Factor X$_{Debrecen}$: Gly204Arg mutation in factor X causes the synthesis of a non-secretable protein and severe factor X deficiency

Zsuzsanna Bereczky,[1] Helga Bárdos,[2] István Komáromi,[3] Csongor Kiss,[4] Gizella Haramura,[1] Éva Ajzner,[1] Róza Ádány,[2] and László Muszbek[1,3]

[1]Clinical Research Center, [2]Department of Preventive Medicine, [3]Hemostasis, Thrombosis and Vascular Biology Research Group of the Hungarian Academy of Sciences; [4]Department of Pediatrics, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary

ABSTRACT

Due to a homozygous Gly204Arg mutation in the factor X (FX) gene no detectable FX antigen was found in the plasma of a one-year old patient with severe bleeding diathesis. The amino acid replacement destabilized the disulfide bond that holds the two FX chains together, decreasing the interaction between the Cys201-Cys206 loop region and the region connecting the EGF2 and serine protease domains. Both Gly204 FX and Arg204 FX were synthesized in transfected cells, but only the wild type protein became secreted. The mutant protein was diverted from the normal secretory pathway and retained at the trans Golgi-late endosome level.

Key words: factor X, factor X deficiency, inherited bleeding diathesis, rare coagulopathy, *F10* mutation.

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Introduction

Blood coagulation factor X (FX) is synthesized in the liver as a single chain protein.[1,2] During maturation, a tripeptide, Arg140-Lys141-Arg142, is cut out from the peptide chain in the Golgi. The resultant 17-kDa light-chain and 45-kDa heavy-chain are held together by a disulfide-bridge between Cys132 and Cys302. The light chain contains a Gla domain, two epidermal growth factor (EGF) domains and an additional connecting region (CR). The heavy chain consists of the serine protease (SP) domain and the N-terminal activation peptide (AP) that is cleaved off upon activation by factors IXa or VIIa. Inherited FX deficiency is a rare (1:1,000,000) coagulopathy with severe bleeding symptoms in homozygous patients.[1] More than 80 mutations have been described in the *F10* gene (*www.med.unc.edu/isth/mutations-databases*), the majority of which are missense mutations. In type I FX deficiency, both functional activity and antigen level are decreased, while in type II deficiency a dysfunctional molecule is present. A few reports have suggested that secretion defects, due to missense mutations in domains of the mature protein, cause type I deficiency. However, this has been proved by expression studies.[3-5] Here we demonstrated that Gly204Arg substitution resulted in severe type I FX deficiency by causing structural changes in the molecule and a secretion defect due to retention at the trans Golgi-late endosome level.

Design and Methods

Patient

A one-year-old boy with subdural hematoma and bruising had highly prolonged prothrombin time and activated partial thromboplastin time. The activity of all clotting factors, except for FX, was in the reference interval. A highly reduced FX activity (1%) together with FX antigen concentration below the limit of detection suggested type I FX deficiency. No bleeding history in the family was reported. The study protocol was approved by the Ethics Committee of the University. Informed consent was given by the patient's mother.

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Correspondence: László Muszbek, Clinical Research Center, University of Debrecen, Medical and Health Science Center, 98 Nagyerdei krt. PO Box 40, H-4012 Debrecen, Hungary. E-mail: muszbek@med.unideb.hu

Procedures

With the exception of exon 8, primers described in the literature were used for amplification of genomic DNA.[3,6,7] The following primers were designed for the amplification of exon 8: 5'-CGTCTGTCCCAGGGGAC-3' (forward), 5'- TGGGATCTCACTTTAATGGAG-3' (reverse). Forward and reverse direct fluorescent sequencing of PCR products were performed by ABI PRISM 310 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). The mutant FX expression vector[8] was generated by site-directed mutagenesis of the wild type FX cDNA inserted in pCMV4 vector using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Human embryonic kidney (HEK293) cells were transiently transfected[8] with 1 Ìg wild type or mutant vector using Effectene transfection reagent (Qiagen). After a 72-hour incubation conditioned media were collected, part of the cells were used for confocal laser scanning microscopy (CLSM), while another part of the cells was lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Roche). Aliquots of the media and cell lysates were used for FX antigen determination by ELISA (Asserachrom X:Ag, Diagnostica Stago, Asnières, France) and for immunoprecipitation by biotinylated goat anti-human FX antibody (Haematologic Technologies Inc, Vermont, VT) and streptavidine agarose (Sigma-Aldrich). Immunoprecipitates were analyzed by Western blotting using rabbit anti-human FX antibody (Dako, Glostrup, Denmark) and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The reaction was visualized by ECL Plus chemiluminescence reagent (Amersham, Little Chalfont, UK).

For pulse chase analysis, transfected HEK 293 cells were preincubated with methionine-, and FCS-free medium for 30 min. Then they were pulse-labeled for 30 min with 0.5 mCi/mL [^{35}S]methionine (Amersham), and chased for 30 min, 2 and 6 hr. FX immunoprecipitated from cell lysates and culture media was analyzed by SDS PAGE and fluorography.

Cells fixed in 96% ethanol, 1% acetic acid were incubated with 5% normal human serum in phosphate buffered saline for 15 min to prevent non-specific IgG binding. Staining for FX was combined with the detection of calnexin (an endoplasmic reticulum marker), or mannosidase II (a cis-Golgi marker), or mannose 6 phosphate receptor (a trans-Golgi-late endosome marker).[9-11] Cell preparations were examined by CLSM (LSM 410, Zeiss, Oberkochen, Germany). The relative amount of FX was determined by measuring integrated fluorescence intensity on 150-200 cells/slide.[12] Molecular modeling and simulations were performed on reconstituted single-chain (des1-45)FX.[13,14] After minimization and heating up dynamics, the geometries of Gly204 and Arg204 EGF2-CR-AP-SP construct were submitted to molecular dynamics simulation using OPLS/AA force field and periodic boundary conditions with TIP4P water solvent model with the GROMACS package.[15]

Results and Discussion

Molecular genetic analysis revealed a homozygous G>A missense mutation at coding nucleotide position c.730 resulting in a Gly204Arg replacement in the amino acid sequence. The mother was heterozygous for the mutation; the father was unknown and other family members were not available for testing. The absence of the mutation in one hundred healthy individuals suggested its causative nature. We have given a preliminary presentation of this mutation[16] and later it was also detected in an Iranian patient.[5]

Using ELISA technique and quantitative fluorescent image analysis, in HEK cells expressing Arg204 FX the concentration of FX was found to be 1.5-fold and 2.2-fold

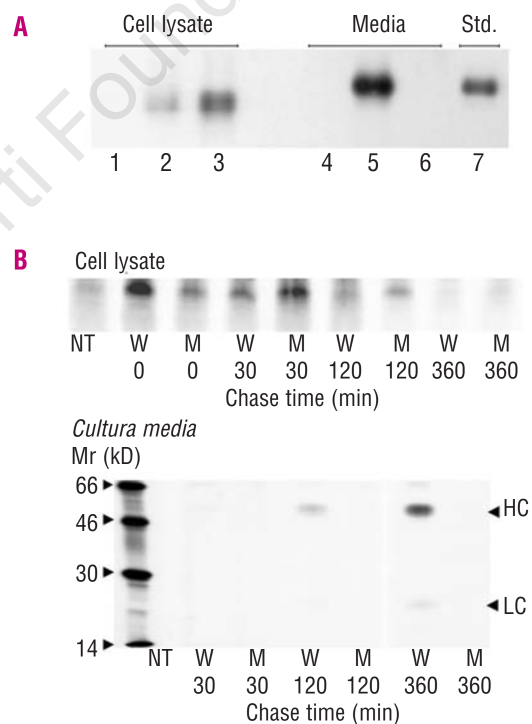

Figure 1. Detection of factor X expressed by HEK293 cells transfected with wild type and mutant factor X constructs. **A.** Detection of FX in the cell lysate (1-3) and culture media (4-6) by Western blotting. SDS PAGE was performed in non-reducing condition. 1,4: non-transfected cells; 2,5: cells expressing wild type FX; 3,6: cells expressing Arg204 mutant FX; 7: purified FX standard. FX in the cell lysate had slightly higher mobility than FX in the culture media or FX prepared from human plasma, the difference probably reflects different stages of glycosylation. **B.** Metabolic labeling of HEK cells expressing wild type and mutant factor X. Non-transfected (NT) HEK293 cells and cells transfected with wild type (W) and Arg204 mutant (M) constructs were pulse labeled with [^{35}S] methionine, then chased for various intervals. Cell lysate samples represent immunoprecipitates from ~5×10^5 cells, while culture media samples were immunoprecipitated from the supernatant of ~2.5×10^5 cells. The immunoprecipitates loaded onto the gels derived from the same amount of proteins in each sample. HC: FX heavy chain; LC: FX light chain.

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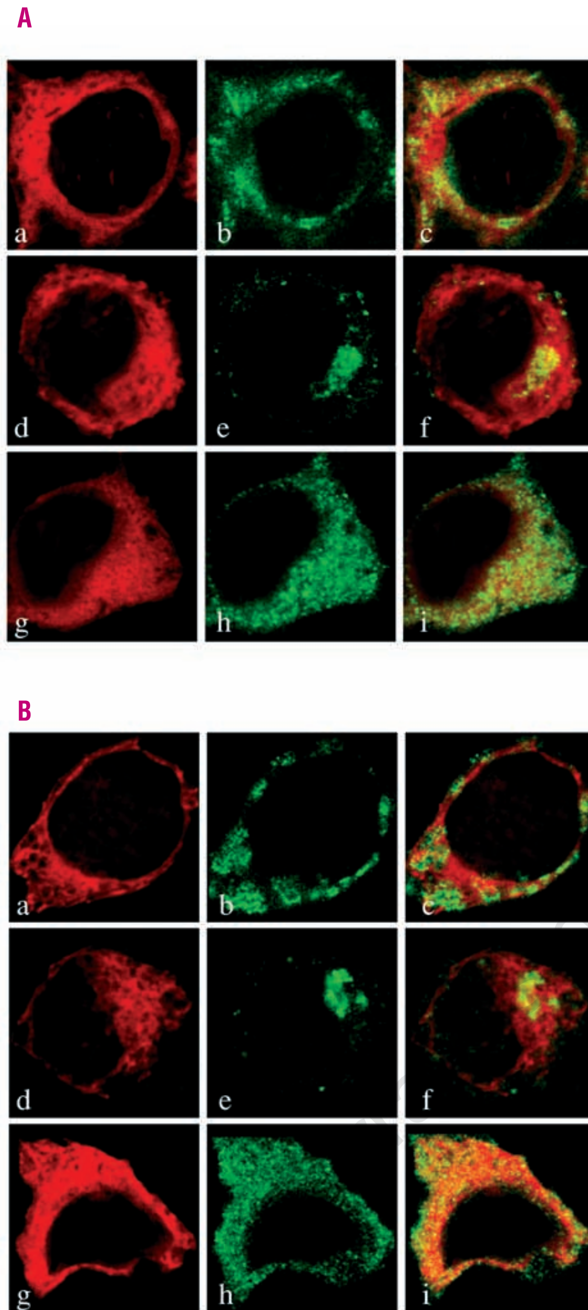

Figure 2. Double immunofluorescent staining for FX and intracellular organelle markers in HEK cells expressing wild type (A) or Arg204 mutant (B) factor X. Goat anti-human factor X antibody (Haematologic Technologies), followed by biotinylated anti-goat IgG (Vector) and Texas Red Streptavidin (Vector) were used to detect FX. Monoclonal primary antibodies against calnexin and mannose 6 phosphate receptor and rabbit antibody against mannosidase II were from Abcam (Cambridge, UK). The respective secondary antibodies (Vector) were labeled with fluorescein. Confocal laser scanning microscopic images (magnification $100 \times 3,2$). a-c: immunostaning for $FX\alpha$ and the endoplasmic reticulum marker, calnexin; d-f: immunostaning for FX and mannosidase II, a cis-Golgi marker; g-i: immunostaning for FX and mannose-6P receptor, a trans-Golgi marker. FX (a,d,g) appears in red; calnexin (b), mannosidase II (e) and mannose 6P receptor (h) appear in green; c,f and i are merged images.

higher respectively than in cells expressing wild type FX. In agreement with these results, the band representing Arg204 FX on the Western blot was more intense than the band corresponding to Gly204 FX (Figure 1A). FX appeared as a double band which is probably due to the different extent of glycosylation.[2,17] In the media of cells expressing wild type FX 93.7 mU/mL FX was measured by ELISA, while FX could not be detected in the media of cells expressing the mutant protein. Immediately after pulse labeling of HEK cells, the band of wild type FX was more intense than the band representing Arg204 mutant (Figure 1B). During the chase period the amount of radio-labeled wild type FX rapidly decreased in the cell lysate, and, in parallel, bands representing FX heavy and light chains appeared in the culture media. In contrast, the intensity of the band corresponding to the mutant protein increased during the first 30 min in the cell lysate and only then started to decay. No mutant FX appeared in the culture media. Evidently, newly synthesized wild type FX became secreted and rapidly disappeared from the cells, while the mutant proteins, which cannot be secreted, accumulated in the cytoplasm and were finally eliminated by intracellular proteases. Double immunofluorescent staining showed that the localization of Arg204 FX in HEK cells neither corresponded to the localization of the endoplasmic reticulum (Figures 2Ba-c) nor to that of the cis-Golgi apparatus (Figures 2Bd-f). However, the staining for Arg204 FX appeared in co-localization with the trans-Golgi-late endosome network (Figures 2Bg-i). No such co-localization was found in the case of wild type protein (Figures 2Ag-i). The results suggest that the mutant FX is diverted from the normal secretory pathway toward the late endosomes, and finally becomes degraded in lysosomes.

The site of the mutation is located in a small disulphide-bridged loop formed between Cys201 and Cys206. Cys201, Gly204 and Cys206 residues were highly conserved during evolution and they are also present in factor VII, the closest relative of FX (SwissProt database). From in silico experiments (Figure 3), it is evident that the replacement of small apolar glycine at position 204 by the bulky positively charged arginine introduces major structural changes into its surroundings. The region between Cys132 and Arg142, which corresponds with the C-terminal part of the light chain plus the tripeptide, has different orientation and lies much farther from the Cys201-Cys206 disulphide bridged loop than in wild type FX. The mean Coulomb interaction energy between the loop and the Cys132-Arg142 peptide segment was lower in the wild type protein than in the Arg204 mutant (-188.6 kcal/mol versus -88.5 kcal/mol), i.e. in the case of the mutant the interaction considerably weakened. The Cys132 residue is involved in the formation of the disulfide bond that keeps the EGF2 and SP domains together, the mutation could prevent the formation of this disulfide bond or make it unstable and might in this way also influence the global folding of the protein. The structural changes could also alter the molecular recognition and/or transport properties of FX and contribute to the secretion defect of Arg204 mutant.

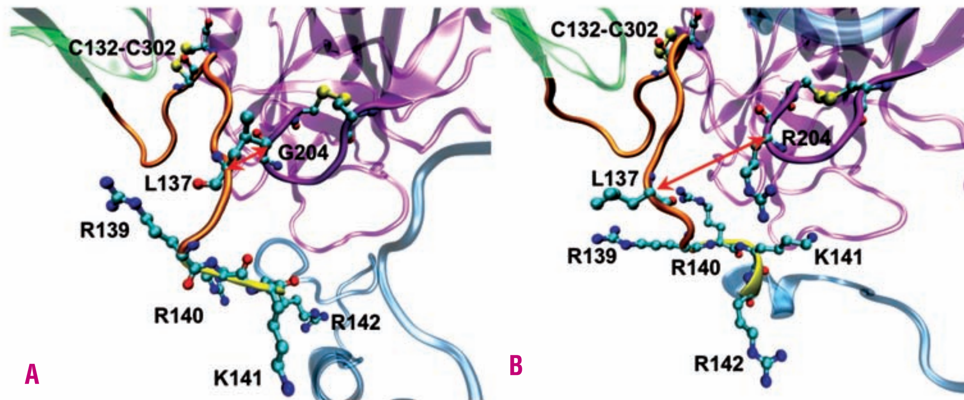

Figure 3. Averaged three-dimensional structure for wild type (A) and Gly204Arg mutant (B) factor X. The starting structure obtained from the Research Collaboratory for Structural Bioinformatics, Protein Data Bank (*www.rcsb.org*) was supplemented according to Venkateswarlu *et al.*[14] The geometries of Gly204 and Arg204 EGF2-CR-AP-SP constructs were submitted to a 7 nanosecond molecular dynamics simulation (6 nanoseconds equilibration and 1 nanosecond sampling). Results were visualized by the Visual Molecular Dinamics software suit.[18] Parts of epidermal growth factor 2 (transparent green), activation peptide (steal blue), serine protease (transparent pink) domains and the whole connecting region (gold) are shown on the figure. The Arg140-Lys141-Arg142 tripeptide is depicted in yellow. Selected amino acid residues as well as the disulfide bonds Cys132-Cys302 and Cys201-Cys206 are shown by ball-and-stick model. The location of Cys201-Cys206 loop is shown in opaque pink. The distance between the 204 C_α and 137 C_α atoms is shown by a double-headed red arrow.

Authorship and Disclosures

ZB was involved in all aspects of research, data analysis and writing the paper; HB and RÁ performed and evaluated immunofluorescent studies; IK performed molecular modeling; CK and ÉA were involved in the laboratory diagnosis and management of the patient; GH was involved in immunoblotting experiments; LM designed research, analyzed the data and wrote the paper.

The authors reported no potential conflicts of interest.

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