Expression of Human Blood Coagulation Factor XI: Characterization of the Defect in Factor XI Type III Deficiency

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Factor XI (FXI) is a zymogen of a serine protease that participates in the intrinsic or contact phase of the blood coagulation cascade. Human FXI is a glycoprotein that consists of two identical polypeptide chains held together by a single disulfide bond in each of the two polypeptide chains. During the activation of FXI, a single internal ArgLeu peptide bond in each of the two polypeptide chains is cleaved. FXIa is composed of two heavy and two light chains, which are held together by three disulfide bonds. Each of the light chains contains the catalytic portion of the enzyme and is homologous to the trypsin family of serine proteases. Each of the heavy chains consists of four amino acids of 90 (or 91) amino acids, with characteristic disulfide bonds. The first apple domains are responsible for the binding of FXI to high molecular weight kininogen and a polyanionic surface. The first intron (intron N) of the gene. This mutation interferes with calcium-dependent binding of FXI to FIX. The FXI deficiency is a disorder characterized by a mild or no bleeding tendency. The vast majority of the FXI deficiencies have been found in the Ashkenazi Jewish population. Recently, the occurrence of three point mutations in the gene for FXI in Ashkenazi Jewish patients has been described. The type I mutation was rare and involved a nucleotide change at an intron/exon splice junction in the last intron (intron N) of the gene. This mutation interferes with the splicing of the messenger RNA (mRNA). The two most prevalent mutations were type II, resulting in the conversion of Glu147 to a stop codon in the second apple domain, and type III, an amino acid substitution of Leu for Phe at position 283 (Phe283Leu) in the fourth apple domain. Although the Phe283Leu conversion was the only change found in the coding region of the gene, it was not certain whether this mutation caused the FXI deficiency directly or was linked to an unidentified mutation. Therefore, in vitro expression of normal human FXI has been investigated and compared with the expression of the Phe283Leu mutant FXI in baboon kidney (BHK) cells. Characterization of both the normal and mutant FXI proteins showed that the Phe283Leu mutation caused an impaired dimerization and secretion of FXI.

MATERIALS AND METHODS

Construction of expression vectors. Human FXI cDNA was subcloned from the original pBR322 vector into the EcoRI site of pZEM229R for expression, and into M13mp18 for mutagenesis. The pZEM229R was kindly provided by Dr E.R. Mulvihill, Zymogenetics Inc, Seattle, WA. The oligonucleotide primer PFXI-M3 (GACACTGATCTCTTGGGAGAA) was synthesized on an Applied Biosystems Synthesizer (Foster City, CA) and used for mutagenesis of amino acid residue 283 from Phe to Leu. Mutagenesis was performed using T7-GEN (US Biochemicals, Cleveland, OH) by the method of Vandeyar et al. The mutation in the cDNA was confirmed by dye sequence analysis and the new construct was then cloned into pZEM229R.

Plasmids were transformed into Escherichia coli RR1 cells and grown in Terrific Broth for large-scale preparation. Alkaline lysis and CsCl banding were performed according to standard procedures, with the addition of phenol-chloroform extractions before and after CsCl banding. The authenticity of the FXI cDNA inserts was verified by dye sequencing of the transfection plasmids.

Cell culture. A thymidine kinase-deficient BHK cell line, BHK-570 (ATCC no. CRL 10314), was used as the host cell for the transfection experiments. Cells were grown in Dulbecco's modified Eagle medium (DMEM), 0.5% fetal bovine serum (FBS), 50 μg/mL penicillin, 100 μg/mL streptomycin, and 0.6 mmol/L Na₂HPO₄ in a 5% CO₂ atmosphere at 37°C. For transfection, BHK-570 cells were plated at 1:25 split ratios in 150-mm plates (Falcon, Oxnard, CA) overnight and transfected for 4 hours in 10 mL of medium with 30 μg of plasmid precipitated with calcium phosphate. After a 1-hour shock in 15% glycerol in Tris-phosphate-buffered saline (PBS) (25 mmol/L Tris-HCl, pH 7.4, 0.14 mol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 0.6 mmol/L Na₂HPO₄), the cells were grown...

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for 24 hours in normal medium. The cells were then subjected to selective medium containing 1 μmol/L methotrexate for 7 to 10 days. Random clones were picked and propagated.

For studies of intracellular biosynthesis and secretion of recombinant FXI (rFXI), the clones were grown to confluence in 100- or 150-mm plates. At confluence, the cells were washed twice with 120 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L sodium phosphate, pH 7.4 (PBS). Serum-free medium was then added, and collected after 24 hours. The cells were washed twice with PBS, and treated with RIPA (10 mmol/L HEPES, pH 7.5, 30 mmol/L NaCl, 5 mmol/L EDTA, 0.3 mol/L sucrose, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate [SDS]) for 1 hour. The RIPA fraction was used as an intracellular pool for protein and FXI determinations.

To purify secreted rFXI, the FXI-secreting cell line rFXI-24 was grown in 20 150-mm plates. Confluent monolayers were washed twice with PBS containing 0.01% CaCl, and 0.01% MgCl. Serum-free medium was added, and collected every 24 hours for 4 to 5 days. Daily collections were made, 10 mmol/L in benzamidine and 0.1 mmol/L in DFP, stirred for 15 minutes at room temperature, and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was diluted with an equal volume of water and then applied to a CM-Sephadex column (2.5 × 10 cm), previously equilibrated with phosphate buffer (20 mmol/L sodium phosphate, pH 7.0, 50 mmol/L NaCl, 0.02% sodium azide). rFXI was eluted with 20 mmol/L sodium phosphate buffer, pH 7.0, containing 0.6 mol/L NaCl. The eluted protein was then applied to a high molecular weight kininogen peptide IV column, as described by Naito and Fujikawa. The yield of purification of rFXI varied between 15% and 30%.

rFXI (300 pmol) was desalted on an HPLC C3 column, and sequenced using a Model 477A Applied Biosystems protein sequencer.

RNA extraction, Northern blotting, and hybridization. Total cytoplasmic RNA was prepared from control or transfected BHK cells by the single-step method of Chomczynsky and Sacchi. RNA samples were denatured in a formamide/formaldehyde solution as described by Sambrook et al for 15 minutes at 55°C, and separated by electrophoresis in a denaturing 1% agarose gel. Transfer of the RNA to nitrocellulose was performed with a Vacuum Blotting System (Pharmacia-LKB, Piscataway, NJ). After transfer, the RNA was crosslinked to the membrane by a UV Stratalinker (Stratagene, La Jolla, CA). Blots were then prehybridized for 4 hours and hybridized for 20 hours at 42°C in a solution containing 50% deionized formamide, 6X SSC (1X SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate), 50 mmol/L sodium phosphate, 0.1 mg/mL yeast total RNA, and 2X Denhardt’s solution (2X Denhardt’s is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin). Posthybridization washes were performed to a final stringency of 0.2X SSC/0.1% SDS at 65°C for the FXI cDNA probe and 0.1X SSC/0.1% SDS at 65°C for the 28S RNA cDNA probe. The density of autoradiographic signals was quantitated with a laser scanning densitometer equipped with an integrator (DU-79 Spectrophotometer; Beckman, Fullerton, CA). Values of 28S RNA were used to correct for differences in the loading of RNA.

Preparation of DNA probes. The FXI cDNA probe was the full-length cDNA obtained from the pB322 plasmid. A 280-bp bovine 28S ribosomal subunit cDNA probe (kindly provided by Dr E. H. Sage, University of Washington) was used for normalization of the RNA concentrations. DNA fragments were nick-translated with a Multiscribe DNA labeling system (Amersham, Arlington Heights, IL) in the presence of [32P]-dCTP (Amersham).

Metabolic labeling studies. Metabolic labeling studies were performed in 6- or 24-well plates in cysteine-free minimum essential medium (MEM) or DMEM, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 50 to 100 μCi/mL [35S]-L-cysteine (Amersham) in a 5% CO2 atmosphere at 37°C for 24 hours. The intracellular proteins were obtained by RIPA treatment as described above. The supernatants and RIPA fractions were preclarified for 1 hour at 4°C with 10 μL of a 1:1 suspension of goat antiserum IgG coupled to agarose (Sigma, St Louis, MO). After centrifugation, the supernatant was transferred to a tube containing 1 μg of an FXI monoclonal antibody (XI-5) and incubated on ice for 1 hour. Ten microliters of a 1:1 suspension of goat antiserum IgG coupled to agarose was then added and the mixture was incubated for 2 hours at 4°C with rotation. The agarose was washed four times by centrifugation (three times with 50 mmol/L Tris, pH 7.4, 0.5 mol/L NaCl, 0.1% NP-40 followed by a wash with 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl). After the last wash, 50 μL of Laemmli-buffer containing 5% SDS was added and incubated for 60 minutes at 37°C before SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was fixed and treated with Amplify (Amersham) according to the instructions of the manufacturer before autoradiography.

Analytical methods. Protein determinations were performed with the BCA-assay (Pierce, Rockford, IL) using bovine serum albumin as reference.

SDS-PAGE and immunoblotting were performed as described by Laemmli and Towbin et al, respectively. Prestained and [35S]-labeled molecular weight markers were obtained from Bethesda Research Laboratories (Bethesda, MD). The loading per lane was adjusted to the amount of cellular protein.

FXI activity was determined in a one-stage clotting assay using FXI-deficient bovine plasma. FXI antigen was determined with an enzyme-linked immunosorbent assay (ELISA), using a monoclonal antibody to FXI as primary antibody, and immunopurified rabbit antibodies to FXI as secondary antibody. Normal human plasma (George King Biomedical, Overland Park, KS) was used as a reference for both the clotting and the ELISA assays.

Oxidation of rFXI was performed according to Hirs and the amino acid composition of the oxidized sample was determined with a Waters PicoTag system (Waters, Milford, MA) as described by Bidlingmeyer et al.

RESULTS AND DISCUSSION

Expression vectors for human rFXI. An expression vector was constructed by cloning the cDNA coding for human FXI into pZEM229R. This plasmid, designated pZEM229R-XI, is a pUC-based vector that includes a dihydrofolate reductase cDNA as selectable marker (Fig 1). The FXI cDNA was placed under the control of a modified version of the metallothionein promoter that was in juxtaposition to the SV40 enhancer. The metallothionein promoter has nearly full induced transcriptional activity in this construct. The SV40 terminator was used to ensure proper termination of the FXI mRNA. The normal FXI cDNA was also subjected to site-specific mutagenesis by converting nucleotide 944 from T to C. This resulted in an amino acid change of Phe283 to Leu that is characteristic of the type III FXI deficiency in Ashkenazi Jews. This construct was also cloned into pZEM229R for expression, and designated pZEM229R-XI-Phe283Leu.
Fig 1. Expression vector for human FXI. The FXI cDNA was cloned into the EcoRI site of pZEM229R under the control of the modified methionylthionin (MT-1) promoter. The dihydrofolate reductase (DHFR) cDNA was used as selectable marker.

Expression of rFXI in BHK cells. The BHK cell line BHK-570 that is deficient in thymidine kinase was used as the host cell line for transfection, because it readily expresses many proteins of the coagulation and fibrinolytic pathways, and allows amplification of expression vectors containing the dihydrofolate reductase selectable marker. Transfection of BHK-570 cells with pZEM229R-XI or pZEM229R-XI-Phe283Leu and selection with 1 μmol/L methotrexate resulted in numerous FXI-secreting colonies as measured by ELISA. The cell lines used in characterization of the proteins were called rFXI and rFXI-Phe283Leu.

rFXI was then isolated from a large-scale cell culture preparation by affinity chromatography using a peptide derived from high molecular weight kininogen. The purified rFXI was indistinguishable from plasma-derived FXI on slab gel electrophoresis, under reducing and nonreducing conditions (Fig 2). In addition, rFXI was biologically active with a specific clotting activity of 250 U/mg.

An amino-terminal sequence analysis of the first eight residues of rFXI showed the following: Glu-X-Val-Thr-Gln-Leu-Leu-Lys. This sequence was identical to the amino-terminal sequence of human FXI, with X being Cys. It was distinctly different from the bovine sequence of Glu-Cys-Val-Thr-Thr-Leu-Phe-Gln. Accordingly, the purified FXI was the human protein and not derived from bovine serum used during tissue culture.

Recently, McMullen et al reported that Cys-11 was not involved in dimer formation of FXI, but was bound to a single Cys residue. To investigate whether rFXI also had a Cys residue bound to Cys-11, the cysteic acid contents of oxidized intact rFXI (without sample hydrolysis) was analyzed. rFXI contained 0.6 moles of cysteic acid per mole of subunit of FXI. These data show that rFXI was essentially identical in structure and function to the plasma-derived FXI.

Characterization of FXI Phe283Leu. Recently, Asakai et al reported the defects in six FXI-deficient patients of Ashkenazi Jewish origin. Three point mutations were identified. Two of the mutations could account for the observed deficiency, because they resulted in the formation of a premature stop-codon, or a change that disrupted an intron-exon splice boundary. The third mutation was an amino acid substitution of Leu for Phe at position 283. This mutant was also characterized using the in vitro expression system. Cells transfected with the FXI-Phe283Leu expression vector secreted about 8% of the level of cells expressing the normal factor XI (Table 1). This level was similar to that observed in patients homozygous for type III FXI deficiency. This secretion level was consistent with the conclusion that the substitution of Leu for Phe at position 283 was responsible for the type III FXI deficiency. It also implies that the in vitro expression system for FXI type III accurately represents the defect found in these patients and provides a good system for studying the molecular mechanism.

Western blot analysis of secreted rFXI and rFXI-Phe283Leu showed that both molecules were dimers with molecular weights identical to plasma-derived FXI on
### Table 1. Characterization of rFXI and rFXI-Phe283Leu—Producing Cell Lines

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<tr>
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<th>Expression Levels (U/mL)</th>
<th>Specific Activity (U/U)</th>
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<tr>
<td>rFXI</td>
<td>0.20 ± 0.09 (n = 9)</td>
<td>0.71 ± 0.24 (n = 9)</td>
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<tr>
<td>rFXI-Phe283Leu</td>
<td>0.017 ± 0.01 (n = 4)</td>
<td>0.68 (n = 1)</td>
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*Expression levels were determined from 24-hour serum-free medium collections (10 mL) of 10-cm confluent plates. Antigen was determined with an ELISA as described in Materials and Methods. Confluent plates contained 0.88 ± 0.07 mg of cellular protein. Data are expressed as mean ± SD, with 1 U being the amount of FXI present in 1 mL of normal plasma. n represents the number of FXI-secreting cell lines.

†Specific activity is expressed as clotting activity per unit of antigen.

SDS-PAGE (Fig 3). The minor band visible in Fig 3 with an apparent molecular weight of 60 Kd represents monomeric FXI under nonreducing conditions. These data suggested that the secreted proteins were fully processed. Both recombinant proteins were also active in clotting assays (Table 1). Normal rFXI had a specific clotting activity to antigen ratio of 0.71 ± 0.24 (mean ± SD of nine different FXI-producing clones) compared with 0.68 for the Phe283Leu mutant protein.

Northern analyses were also performed to investigate whether the difference in secretion between normal rFXI and rFXI-Phe283Leu was caused by a decrease in the mRNA level. Figure 4 shows that comparable levels of FXI mRNA were present in both the FXI and FXI-Phe283Leu-producing cell lines. BHK-570 cells, which were not transfected, did not show any detectable mRNA for FXI. When the mRNA was normalized to 28S ribosomal RNA (Fig 4, lower panel), the level in the FXI-Phe283Leu clone was 34% higher than the mRNA level of the normal FXI clone. This indicates that the decreased secretion by the FXI type III mutant clone was not due to differences in steady-state levels of FXI mRNA.

Metabolic labeling studies of intracellular, normal FXI also showed that the major portion of FXI was present as a dimeric molecule (Fig 5). The molecular weight, however, was slightly less than the secreted FXI, suggesting that a posttranslational step, such as glycosylation, was still necessary before secretion would occur. The mutant protein showed a marked decrease in the amount of dimeric FXI, together with an increase in monomeric FXI. Densitometric scanning of the autoradiographs showed that the intracellular mutant FXI was about equal in dimeric FXI compared with monomeric FXI, while in the normal cell line the amount of dimeric FXI was 30-fold higher than monomeric FXI.
Pulse-chase labeling experiments with the FXI and FXI-Phe283Leu-producing cell lines indicated that secretion of normal FXI was relatively slow, resulting in cellular accumulation of the dimeric form of FXI (Fig 6). In the Phe283Leu mutant a significant amount of FXI was monomeric. In time, however, the monomer was converted to a dimer and was secreted (Fig 6). The presence of an increased amount of monomeric FXI in the type III mutant implies that dimerization of the mutant protein was impaired. Once the dimer was formed, it was secreted and was biologically active. The increased amount of monomeric FXI in the mutant suggested that some accumulation occurred before the formation of the dimer. It was also possible that a portion of the monomer was degraded, because no extensive accumulation occurred. These data further indicate that the type III mutation can account for the defect in FXI-deficient patients. It is also in agreement with studies that found a strict correlation between antigen and activity levels in FXI-deficient individuals.27,28

There are several possible reasons for a decreased secretion of the mutant FXI compared with wild-type FXI.

For instance, the mutation may interfere with the correct folding of the fourth apple domains. Also, it may retard the dimerization of the two chains, which involves the formation of a disulfide bond between the fourth apple domains. This explanation seems quite likely, because the intracellular level of dimeric FXI was markedly reduced in the mutant as compared with that of the normal molecule.

The in vitro expression system provides a unique tool for studying the assembly and processing of FXI. Furthermore, it provides a system by which mutations in the FXI molecule may be selectively introduced to study structure-function relationships.

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Fig 5. Characterization of intracellular rFXI and rFXI-Phe283Leu. Cells were metabolically labeled with [35S]-cysteine in cysteine-free MEM medium. After 24 hours, the intracellular proteins were obtained by RIPA treatment of the cells and immunoprecipitated. The proteins were separated by SDS-PAGE on a 7.5% gel. Lane 1, BHK cells; lane 2, rFXI-secreting cells; lane 3, rFXI-Phe283Leu-secreting cells. The molecular weights of the marker proteins are indicated in kilodaltons.

Fig 6. Pulse-chase labeling of cells secreting rFXI and rFXI-Phe283Leu. Cells were metabolically labeled with [35S]-cysteine in cysteine-free DMEM for 16 hours. Then the cells were washed and chased with regular medium for the indicated times in hours. Cells secreting rFXI (A and B), and cells secreting rFXI-Phe283Leu (C and D) were used. The secreted proteins were immunoprecipitated from the supernatants (B and D), while the intracellular proteins were obtained by immunoprecipitation of the RIPA-treated cells (A and C). The proteins were separated by SDS-PAGE on 7.5% gels. The molecular weights of the marker proteins are indicated in kilodaltons.
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