Molecular Defect (Gla$^{+14} \rightarrow \text{Lys}$) and Its Functional Consequences in a Hereditary Factor X Deficiency (Factor X “Vorarlberg”)*

(Received for publication, November 21, 1989)

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Factor X (FX) “Vorarlberg” is a congenital FX deficiency characterized clinically by a mild bleeding tendency. Homozygous individuals have a FX activity of less than 10% in the extrinsic system and 25% in the intrinsic system. FX antigen is 20%. Using molecular techniques, two point mutations were detected in the coding sequence of the FX Vorarlberg gene: a G→A at base pair 160 in exon II resulting in a change of Gla→Lys (AAA); a G→A at base pair 424 in exon V resulting in a change from Glu to Lys (AGA) to Lys (AGG). The mutations abolished a TaqI restriction site in exon II and an MnlI site in exon V. To determine whether these mutations are present on one or both alleles, restriction analyses of amplified exon II and exon V fragments were performed. Analysis of the pedigree showed that the genotype for the mutation on exon II (homozygous versus heterozygous) correlates with the severity of the phenotypic coagulation defect. We therefore conclude that the mutation in exon II is responsible for the functional defect in FX Vorarlberg. We have also purified the mutant FX protein from patient plasma. Purified FX Vorarlberg is indistinguishable from normal FX on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its activity is 15% of normal FX upon activation with factor VIIa/tissue factor, 75% upon activation with factor IXa/factor VIIIa, and 100% upon activation with RVV. Activation at varying Ca$^{2+}$ concentrations shows that the affinity of FX Vorarlberg for Ca$^{2+}$ is decreased for Ca$^{2+}$; Factor Xa Vorarlberg is able to convert prothrombin at a normal rate but also shows decreased affinity for Ca$^{2+}$ in this interaction. Upon addition of Ca$^{2+}$, FX Vorarlberg does not undergo the same conformational change as normal FX. Our data show that FX Vorarlberg has a decreased affinity for Ca$^{2+}$ which impedes a normal conformational change. This leads to a decreased rate of activation by factor VIIa/tissue factor and by factor IXa. The decrease is much more marked for the extrinsic than for the intrinsic pathway.

Factor X (FX) is a plasma glycoprotein required in both the intrinsic and extrinsic pathways of blood coagulation (1). It is synthesized in hepatocytes as a single polypeptide chain; following several post-translational modifications, including glycosylation, vitamin K-dependent carboxylation of the y-carbon of the first 11 glutamic acid residues, and cleavage of the leader sequence, the mature protein is secreted into the circulation. FX circulates as a two-chain zymogen composed of a light chain (Mr 16,200) and a heavy chain (Mr 42,000) joined by a single disulfide bond (2). The light chain of FX contains the 11 y-carboxyglutamic acid residues, 1 residue of $\beta$-hydroxyaspartic acid, and two growth factor-like domains, which show sequence similarity with epidermal growth factor. The heavy chain contains the activation peptide and the catalytic domain. A single arginine-isoleucine bond is cleaved in the heavy chain of FX by both the intrinsic and the extrinsic pathways of blood coagulation, generating factor Xa (FXa) and a small activation peptide (3). This reaction is catalyzed by factor IXa (FIXa) and its cofactor VIIIa (FVIIa) in the intrinsic system and by factor VIIa (FVIIa) and its cofactor tissue factor (TF) in the extrinsic system. Both reactions require calcium ions and phospholipids (4, 5).

The amino acid sequence for human FX has been derived from FX cDNAs and direct amino acid analysis (6-9). The gene has also been isolated and partially characterized. It has been mapped to chromosome 13q32-pter (10), where it spans approximately 25 kilobases. Although still incompletely characterized, it consists of seven introns and eight exons and shows considerable structural homology with the genes encoding the other vitamin K-dependent clotting factors (11). The functional domains of the protein are represented by different exons: exon I codes for the signal peptide region, exon II for the propeptide and the y-carboxyglutamic acid-rich domain, exon III for the short aromatic acid-rich stack, exon IV for the activation peptide, and exons VII and VIII for the catalytic domain. The splice junction sites follow the GT/AG rule, include type 1 and type 0 junctions, and delineate seven introns ranging in size from 950 to 7400 nucleotides (11). The human FX mRNA consists of approximately 1500 nucleotides including a coding region of 1475 bases and a short 3’-untranslated region of 10 nucleotides (8). The polyadenylation signal (ATTAAA) is located in the coding sequence and
precedes the stop codon by one nucleotide. The 5' end of the mRNA has not been characterized.

Congenital FX deficiency is inherited as an autosomal recessive trait (12). Considerable phenotypic heterogeneity exists among factor X variants. This is evident both structurally, with patients reported to have normal, reduced, or absent antigen levels (13, 14), and functionally, as evidenced by the variety of patterns seen following activation of abnormal Factor Xs in either the intrinsic or the extrinsic system (15). FX "Vorarlberg" has different activities in the extrinsic versus the intrinsic system. Although little is known about the mechanisms by which Factor X is activated by IXa/VIIIa or VIIa/TF. In this work, we report the molecular basis of a new FX variant, FX "Vorarlberg," using DNA sequence analysis. Further, we characterize the mutation at the Gla residue results in a protein with decreased ability to bind calcium. This decrease appears to account for its altered function.

**EXPERIMENTAL PROCEDURES**

**Gene Analysis**

*Patient Data—* Blood samples were obtained after informed consent from two members (Fig. 1) of a FX-deficient family living in a remote valley in the mountain area of Vorarlberg, Austria. FX deficiency was detected in III/9 at a routine preoperative coagulation screening. Past history was notable only for mild bruising since childhood. Tooth extractions, an appendectomy, and a hysterectomy had been performed without any apparent bleeding tendency.

**Isolation and Characterization of Mutant Factor X Gene—** Genomic DNA was prepared from the patients' peripheral blood using standard methods (16), and Southern blots were performed to determine whether the mutant gene was grossly intact (17). Ten µg of genomic DNA from a patient (III/4 in Fig. 1) and from a nonrelated normal control were digested with 30 units of EcoRI at 37 °C for 3 h. The DNA was electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane according to the method of Southern (17). A FX cDNA was radiolabeled with [32P]dCTP by priming with random hexanucleotides, using the manufacturer's conditions (Boehringer Mannheim). Twenty ng of labeled probe with a specific activity of 1.8 x 10⁶ were added to the filter, incubating in 6 x SSC (where SSC is standard saline citrate), 0.01 M EDTA, 0.1 M KPO₄, 5 x Denhardt's solution, 0.5% SDS, and 250 µg/ml salmon sperm DNA. After incubation for 12 h at 68 °C, the filter was washed at 0.5 x SSC twice for 15 min at room temperature, in 0.1 x SSC, 0.5% SDS for 45 min at 68 °C and exposed to x-ray film at −70 °C for 24 h.

All eight exons of FX were isolated using the enzymatic amplification technique (18). Synthetic oligonucleotides derived from previously published intron sequences flanking the 5' and 3' ends of each exon (19) were used to prime amplification (Table I). HindIII restriction sites were built in at the 5' ends of each oligonucleotide (underlined in Table I). Target sequences were amplified in a 100-µl volume containing 1 µg of genomic DNA, 10 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1% (w/v) gelatin, 1.5 µM of each deoxynucleotide (dNTP:dCTP, dGTP, dATP, dTTP), 1 µM of each primer, and 1 unit of Taq polymerase. The samples were overlaid with 100 µl of mineral oil to prevent condensation and subjected to 30 cycles of denaturation at 94 °C for 2 min, annealing at 55 °C for 2 min and primer extension at 70 °C for 3 min in a DNA Thermal Cycler R (Perkin-Elmer Cetus Instruments). The amplified DNA was electrophoresed through a 2% agarose gel in Tris-borate-EDTA buffer (TBE) and the gel fragment containing the amplified sequence cut out. The recovered gel slice was ground and incubated with 50 µl of TBE, 50 µl of phenol, and 50 µl of chloroform:isoamyl alcohol (24:1) for 15 min at −70 °C. After centrifugation for 15 min in a microcentrifuge, the supernatant was recovered and the DNA precipitated with ethanol. After digestion with HindIII, the fragments were subcloned into M13 sequencing vectors and sequenced as previously described (20). Exons I, III, IV, VI, VII, and VIII were sequenced in both orientations from a single PCR amplification for each exon. Exons II and V were sequenced twice in each orientation using four different subclones from three different amplifications. Exon II and V amplified twice in each orientation using four different subclones from three different amplifications. Exon II and V were also amplified as described above to perform restriction analysis. Thirty-base synthetic oligonucleotides derived from the exon and part of the intron were used in this amplification to yield an exon II fragment of 185 bp containing a single TaqI site (Fig. 2) and an exon V fragment of 149 bp containing a single MnlI site (Fig. 3). Fragments were electrophoresed and purified as described above. Digestions were carried out according to the manufacturers' conditions with TaqI for the amplified exon II fragment and with MnlI for the exon V fragment. Digests were electrophoresed on a 6% agarose gel (NuSieve GTG, FMC Bio Products, Rockland, ME) in TBE buffer. Exon II and V amplified from normal DNA were used as controls in all restriction digests.

**Proteins**

*Protein Purification and Analysis*—FX Vorarlberg was isolated after informed consent from patient III/9 (Fig. 1), who is homozygous for the causative mutation in the Gla domain (Gla<sup>14</sup>—Lys) and heterozygous for the presumed polymorphic site in the second epidermal growth factor-like domain (Gla<sup>494</sup>—Lys). Normal FX was isolated from outdated frozen normal human plasma. Plasma was thawed at 37 °C and barium chloride (1 mM) was added to a final concentration of 0.1 M. Barium precipitate was collected after centrifugation and washed twice in 0.1 M BaCl₂, 1 mM benzamidine-HCl (three times the original plasma volume). The barium precipitate was eluted with 45% saturated ammonium sulfate, 1 mM benzamidine (one-tenth original plasma volume). The eluate was dialyzed against 3 x 1 liter of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% polyethylene glycol 8000 (PEG), 1 mM benzamidine overnight. FX was isolated from the Ba<sup>2+</sup> citrate eluate by immunoaffinity chromatography using either a mouse monoclonal (gift of Dr. William Church and Dr. Kenneth Mann, University of Vermont (Ref. 21)) or a rabbit polyclonal anti-human FX antibody coupled to an Affi-Gel 10 matrix. The rabbit calcium-dependent anti-human factor X antibody was made from the plasma of a rabbin injected with human factor X. The calcium-dependent antibody was purified by

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; bp, base pair; RVV, Russell's viper venom; PT, prothrombin time; aPTT, activated partial thromboplastin time; DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amine.
passing rabbit serum through a column of factor X-Affi-Gel 10 in the presence of 10 mM EDTA. Antibody was eluted from the column with 10 mM EDTA. The Bs3 eluate was passed through the column at 10 ml/h. The column was washed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG, until the 280-nm absorbance returned to baseline. Factor X was eluted with 3 M sodium thiocyanate, 50 mM Tris-HCl, pH 7.4. The purified protein was dialyzed into 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG, and stored frozen.

Concentration of FX—Concentration of FX (Vorarlberg and normal) was determined by absorbance at 280 nm (extinction coefficient: 1.16 ml x mg⁻¹ x cm⁻¹) corrected for light scattering by measuring absorbance at 320 nm.

Active Site Titration of Factor Xa—This was performed as previously described (22) using p-aminobenzamidine. After activation of FX, 10 ng/ml heparin was added. Small microliter additions of antithrombin III were made, and the fluorescence decrease at 276 nm (excitation at 336 nm) was monitored. The amount of antithrombin III required to displace all of the p-aminobenzamidine from the active site is equal to the concentration of the active enzyme.

Factor IX—FIX and prothrombin were isolated from barium chloride-precipitated plasma using DEAE-cellulose and dextran-sulfate agarose columns as previously described (23).

Factor VIIIa (FVIIIa)—Two different procedures were used to generate FVIIIa from a commercially available FVIII preparation (Monoclate, Armour Pharmaceuticals). In one procedure, 86 nM FVIII was incubated with 5 nM thrombin at room temperature for 10 min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG. The reaction was stopped by addition of the thrombin inhibitor Phe-Pro-Arg-chloromethyl ketone to a final concentration of 20 μM. Thrombin-activated FVIII was used when the consecutive activation of FX (by FIXa and FVIIIa) was monitored by a chromogenic substrate. To avoid any carryover of Phe-Pro-Arg-chloromethyl ketone to the final activation, FVIII was activated with FXa when the consecutive activation of FX was monitored by the conversion of prothrombin to thrombin. In this activation 86 nM FVIII was incubated with 1.5 nM of FXa (activated with RVV) at room temperature for 15 min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG. Thrombin—Human prothrombin was activated by the thrombin activator of E. carinatus coupled to an Affi-Gel 10 matrix. Thrombin was then isolated using an SF ZetaPrep 1 chromatography disc as described elsewhere (24).

Human Factor V and Factor V Coagulant Protein from Russell's Viper Venom—These proteins were a generous gift of Drs. Barry Lentz, Daniel Powers, and Gwyn Cutsforth, University of North Carolina, Chapel Hill.

Human (Recombinant) FVIIa (Niastase)—This was a generous gift from Novo Industries.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed using the techniques of Laemmli as modified by LeStourger and Beyer (25). The gels were stained by a silver stain procedure (26).

Coagulation Assays—One-stage clotting assays were used to determine FX activity of FX Vorarlberg plasma and isolated FX Vorarlberg (27). All clotting assays were performed on a Fibrosystem fibrometer. FX activity was determined using assays based on the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) using standard methods. Activation with Russell's viper venom was performed according to previously published methods (28) using a crude RVV preparation (Sigma).

Coagulant Activity of FX (Vorarlberg and Normal)—For the analysis of FX activity in the extrinsic pathway, 100 μl of FX was incubated for 3 min at 37°C. At the end
of the incubation time, 200 μl of 37 °C thromboplastin (rabbit brain) was added, and simultaneously, the fibrometer timer was started. FX clotting activity was measured in plasma (normal or Vorarlberg) that was serially diluted (1:20 to 1:320) into FX-deficient plasma. For assays of purified factor X, samples were prepared by incubating 60 μM of FX-deficient plasma and 40 μl of various concentrations of pure FX (normal or Vorarlberg) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG. A standard curve was generated with normal FX and the activity of FX Vorarlberg was expressed as a percentage of normal FX.

**uPTT**—For the analysis of the FX activity in the intrinsic pathway, 100 μl of plasma (normal or FX-deficient Vorarlberg) was incubated with 100 μl of human FX-deficient plasma and 100 μl of aPTT reagent for 3 min at 30 °C. At the end of the incubation time, 100 μl of CaCl₂ (25 mM) was added, and simultaneously, the fibrometer timer was activated. FX was measured in plasma (normal or Vorarlberg) that was serially diluted (1:20 to 1:320) into FX-deficient plasma. For assays of purified FX, the concentration of FX (normal or Vorarlberg) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG was varied. A standard curve was generated with normal FX and the activity of FX Vorarlberg was expressed as a percentage of normal FX.

**RVV Time**—For the analysis of FX activity after activation with RVV, FX was incubated with 100 μM of FX-deficient plasma and RVV (0.1 μg/ml) for 30 s at 37 °C. At the end of the incubation time, 0.1% PEG for 30 s at room temperature. The activation of FX was started by the addition of 0.7 nM recombinant FXa. Activation of FX (Normal and Vorarlberg) by RVV/FVIIa—Fifteen nm FX (normal or Vorarlberg) were incubated with 250 μM Spectrozyme FXa and 5 μl of Thromtest (Ortho Diagnostics) as a source of tissue factor in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 0.1% PEG for 30 s at room temperature. The activation of FX was started by the addition of 0.7 nM pure FVIIa.

**Activation of FX (Normal and Vorarlberg) by RVV**—Fifteen nm FX (normal or "Vorarlberg") was incubated with 250 μM Spectrozyme FXa, 5 nm FVIIa, 10 μM phospholipid vesicles in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG for 3 min at room temperature. Phospholipid vesicles (50% phosphatidylycerine, 50% phosphatidylcholine) were prepared by reverse phase evaporation (30). The activation of FX was started by the addition of 5 mM CaCl₂.

**Activation of FX (Normal and Vorarlberg) by RVV**—Fifteen nm FX (normal or Vorarlberg) was incubated with 250 μM Spectrozyme FXa, 0.5 ng/ml RVV, in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG for 30 s at room temperature. The activation of FX was started by the addition of 5 mM CaCl₂.

**All activations** were performed at a CaCl₂ concentration of 5 mM (as described above) and at CaCl₂ concentrations ranging from 5 to 0.5 mM.

**Prothrombin Conversion by FXa**

The thrombin-specific fluorescent substrate DAPA was synthesized by the procedure of Nesheim et al. (31) and was used to monitor the conversion of prothrombin to thrombin by FXa. FX (normal and Vorarlberg) was first activated to FXa by FVIIa/TF, by FIXa/FVIIIa, and by RVV as outlined above (without the addition of Spectrozyme FXa). To allow complete activation of FXa, reactions were run for 60 min at room temperature. One-hundredth nm of FXs (normal or Vorarlberg) was incubated with 1.2 nM FV, 0.10 ng/ml Factor V-coagulant protein from Russell's viper venom, 1 μM phospholipid vesicles (phosphatidylycerine/phosphatidylcholine = 1:1), 0.5 μM DAPA in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM CaCl₂, 0.1% PEG for 2.5 min at room temperature. The reaction was started by the addition of prothrombin to a final concentration of 120 nM. The increase in fluorescence when DAPA binds to the thrombin generated by the subsequent activation of prothrombin by FXa was measured with a Perkin-Elmer LS-5 spectrophotometer with an excitation wavelength of 380 nm (10 mm slit) and an emission wavelength of 565 nm (20 mm slit).

**Intrinsic Fluorescence Quenching**

Quenching of the intrinsic fluorescence upon addition of CaCl₂ was measured according to the method of Nelson et al. (32) and Prendergast and Mann (33). Small microliter additions of 100 μM CaCl₂ were made to 100 μg/ml of FX (normal or Vorarlberg) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% PEG. The emission spectrum of the FX (normal or Vorarlberg) upon addition of CaCl₂ was monitored at each CaCl₂ concentration (after a 2-min incubation) using a Perkin-Elmer LS-5 spectrophotometer with an excitation wavelength of 380 nm (10 mm slit) and an emission wavelength of 340 nm (20 mm slit).

**Gla Analysis**

Gla analysis was done according to the method of Price (34).

**Data Analysis**

Both the Perkin-Elmer Lambda 3 dual-beam spectrophotometer (Spectrozyme FXa assays) and the Perkin-Elmer LS-5 fluorometer (p-amino-benzamidine and DAPA assays and intrinsic fluorescence measurements) were coupled to a dedicated HP 85 microcomputer. Time course data were sampled using a Hewlett-Packard HP 3490A analog-digital converter. For coupled assays using Spectrozyme FXa, the rate of factor X activation was determined from the slope of the line obtained by plotting the change in the 404-nm absorbance (A₄₀₄) versus the time of the reaction (23). For prothrombin activation assays using DAPA, the rate of thrombin-activation was determined from the slope of the line fit to the initial values of a plot of fluorescence versus time (33).

**RESULTS**

**Screening Coagulation Assays in the Kindred**

The index case (III-9 in Fig. 1) was found to have a FX activity level of 5% using a PT based assay. Therefore, other family members were screened: III-1 and III-4 also had low levels, 4.5 and 7.5%, respectively; members of generation IV had higher FX activity levels, all approximately 50% (IV-1, 51%; IV-5, 54%; IV-9, 54%; IV-10, 48%; IV-11, 65%). Family members with FX activity levels of <10% (III-1, III-4, III-9) are considered phenotypically homozygous, and those with FX activity levels of approximately 50% are considered phenotypically heterozygous.

**Isolation and Characterization of the Mutant FX Gene**

Southern blot analysis of a patient's (III/4) EcoRI-digested genomic DNA probed with a cDNA containing the entire coding sequence showed bands at 7.5, 5.2, 2.8, and 2.5 kilobase. The same bands were seen with genomic DNA from a non-related control (data not shown). Thus, there was no evidence of gross gene deletion or rearrangement in the patient's FX gene. The nucleotide sequence for all exons and for the exon/intron junctions was determined from patient's III/4. Sequence analysis revealed two differences between this sequence and the normal FX cDNA sequence. One mismatch was found at bp 91 of exon II (bp 160 of the coding region of the cDNA) (Fig. 4). The G-A change at the first nucleotide of codon 14 results in the substitution of a lysine (AAA) for a glutamic acid residue. The mutation abolishes a naturally occurring TaqI restriction site with the sequence 5' GGAA TAAT 3' (Fig. 2). Thus, genomic exon
II fragments amplified from the mutant allele cannot be cut with TaqI. Enzymatically amplified and digested exon II fragments (Fig. 1) from the phenotypically homozygous family members III/1, III/4, and III/9 uniformly show a single band at 185 bp representing the uncut exon II fragment. Therefore, these patients are genetically homozygous for the defect in exon II.

Phenotypically heterozygous family members (IV/1, IV/5, IV/9, IV/10, and IV/11) display three bands: a band of 185 bp which is also seen in homozygous patients and represents DNA amplified from a mutant allele, and bands of 84 and 101 bp representing the cut exon II fragment amplified from a normal allele. Thus, these patients are heterozygous for the defect in exon II. Complete cutting occurs when exon II is amplified from a normal control.

A second mismatch was detected at bp 57 of exon V (bp 424 of the cDNA coding sequence). The G→A change at the first bp of codon 102 results in the replacement by lysine of the normal allele. Thus, these patients are heterozygous for the defect in exon V. Complete cutting occurs when DNA is amplified from a normal control.

Analysis of the pedigree (Fig. 1) shows that there are at least two mutant alleles in this family. One mutant allele carries the defect in exon II and the defect in exon V. The other mutant allele is defective in exon II but has a normal sequence in exon V. The possibility of a third mutant allele, normal in II but carrying the mutation in exon V, exists but cannot be proved or disproved based on the available kindred data. Family members who are phenotypically heterozygous for the defect in exon II may be X(XVorarlberg) heterozygous for the defect in exon V. Complete cutting occurs when DNA is amplified from a normal control.

Analysis of the pedigree (Fig. 1) shows that there are at least two mutant alleles in this family. One mutant allele carries the defect in exon II and the defect in exon V. The other mutant allele is defective in exon II but has a normal sequence in exon V. The possibility of a third mutant allele, normal in II but carrying the mutation in exon V, exists but cannot be proved or disproved based on the available kindred data. Family members who are phenotypically heterozygous for the defect in exon II are heterozygous for exon V.

Isolation of FX (Normal and Vorarlberg)

FX Vorarlberg and normal FX were isolated from BaCl2-precipitated plasma using three different antibody columns: 1) a rabbit polyclonal anti-human FX antibody; 2) a monoclonal anti-FX antibody; and 3) a calcium-dependent rabbit polyclonal anti-human FX antibody. FX Vorarlberg and normal FX from any of the three antibodies ran as a single band on unreduced SDS-polyacrylamide gel electrophoresis with an apparent Mr of 70,000 and were both processed into their two-chain forms under reducing conditions with an apparent Mr for the heavy chain of 47,000 (Fig. 5). Amounts of material loaded onto the gel were insufficient to allow visualization of the light chain, which stains poorly with silver stains. While not quantitative precisely, the yields from patient plasma for the three columns were similar, suggesting that factor X Vorarlberg exists as one population in patient plasma rather than two, i.e., one responsive to Ca2+-dependent conformational changes and the other refractory to such changes. Still, to avoid isolating a subpopulation of factor X Vorarlberg, the non-Ca2+-dependent antibody columns were used for purification.

Gla Analysis

Amino acid analysis showed that neither the Gla content nor the ω-hydroxyaspartic acid content of FX Vorarlberg differed significantly from the content of normal FX (isolated by the identical procedures). FX Vorarlberg analyzed (as described under “Experimental Procedures”) for 5.9 mol of Gla/mol of protein as compared with 6.5 mol of Gla/mol of normal factor X. The ratio of Gla in Vorarlberg to Gla in normal FX of 0.83 is almost exactly the value that would be expected (0.91) if a single Gla residue were removed from normal factor X. These results indicate that the substitution of Lys for Glu14 does not lead to a marked reduction in the Gla content of circulating factor X Vorarlberg.

FX-dependent Coagulant Activity of FX Vorarlberg Plasma

To determine coagulant activity of FX Vorarlberg plasma, human FX-deficient plasma was mixed with FX Vorarlberg (or normal) plasma and the PT, APTT, and RVV-times determined (Table II). Using the PT, FX Vorarlberg plasma has a FX clotting activity of 5% of normal. Using the APTT, FX Vorarlberg plasma has a FX clotting activity of 25% of normal. When RVV is used to activate FX in plasma, FX Vorarlberg has a FX clotting activity of 15% of normal FX.

Table II

| Assay       | Patient plasma | Reconstituted plasma |
|-------------|----------------|----------------------|
| Antigen     | 0.20           | 1.00                 |
| PT          | 0.05           | 0.15                 |
| aPTT        | 0.20           | 1.00                 |
| RVV         | 0.15           | 1.00                 |

* Purified factor X (normal or Vorarlberg) was added to factor X-deficient plasma.
FX antigen was reduced to 20% of normal in the FX Vorarlberg plasma.

Coagulant Activity of Purified FX Vorarlberg

Since the antigen levels were reduced in patient plasma, to determine the coagulant activity of the purified FX Vorarlberg, identical amounts of the purified FX Vorarlberg protein or normal FX were added to human FX-deficient plasma and the PT, the aPTT, and the RVV-times of these plasmas were measured (Table II). Using the PT as a measurement of the FVIIa/TF-dependent, extrinsic pathway of blood coagulation, purified FX Vorarlberg has a clotting activity of 15% of normal. Using either the aPTT or RVV assays, purified FX Vorarlberg has a FX clotting activity of 100% of normal.

Activation of Purified FX Vorarlberg

The reduced activity of FX Vorarlberg in the PT could be due either to a defect in the activation of FX Vorarlberg by FVIIa/TF or to a defect of the activated factor thus formed. To characterize the interaction of purified FX Vorarlberg with the enzymes FVIIa, FIXa, and RVV, the initial rate of activation of purified FX Vorarlberg was compared with the initial rate of activation of normal FX using the chromogenic substrate Spectrozyme FXa. The increase in absorbance when FXa cleaves the chromogenic substrate is proportional to the FXa generated by the cleavage of the enzymes. All activations were performed at room temperature with Ca++ concentration (the same Ca++ concentration used in the coagulant assays).

Activation with FVIIa—Using a molar excess of human recombinant FVIIa in the presence of a tissue factor source (Ortho brain thromboplastin), the rate of activation of FX Vorarlberg was 15% of the rate of activation of normal FX (Table III).

Activation with FIXa—Using a molar excess of human FIXa/FVIIa and phospholipid vesicles, the rate of activation of FX Vorarlberg was 75% of the rate of activation of normal FX (Table III).

Activation with RVV—When RVV was used to activate FX, no difference was observed in the rate of activation between FX Vorarlberg and normal FX (Table III).

Ca++ Dependence of FX Activation Activation of FX by FIXa, FVIIa, and RVV is Ca++-dependent. Lowering the Ca++ concentration impedes the rate of activation in a characteristic, non-linear fashion. The molecular defect in FX Vorarlberg affects a potential Ca++-binding residue. We therefore compared the rate of activation of FX Vorarlberg and normal FX at different Ca++ concentrations ranging from 0.5 to 5 mM. Fig. 6 shows the Ca++ dependence of FX activation by FVIIa, FIXa, and RVV. The highest rate of activation of normal FX is set as 100%.

Activation by FVIIa—Normal FX showed a loss in the rate of activation below 5 mM Ca++ and was completely inactive at 0.5 mM Ca++. FX Vorarlberg showed a rate of activation of 15% of normal at 5 mM Ca++ and 3.5 mM Ca++ and had an immediate loss of activity below 3.5 mM Ca++ (Fig. 6B). When the relative rates of the FX activation were used (normalized to 100% at 5 mM Ca++), no difference in the Ca++ dependence of FX Vorarlberg and normal FX was observed.

Activation by FIXa—Beginning at a concentration of 5 mM Ca++, normal FX showed a slightly increasing rate of activation until a Ca++ concentration of 2 mM, with an immediate loss of activity below 2 mM Ca++. FX Vorarlberg had a rate of activation that was slightly reduced compared with normal at 5 mM Ca++ (Fig. 6A). Increasing the Ca++ concentration to 7 mM resulted in a slight loss of activity. Below a Ca++ concentration of 5 mM the rate of activation dropped immediately and was 10% at 3 mM Ca++. Comparison of the relative rates of FX activation showed that the Ca++ affinity of FX Vorarlberg is decreased.

Activation by RVV—Normal FX showed a slight increase in the rate of activation from 5 mM Ca++ to 2 mM and lost activity immediately below that. FX Vorarlberg was normally active at 5 mM but lost activity below 5 mM Ca++ (Fig. 6C). The slopes of normal FX and FX Vorarlberg roughly paral-
led each other but were separated by a difference in the Ca\(^{2+}\) concentration of approximately 3 mM.

Conversion of Prothrombin by FXa

To determine the activity of activated FX Vorarlberg (FXa Vorarlberg) toward prothrombin, normal FX and FX Vorarlberg were activated with FVIIa, FIXa, or RVV. Identical amounts of the activated normal FX or FX Vorarlberg were then used to convert prothrombin to thrombin. At a Ca\(^{2+}\) concentration of 5 mM, the activity of FX Vorarlberg was 100% of normal FX, irrespective of the method of activation of FX (Table III).

Ca\(^{2+}\) Dependence of the Conversion of Prothrombin by FXa

To determine the activity of FXa Vorarlberg and normal FXa at different Ca\(^{2+}\) concentrations, FX (Vorarlberg and normal) was activated with RVV and then incubated with prothrombin at various Ca\(^{2+}\) concentrations (Fig. 7). Normal FXa showed a slight increase in the rate of activation of prothrombin from 5 to 1 mM Ca\(^{2+}\). Below a Ca\(^{2+}\) concentration of 1 mM, normal FXa showed an immediate loss of activity. FXa Vorarlberg showed a slight increase in the rate of activation of prothrombin from 5 to 2 mM Ca\(^{2+}\). Below a Ca\(^{2+}\) concentration of 2 mM, normal FXa showed an immediate loss of activity. The slopes of normal FX and FX Vorarlberg paralleled each other. However, FXa Vorarlberg had an apparent Ca\(^{2+}\) affinity which was 0.5 mM lower than that of normal FX.

Intrinsic Fluorescence Quenching

The intrinsic fluorescence quench of FX (normal and Vorarlberg) is shown in Fig. 8. Addition of Ca\(^{2+}\) to normal FX resulted in a continuous loss of intrinsic fluorescence until the Ca\(^{2+}\) concentration reached 2.5 mM. No further change in fluorescence was observed above this Ca\(^{2+}\) concentration. FX Vorarlberg showed the same loss of intrinsic fluorescence as normal FX until the Ca\(^{2+}\) concentration reached 1.5 mM. While further additions of Ca\(^{2+}\) decreased the fluorescence of normal FX, no further change was observed in FX Vorarlberg.

DISCUSSION

Analysis of the gene coding for FX Vorarlberg disclosed that a point mutation in exon II, which encodes the Gla domain, generates the coagulation defect in these patients. As the mutation is located within a naturally occurring TaqI restriction site, a clear distinction can be made between homozygous and heterozygous genotypes by checking for the TaqI restriction site in the amplified exon II fragments. We were able to show that family members who are genetically homozygous for the defect display a much more severely affected phenotype (i.e. homozygous phenotype) than family members who are genetically heterozygous. Thus, the two distinct classes of phenotype correspond to defects in either one or both alleles of exon II.

The point mutation in exon V, present on one allele, does not appear to be the causative mutation. First, its appearance bears no relationship to the affected phenotype. Second, the affected residue (amino acid 102), in contrast to the one in the Gla domain, is not conserved at all among the vitamin K-dependent coagulation factors. The most likely possibility is that this amino acid change represents a polymorphism.

The mutation on exon II affects the codon for Glu\(^\text{14}\), a \(
\)carboxylated glutamic acid (Gla) residue, and results in the substitution of a lysine at this position. Gla analysis indicates that this substitution results in the loss in circulating FX Vorarlberg of only a single Gla residue. Thus, residue 14 in factor X does not appear to be critical for carboxylation of the remaining Gla residues.

As is the case for the other vitamin K-dependent procoagulant proteins, \(\gamma\)-carboxylation of glutamic acid residues in the amino-terminus portion of the molecule is required for the protein to have coagulant activity (1). It is not yet clear, however, how much of the Gla domain must be intact in order to preserve normal function; nor is it clear whether the intrinsic and extrinsic systems, which both result in the cleavage of a single bond in the heavy chain to generate FXa, depend in an equivalent manner on a fully carboxylated Gla region of X. It is known that complete removal of the Gla domain from factor X results in a species that cannot be activated by either VIIa/TF or IXa/VIIIa (35). Studies of the Gla domain of prothrombin have shown that loss of as few as 3 Gla residues results in a deficient Ca\(^{2+}\)-dependent conformational change associated with poor phospholipid binding (36). Hiskey et al., modifying specific Gla residues in prothrombin fragment 1, also reported poor phospholipid binding (37). The study of factor X Vorarlberg provides a method for defining the functional characteristics of a single specific Gla residue.

Our initial characterization of the mutant protein was by clotting assays on patient plasma. Even this relatively crude measure demonstrated that activity in the extrinsic system (5% of normal), as measured by the PT, was markedly reduced.

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**Fig. 7.** Calcium ion dependence for prothrombin activation by factor X Vorarlberg. Prothrombin was incubated with factor Xa (●) or factor Xa Vorarlberg (■), factor Va, phospholipid, and the indicated concentration of calcium ion as described under “Experimental Procedures.” The rate of thrombin formation (relative to the highest value) is plotted versus the concentration of calcium ion.

**Fig. 8.** Intrinsic fluorescence of factor X Vorarlberg. Factor X (●) or factor X Vorarlberg (■) was titrated with the indicated concentration of calcium and the intrinsic fluorescence measured as described under “Experimental Procedures.” The fluorescence at 320 nm (290 nm excitation) was plotted against the concentration of calcium added. The fluorescence is expressed as a ratio of the initial value (no calcium present).
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carboxylation at Glar4 and the resulting conformational change are more critical for VIIa/TF-mediated activation of X than for IXa-VIIIa-catalyzed activation. This in turn suggests that the two complexes almost certainly have different sites of interaction with FX, despite the fact that they cleave FX at the same bond. It is not possible to determine from these data whether the reduced rate of activation by VIIa-TF depends specifically on the absence of the Gla at residue 14, or whether it results because Ca$^{2+}$ binding is less than maximal (i.e. a point mutation at any other Gla residue would have a similar effect). Further study of this and of other proteins with mutations in the Gla region should prove informative from this standpoint.

Acknowledgments—We thank Dr. Darrel W. Stafftord for providing the FX cDNA, and Gwyn Cutsforth and Dr. Dan Powers for the generous gift of factor V and RVV. We also thank Dr. Kenneth Mann and Dr. William Church for supplying immobilized monoclonal anti-factor X antibody.

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