Factor X Shanghai and disruption of translocation to the endoplasmic reticulum

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Background and Objectives. Most secreted proteins, including coagulation factor X (FX), are synthesized with a signal peptide, which is necessary for targeting the nascent polypeptide into the endoplasmic reticulum. Characterization of naturally occurring mutations may provide insights into the functional roles of the amino acids in the signal peptide.

Design and Methods. A 52-year old male patient with type I FX deficiency was studied. Mutations were searched for by FX gene (*F10*) sequencing. The wild-type and the mutant FX proteins were expressed in transfected cells and then immunological assays were performed. Pulse-chase experiments and cell-free expression studies were conducted to determine the cellular fate of the mutant FX molecules.

Results. The patient we studied was homozygous for a substitution of arginine for serine at codon –30 in the signal sequence of *F10*. Immunoassays detected low FX antigen levels in both the conditioned media and lysates of the cells expressing the mutant protein. Pulse-chase analysis showed that only trace amounts of the mutant FX protein were detectable in the conditioned media, and that the mutant molecules did not accumulate inside the cells either. The results of cell-free expression studies showed that although the transcription and translation of the mutant construct were normal, no post-translational processing, such as N-linked glycosylation, occurred in the presence of microsomes.

Interpretations and Conclusions. These findings suggest that substitution of a neutral polar amino acid, serine by arginine, in the hydrophobic core of FX signal peptide severely impairs the ability of the protein to enter the endoplasmic reticulum and results in FX deficiency.

Key words: factor X, deficiency, mutation, signal peptide, translocation.

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Factor X (FX) is a vitamin K-dependent plasma glycoprotein that plays a crucial role in the blood coagulation cascade, as it can be activated to FXa, the only known endogenous activator of prothrombin, through either the intrinsic pathway or extrinsic pathway in the presence of calcium and phospholipids.[1,2] FX is synthesized in hepatocytes with a 40-residue pre-pro leader sequence (residues –40 to –1, numbering the N-terminus alanine of the mature protein as +1) containing a hydrophobic signal sequence (residues –37 to –22). After several modifications, FX is secreted into plasma as a two-chain zymogen composed of a 17-kDa light chain disulphide linked to a 45-kDa heavy chain.[1] The gene for human FX (*F10*) contains eight exons and spans more than 27 kilobases (kb) on chromosome 13q34-ter, approximately 2.8 kb downstream of the polyadenylation site of the factor VII gene.[1,3] Frequently associated with consanguinity, hereditary FX deficiency is one of the most severe autosomal recessive clotting disorders.[4] Depending on the phenotype, two major forms of FX deficiency have been classified: type I deficiency, which is characterized by concordantly low circulating levels of both functional and immunological FX, whereas type II corresponds to low coagulant activity contrasting with normal or low borderline antigen values due to dysfunctional FX molecules in plasma. The most frequent symptoms of FX deficiency are epistaxis, hemarthrosis, muscle hematomas, umbilical stump bleeding and gastrointestinal bleeding.[4,5] The genetic basis of FX deficiency is heterogenous, and to date, more than 60 distinct mutations have been identified.[6-10] However, only in

a relatively small number of studies have the molecular and cellular defects of mutated FX been examined.[11-19] We identified a patient with type I FX deficiency carrying a homozygous missense mutation (Ser–30Arg) in the signal sequence of FX and here describe the molecular characterization of a new FX variant designated as FX Shanghai.

Design and Methods

Case report

A pedigree with inherited FX deficiency from Shanghai, China, was studied. The propositus, a 52-year old male, was admitted for severe gastrointestinal bleeding which was controlled by fresh-frozen plasma and prothrombin complex concentrate transfusions for a couple of days. He had a past history of moderate bleeding after hemostatic challenge (e.g. trauma). His parents are first cousins, and none of the other members of the pedigree had a history of bleeding disorders.

Coagulation assays

After informed consent, blood samples were taken from the propositus and ten other family members. Plasma FX activity (FX:C) was determined using a one-stage clotting assay (based upon prothrombin time) on an ACL 3000 (Instrumentation Laboratory, Milan, Italy), and the FX antigen levels (FX:Ag) were measured with an enzyme-linked immunosorbent assay (ELISA), using a rabbit anti-human FX polyclonal antibody (Dako, Glostrup, Denmark) as a capture antibody and horseradish peroxidase -conjugated rabbit anti-human FX antibody as a detection antibody. Both FX:C and FX:Ag levels were expressed as a percentage of the corresponding levels in normal plasma pooled from 30 healthy individuals.

Genetic analysis

Genomic DNA was extracted from whole blood using the standard phenol-chloroform protocol and the primers for polymerase chain reaction (PCR) were designed according to the published sequence of *F10* (GenBank accession number AF503510). The PCR products of all eight exons and intron-exon boundaries were purified from agarose gel and then sequenced using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA).

Construction of expression vectors and site-directed mutagenesis

A 1.5-kb complete human FX complementary DNA (cDNA) was isolated from a human fetal liver cDNA library and cloned into pcDNA3.1(–) (Invitrogen, Carlsbad, CA, USA) to obtain the pcDNA/FXwt as already described.[10] Site-directed mutagenesis was performed using a *megaprimer* method as described elsewhere.[20] Briefly, oligonucleotides P1 (5'CCACTGCTTACTGGCTTATCG3') and P2 (5'GGAGG GGCAAACAACAGATG3') are located just outside the XbaI site and KpnI site of pcDNA3.1 (–), respectively. The mutagenic primer 5'GGAGGCCCTGAGCAGGA3' (underlined letter indicates the mismatch), corresponding to nt 48 to 64, was used to replace the Serine–30 with Arginine (pcDNA/ FXS–30R). The entire FX cDNA was sequenced to confirm the presence of the mutation and to exclude PCR-induced errors.

Cell culture and transfection assays

Human embryonic kidney cells (HEK293) were cultured in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, antibiotics (100 μg/mL streptomycin and 100 IU/mL penicillin) in a 5% CO_2 humidified atmosphere at 37°C. Transfection was performed using PolyFect reagent (Qiagen, Hilden, Germany) and 2 μg plasmid DNA (pcDNA/FXwt and pcDNA/FXS–30R) in 6-well plates according to the manufacturer's instructions. After 16 h, the culture media were replaced with serum-free fresh media, supplemented with L-glutamine, antibiotics and vitamin K1 (5 μg/mL), and then cells were cultured for an additional 36 h. Conditioned media were collected in pre-chilled tubes containing a protease inhibitor cocktail (for use with mammalian cell and tissue extracts (Sigma, St. Louis, MO, USA), and cell debris was removed by centrifugation. Proteins were extracted with M-PER® mammalian protein extraction reagent (Pierce, Rockford, IL, USA) and a protease inhibitor cocktail. Three independent transfection experiments were performed, and for each transfection experiment a mock, with the unrelated pcDNA3.1(–) plasmid as a negative control, was set up.

Measurements of recombinant FX in conditioned media and cell lysates

As described above, FX activities of recombinant FX were measured in conditioned media without sample dilution, using a one-stage prothrombin time based method. FX antigen levels were evaluated both on conditioned media and cell lysates with a sandwich ELISA. Standard curves were constructed with reference plasma diluted 1:200 to 1:6400 in Tris-buffered saline (TBS; pH 7.4, 10 mmol/L Tris-HCl, 150 mmol/L NaCl) with 1% bovine serum albumin (BSA). Samples were diluted 1:20 (cell lysates) and 1:40 (conditioned media) with the same buffer.

Pulse-chase, immunoprecipitation and gel electrophoresis

For metabolic labeling studies, African green monkey kidney COS-7 cells were transfected in 60-mm dishes using PolyFect. Approximately 24 h after transfection, pulse-chase experiments were performed using EasyTag [^{35}S]-labeled methionine (Pekin Elmer Life Science, Boston, MA, USA) as previously described.[21] Labeled proteins were analyzed at 0, 30, 60, 120, and 180 min, and at each time, conditioned media and cell lysates were processed as described in the transfection assays.

To immunoprecipitate recombinant FX either in cell lysates or in conditioned media, rabbit anti-human FX polyclonal antibody and protein-A sepharose (Sigma) were used. The immunoprecipitated proteins were released from protein-A by boiling for 5 min in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) non-reducing loading buffer. Samples were analyzed by 10% SDS-PAGE and gels were dried under vacuum at 70°C for 90 min. Labeled proteins were visualized by exposing the gels to a storage photophor screen for 72 h and analyzed using a Molecular Image FX Multiimage System (Bio-Rad, Hercules, CA, USA).

Cell-free expression of FX proteins and translocation assays

Cell-free expression of FX proteins was performed using a TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). According to the manufacturer's instructions, the wild-type and mutant FX proteins were produced in a total volume of 50 μL containing 40 μL TNT Quick Master Mix, 2 μL [^{35}S]-labeled methionine, 2 μg plasmid DNA in the presence (3 μL) or absence of Canine Pancreatic Microsomal Membranes (Promega) at 30°C for 1 h. Furthermore, to remove any N-linked carbohydrate, some products were digested with 500U endoglycosidase H (New England Biolabs, Beverly, MA, USA) at 37°C for 1 h. Electrophoresis and autoradiography were performed as described above.

Results

Coagulation laboratory data and genetic studies

The proband showed a prolonged activated partial thromboplastin time (APTT, 53.3s vs. 37.6s in the control) and prothrombin time (PT, 30.0s vs. 12.5s). Expressed as percentages of levels in normal plasma, the proband's levels of FX:Ag and FX:C were 8.2% and 2.6% respectively. The levels of the proband's other coagulation factors (fibrinogen, prothrombin and factor V, VII and IX) were within normal ranges. Sequencing of the entire FX coding sequence and the exon-intron boundaries revealed that the propositus was homozygous for a T to G transversion at nucleotide 58 in exon 1 of the *F10* gene, resulting in an amino acid substitution of serine (AGT) to arginine (AGG) at codon –30 in the signal peptide of FX. The proband's mother and his two sons were heterozygous for the same missense mutation, and no other mutation was found in the pedigree. Table 1 summarizes the coagulation data and genetic alterations in the pedigree.

Table 1. Coagulation data and genetic mutations in the pedigree.

Subject	APTT(s) (26.3~ 43.2)*	PT(s) (11.2~13.6)*	FX: C (%) (68.5~126.9)*	FX: Ag (%) (69.8~132.6)*	Ser-30Arg
Mother	30.2	12.9	49.9	53.4	+/-
Propositus	53.3	30.0	2.6	8.2	+/+
Sister	25.5	11.9	94.0	98.6	-/-
Brother	26.9	12.1	87.8	92.2	-/-
Son I	27.2	11.9	71.2	75.8	+/-
Son II	26.7	12.5	69.8	72.0	+/-

**normal range (n=30).*

Table 2. Transient expression assays of pcDNA/FXwt and pcDNA/FXS–30R in HEK293 cells. FX levels were measured in cell lysates and conditioned media after three independent transfection assays. Results are expressed as the percentage of the FX (mean ± SE) produced by the wild-type construct.

	pcDNA/FXwt (%)	pcDNA/FXS–30R (%)
FX: Ag (cell lysate)	100	17.6±1.4
FX: Ag (conditioned media)	100	10.8±1.1
FX: C (conditioned media)	100	5.8±0.6

Transient expression assays in HEK293 cells

To investigate the molecular basis of the FX activity and antigen deficiency observed in the propositus, transient transfection assays were performed in HEK293 cells using pcDNA3.1(–) vectors containing either wild-type or mutant FX cDNA. ELISA assays of the cell lysates and the conditioned media showed that FX:Ag of pcDNA/FXS–30R transfectants was reduced to 17.6% and 10.8%, respectively, as compared to the cells transfected with pcDNA/FXwt. The recombinant mutant FX activity was measured in conditioned media by a one-stage assay, and the result was about 5.8% of the wild-type FX (Table 2).

Pulse-chase analysis of the wild-type and mutant FX molecules

To determine the intracellular and extracellular fate of the mutant FX, pulse-chase experiments using FXwt and FXS–30R were performed. In these exper-

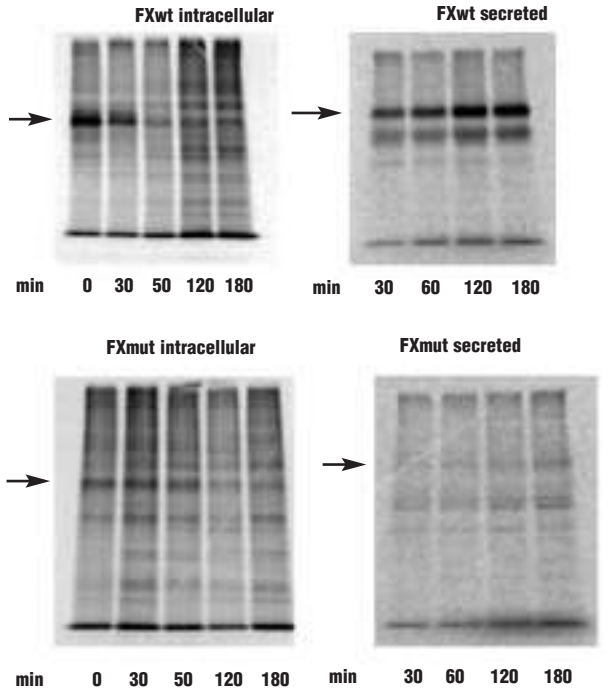

Figure 1. Pulse-chase analysis of wild-type and mutant FX protein in COS-7 cells. COS-7 cells, transiently transfected with pcDNA/FXwt and pcDNA/FXS-30R, were pulse-labeled with [^{35}S]-methionine for 30 min, and then chased by methionine for various periods of time up to 180 min. At the specified chase period, labeled FX proteins were immunoprecipitated from cells lysates and from the corresponding conditioned media, electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels, and detected by a phosphor imager.

iments, the recombinant FX proteins in cell extracts and in the conditioned media were immunoprecipitated, and then analyzed by SDS-PAGE and autoradiography. As shown in Figure 1, after a 30 min pulse with [^{35}S]-methionine, the recombinant FXwt in cell lysates was maximal at 0-30 min, and then decreased gradually as the protein was secreted. At 180 min after labeling, nearly all labeled FXwt was found in the medium. In contrast, during the entire chase period, only trace amounts of the mutant FXS–30R protein were detectable in the conditioned media, and the mutant FX did not accumulate inside the cells either.

Cell-free expression of recombinant FX protein

To further investigate the biochemical effect of this mutation on the biosynthesis of FX, a cell-free expression system, involving rabbit reticulocyte lysate, was used for coupled transcription and translation of the wild-type and the mutant FX proteins. Translation of both these mRNA resulted in a protein of 64-kDa, and the translation efficiency of the mutant appeared similar to that of the wild-type FX protein. Further cell-free translation reactions were performed in the presence of canine pancreatic

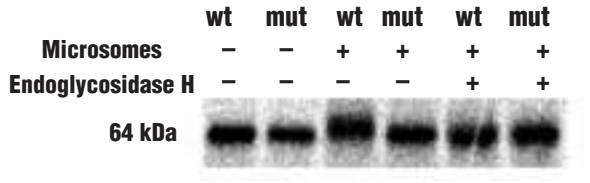

Figure 2. Cell-free translation of FX cDNA constructs and endoglycosidase H digestion. The wild-type FX protein undergoes modification in the presence of microsomes to generate a higher molecular weight band, consistent with N-linked glycosylation. In contrast, no post-translational processing was observed in the mutant protein.

microsomal membranes, which were used as endoplasmic reticulum equivalents to assess the efficiency of co-translocation and initial post-translational processing of recombinant FX proteins. As shown in Figure 2, in addition to the 64-KDa band, a higher molecular weight band was observed in the FXwt translation products when microsomes were added, which suggested the FXwt protein had undergone N-linked glycosylation within the lumen of the microsomes. This modification was confirmed by digesting the protein with endoglycosidase H, which resulted in the loss of the higher molecular weight product, but left the 64-kDa band intact. In contrast, the FXS–30R-generated product did not result in any detectable modification of the protein in the presence of microsomes.

Discussion

The vast majority of eukaryotic proteins are synthesized as precursor forms having an NH_2-terminal signal peptide, which plays important roles in translation by ribosomes, targeting and translocation of the protein to the endoplasmic reticulum. Translocation of the protein into the endoplasmic reticulum is followed by post-translational processing including disulfide bond formation, protein folding, glycosylation and cleavage of the signal peptide.[22] The sequences of signal peptides are heterogeneous, typically including three distinct regions: an N-terminal region of 1-5 positively charged residues, a central region encompassing 7-15 hydrophobic residues, and a polar C-terminal region of 3-7 amino acids containing the signal peptidase cleavage site.[23]

The central hydrophobic core is supposed to fulfill a critical role in initiating translocation through interaction with the endoplasmic reticulum membrane components. Several mutations occurring in the hydrophobic core of the signal peptide have been implicated in reduction of translocation to the endoplasmic reticulum *in vitro*.[24-27]

In a patient with type I FX deficiency, we identified a missense mutation, Ser–30Arg, in the hydrophobic region of the FX signal peptide. Expression studies showed little FX protein in the conditioned medium, which was consistent with the clinical findings. According to the pulse-chase analysis, the synthesis of the FX mutant was not affected and there was no accumulation of the mutant FX intracellularly either, which suggests that the reduced FX level in this patient may be attributed to either reduced FX secretion and/or stability.

As far as we know, the FX Shanghai variant (Ser–30Arg) is the second FX mutation in the signal peptide for which molecular characterization has been performed. FX Santo Domingo is a variant that arises from a Gly–20Arg substitution in the signal peptide of FX.[28] The proband experienced a severe bleeding diathesis and manifested FX:C and FX:Ag of <1% and 5%, respectively. Subsequent studies have shown that this mutant protein is synthesized normally and then translocated into the endoplasmic reticulum successfully, but not processed by the signal peptidase.[15]

The hydrophobic region is rich in Leu, Ala, Met, Val, Ile, Phe, and Trp but may contain an occasional Pro, Gly, Ser, or Thr amino acid residue.[29] Several studies have proven that the replacement with a less hydrophobic residue, such as the mutation Cys–8Arg identified in the signal peptide hydrophobic core of pre-pro-parathyroid hormone (PTH), impairs co-translational translocation as well as post-translational cleavage by isolated peptidase;[24] however, the substitution of an amino acid with higher hydrophobicity, such as leucine for cystine 18 of bovine PTH, is ineffective in modifying translocation.[30]

In this study, no post-translational process, such as N-linked glycosylation, was observed for the FX mutant protein in the presence of microsomes in the cell-free expression studies. The serine to arginine substitution would increase the hydrophilicity value from 0.2 to 3.0.[31] Although serine is a neutral polar amino acid, substitution by arginine may exert a dramatic effect on the translocation function of signal peptide by introducing a positive charge at position –30. Therefore, total hydrophobicity of the hydrophobic region in the signal peptide appears to be a crucial factor. If the signal peptide emerging from the ribosome is not sufficiently hydrophobic, it may not be recognized by the 54-kDa subunit of the signal recognition particle (SRP54), which binds to nascent protein and directs the entire synthetic complex to the endoplasmic reticulum.[32]

Our data provide some evidence that a change in the hydrophobicity of the signal peptide is responsible for the observed disruption of the usual fate of FX protein. The facts that low levels of FX antigen were measured both in the proband's plasma and in the cells expressing the mutant construct indicate that a small portion of the mutant proteins could be translocated into the endoplasmic reticulum and escape degradation. However, for some hitherto unknown reasons, these proteins may not acquire full activity and lead to the discrepancy of antigen and activity levels in plasma, as also occurs in the patient with the FX Santo Domingo variant.[28]

W-BW and Q-HF contributed equally to this work and should be considered as joint-first authors. W-BW, Q-HF, JY and H-LW contributed to the conception and design of the study and analysis and interpretation of data. W-BW and Q-HF wrote the first draft of the paper, which was critically revised by Z-YW and H-LW. W-MW and Q-LD participated in discussions on the project. R-FZ, Y-QH and X-FW were involved in collecting the samples and their phenotypic analysis. We thank all the family members for their generous collaboration and Dr Ji-Ning Lu (MED Pulmonary Center, Boston University, USA) for critical reading of the manuscript. The authors declare that they have no potential conflicts of interest.

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