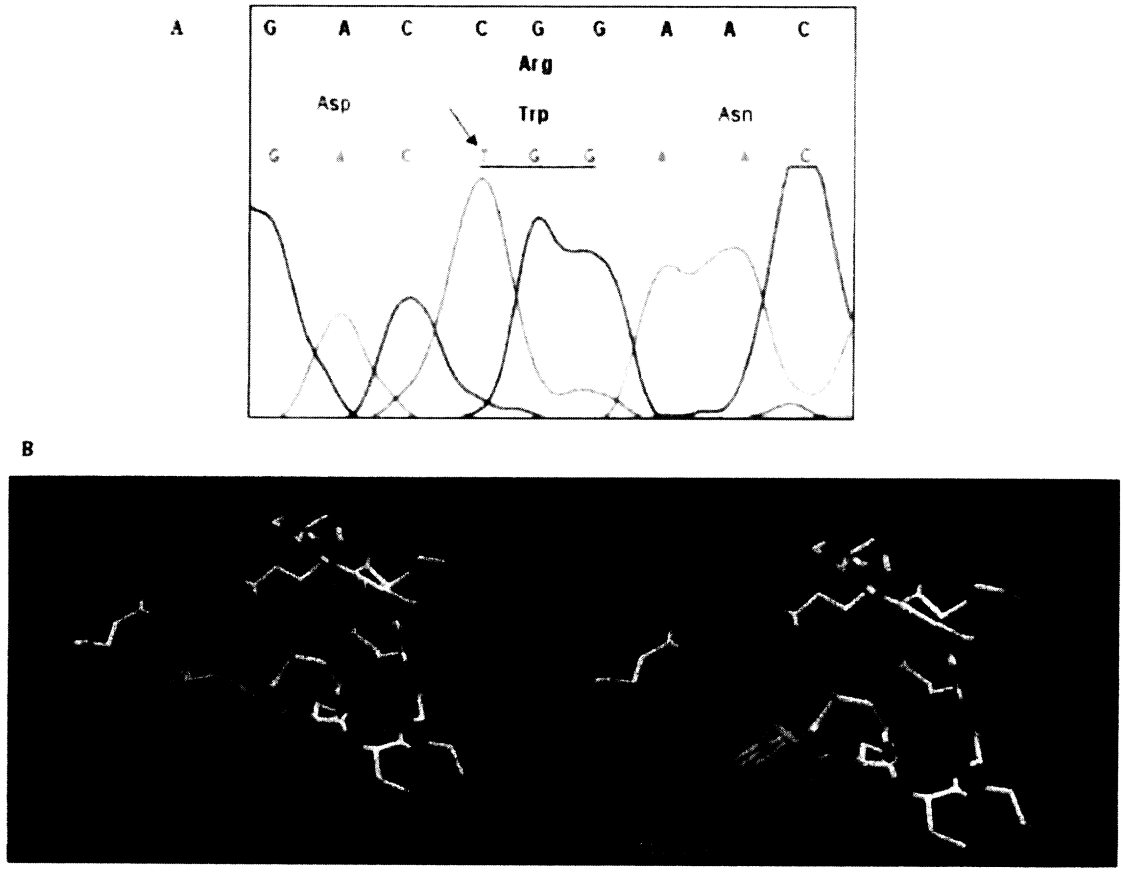

FIG. 1. A. Sense DNA sequence of exon VIII of the F.X gene around codon 251 showing the homozygous transversion 875C>T in the FX Padua proband, resulting in the substitution replacement of Arg with Trp. **B.** Three-dimensional structure of wild-type (*left*) and mutant (*right*) factor X focusing on $Arg^{251} \rightarrow Trp$ substitution (residues in yellow). Dotted lines refer to hydrogen bonds. White ribbon draws the 250–260 Ca^{2+} binding loop.

brother, two sons, and two nieces, although to a smaller extent. The proposita was considered to be homozygote for the abnormality whereas her relatives were considered to be heterozygotes.

PCR amplification and direct sequencing of the entire FX coding sequence and of exon-intron junctions detected in homozygous condition a C to T transversion in exon 8 at nucleotide 875, resulting in the substitution of tryptophan for arginine 251 (Fig. 1A). The only niece available for genotyping disclosed a heterozygous condition, thus confirming the interpretation based on clotting tests. SDS-PAGE and immunoblot analysis of FX mutant did not show heterogeneity in the FX light or heavy chain when compared to FX from pooled normal plasma (data not shown). In the Arg251Trp mutation, a polar charged amino acid mutation is replaced with a non-polar one. Molecular modeling of the mutation (Fig. 1B) showed that replacement of arginine 251 is responsible for the loss of a hydrogen bond between Arg251and Asp203. The force field energy computation disclosed only a minor increase in the total energy of the mutant molecule compared to the wild-type protein. The energy computation, the normal antigen level, the normal electrophoretic pattern, and asymptomatic clinical history do not suggest an incorrect formation of folded mutant protein. However, the new mutation Arg251Trp is comprised in the loop region Asp250-Glu260 of FX, a high-affinity Ca^{2+} binding site common to several serine proteases. Although not highly conserved in this group of clotting proteins, Arg251 and corresponding residues His in FVII and FIX and Tyr in Protein C, are all hydrophilic amino acids flanking the coordination site of oxygen atoms to calcium ion through two water molecules in this loop. The introduction of the hydrophobic residue Trp in the highly hydrophilic region significantly changes the local electrostatic potential. Finally, the mutation might modify the corrent main-chain conformation of this calcium binding site (10). Binding of Ca^{2+} in the protease domain protects proteolytic cleavage in the autolysis loop, which would lead to loss of catalytic efficiency as reported for factor VII by Sabharwal and colleagues (11). Moreover, autolysis-loop cleaved FXa has a marked reduction in the FVa affinity. This new genotype is associated to a peculiar functional defect detected only in the extrinsic phase of FX activation where the interaction between FVIIa/TF and FX does occur. Although we were unable to clearly define the relationship between Ca^{2+}-binding defect and an abnormal activation of FX via the extrinsic pathways, previous *in vitro* studies suggest that a mutation in this loop abolishes the high-affinity Ca^{2+} binding site of FX, potentially interfering with FX activation (12). Moreover, it is of interest to note that other FX mutations involving the low-affinity Ca^{2+} binding site showed an impairment in the extrinsic pathway activation (13,14). This is the case of two mutations in the Gla-domain Ca^{2+} binding site occurring in the variant Gla+14→Lys (FX Vorarlberg) and in the mutant Gla19Ala. When these FX mutants were tested for *in vitro* activation, they showed a more pronounced decrease in the rate of activation by FVIIa/TF compared to FIXa/FVIIIa. In conclusion, an impairment in the protein conformational change due to decreased affinity for Ca^{2+} could explain the abnormal activation of FX by the complex TF/FVIIa in factor X Padua.

The structure-function relations in factor X defects are becoming, little by little, clarified (15).

REFERENCES

1. James HL. Physiology and biochemistry of factor X. In: Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. *Haemostasis and Thrombosis*. Edinburgh: Churchill Livingstone; 1994, p. 439.
2. Hoffman M, Monroe DM III. A cell-based model of hemostasis. *Thromb Haemost* 2001;85:958.
3. Girolami A, Vicarioto M, Ruzza G, Cappellato G, Vergolani A. Factor X Padua: A 'new' congenital factor X abnormality with a defect only in the extrinsic system. *Acta Haematol* 1985;73:31.
4. Girolami A, Molaro G, Lazzarin M, Scarpa R, Brunetti A. A "new" congenital haemorrhagic condition due to the presence of an abnormal factor X (factor X Friuli): Study of a large kindred. *Br J Haematol* 1970;19:179.
5. Simioni P, Vianello F, Kalafatis M, et al. A dysfunctional factor X (factor X San Giovanni Rotondo) present at homozygous and double heterozygous level: Identification of a novel microdeletion (delC556) and missense mutation (Lys(408)→Asn) in the factor X gene. A study of an Italian family. *Thromb Res* 2001;101:219.
6. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680.
7. James HL, Girolami A, Fair DS. Molecular defect in coagulation factor X Friuli results from a substitution of serine for proline at position 343. *Blood* 1991;77:317.
8. Vianello F, Lombardi AM, Dal Bello F, Zanon E, Cabrio L, Girolami A. Conformation sensitive gel electrophoresis for a simple and accurate detection of factor X mutations. *Thromb Res* 2002;107:51.
9. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* 1997;18:14.

10. Venkateswaren D, Perera L, Darden T, Pedersen LG. Structure and dynamics of zymogen human blood coagulation factor X. *Biophys J* 2002;82:1190.

11. Sabharwal AK, Birktoft JJ, Gorka J, Wildgoose P, Petersen LC, Bajaj SP. Affinity Ca^{2+}-binding site in the serine protease domain of human factor VIIa and its role in tissue factor binding and development of catalytic activity. *J Biol Chem* 1995;270:15523.

12. Rezaie AR, Esmon CT. Asp-70→Lys mutant of factor X lacks high affinity Ca^{2+} binding site yet retains function. *J Biol Chem* 1994;269:21495.

13. Watzke HH, Lechner K, Roberts HR, et al. Molecular defect (Gla+14→Lys) and its functional consequences in a hereditary factor X deficiency (factor X "Vorarlberg"). *J Biol Chem* 1990;265:11982.

14. Pinotti M, Marchetti G, Baroni M, Cinotti F, Morfini M, Bernardi F. Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathways results in symptomatic CRM^{red} FX deficiency. *Thromb Haemost* 2002; 88:236.

15. Cooper DN, Millar DS, Wacey A, Pemberton S, Tuddenham EG. Inherited factor X deficiency: Molecular genetics and pathophysiology. *Thromb Haemost* 1997;78: 161.