Purification and Characterization of Factor VII 304-Gln: A Variant Molecule With Reduced Activity Isolated From a Clinically Unaffected Male


Factor VII (FVII) is the plasma serine protease zymogen which, on binding to its cellular receptor tissue factor (TF), initiates blood coagulation. A 47-year-old man with no clinical bleeding tendency was found to have undetectable plasma FVII activity when tested in a one-stage assay using rabbit brain TF, but 0.3 U/mL using recombinant human TF and 1.04 U/mL FVII antigen. Variant FVII purified from his plasma showed an identical migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis to wild-type zymogen. By enzyme kinetic analysis the Km of the variant using FX as a substrate was 12-fold higher than that of normal FVII. Also, the variant FVII was unable to compete with wild-type FVII for limited rabbit TF binding sites. A ligand blot procedure was used to directly demonstrate reduced binding of recombinant human TF to the variant FVII compared with normal FVII. Genetic analysis of leukocyte DNA showed a G to A mutation at codon 304 that results in the substitution of a glutamine for an arginine residue in the catalytic domain of the protease. We conclude that this region of the FVII molecule is important for its function.

MATERIALS AND METHODS

Approval was obtained from the Ethics Committee of the Harrow Health District for these studies. The subject was informed that blood samples were obtained for research purposes only. Consent was given for the withdrawal of 500 mL of blood. His variant FVII was detected during the screening stage for the Thrombosis Prevention Trial, which includes FVII measurements.

Monoclonal antibody (MoAb) to human FVII, RFF VII-2, was the gift of Dr. F. Ofosu (McMaster University, Hamilton, Ontario, Canada). A MoAb to human FIX was the gift of Dr. K. Smith (University of New Mexico School of Medicine, Albuquerque). Recombinant human TF apoprotein was the gift of Dr. D. Higgins (Genentech Inc., South San Francisco, CA). For clotting assays this material was relipidated as described previously. Rabbit TF (thromboplastin) was from Sigma (Poole, Dorset, UK). Purified human FX was from Enzyme Research Laboratories (South Bend, IN). Sulpho-NHS biotin and streptavidin-horseradish peroxidase (HRP) were from Pierce Europe (Luton, UK). Prestained molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from BRL Uxbridge (Middlesex, UK). Superoxide 12 and TSK DEAE 5PW HPLC columns were from Pharmacia LKB (Milton Keynes, UK). The enhanced chemiluminescent detection reagent (ECL) was from Amersham International (Amersham, UK). Triton X-100 was from Calbiochem (San Diego, CA). All other reagents were from Sigma and were reagent grade or better.

Protein estimations were performed by the method of Bradford using bovine serum albumin as standard. One-stage FVII coagulation assays were performed with immunodepleted FVII-deficient plasma as described previously. The coupled amidolytic assay for FVII was performed by the method of Seligsohn et al; FVII antigen (FVII:Ag) was measured using an enzyme-linked immunosorbent assay (ELISA) (Asserchrom FVII:Ag; Diagnostica Stago, Asnières-sur-Seine, France) according to the manufacturer's instructions. FIX was purified according to the method of Smith from pooled normal human plasma donations. After elution from the antibody column, the peak FIX-containing fractions were concentrated and applied to a TSK DEAE 5PW column in 0.02 mol/L Tris HCl pH 7.4 at a flow rate of 1 mL/min. The column was then developed with a linear gradient from 0% to 75% buffer B (A + 1 mol/L NaCl). The purified protein was homogenous by SDS-PAGE and was stored at −70°C in 50% glycerol.

For the biotinylation of coagulation factors, purified proteins were dialyzed into 100 mmol/L NaHCO3 pH 7.8. Ten micrograms of protein in a volume of 250 μL was biotinylated by the addition of
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10 mmol/L sulpho-NHS biotin. The mixture was incubated for 2 hours at room temperature, dialyzed against 0.15 mol/L NaCl, 0.05 mol/L Tris HCl pH 7.4 (TBS), and stored at -20°C.

**Purification of FVII.** Five hundred milliliters of normal or patient blood was collected into 1/10 vol 3.8% trisodium citrate/5 mmol/L benzamidine hydrochloride and the plasma separated by centrifugation as described previously.10 BaCl2, 1 mmol/L, was then added dropwise to a final concentration of 50 mmol/L and the solution mixed for 1 hour on ice before centrifugation at 15,000g for 1 hour at 4°C. The precipitate was suspended in 50 mL 0.05 mol/L Tris HCl pH 7.4 containing 0.15 mol/L NaCl (TBS) and recentrifuged. The pellet was then eluted in 50 mL TBS containing 0.2 mol/L Na2EDTA. This solution was then dialyzed for 16 hours at 4°C against 0.02 mol/L Tris HCl pH 7.4 containing 0.25 mol/L NaCl (TII buffer). The dialysate was then applied at a flow rate of 30 mL/h to a 5 mL controlled pore glass-coupled RFFVII-2 antibody column equilibrated in FVII buffer. Five milligrams of protein A-purified IgG was coupled to this column as described previously.10 One milliliter of protein solution was added to 50 mL of a 1:5 dilution rabbit brain thromboplastin preparation that was previously activated with purified human factor XIa and then developed isocratically with FVII buffer containing 1 mol/L NaCl and 0.1% sodium isothiocyanate adjusted to pH 8.5. Two-milliliter fractions were collected at a flow rate of 0.5 mL/min. One-milliliter fractions were collected. Peak activity-containing fractions were pooled, reconcentrated, and stored in 50% glycerol at -70°C.

**Kinetic analysis.** Steady state kinetic parameters were derived for wild-type and CRM+ FVII using the modification of the methods of Seligsohn et al12 and Osterud et al.13 Briefly, 0.1 mL of CRM+ or normal diluted plasma containing 0.0125 U FVII:Ag was added to 50 pL of a 1.5 dilution rabbit brain thromboplastin (Sigma) or repurified human recombinant TF. Both of these TF preparations gave clot times of exactly 17 seconds in a prothrombin time assay using 20-normal pooled plasma. All proteins were diluted in TBS pH 7.4 containing 0.1% bovine serum album (TBSA). Purified FX diluted in TBSA was then added at various concentrations ranging from 300 to 37 mmol/L in a final volume of 50 mL. The mixture was incubated at 37°C for 1 minute at which time 25 mL 25 mmol/L CaCl2 was added. Following a further incubation for 3 minutes at 37°C the reaction was stopped by the addition of 25 mL of 0.3 mol/L Na2EDTA. FXa generation was linear over this time period. TBSA, 600 JLL, was then placed in a cuvette with 100 mL of the chromogenic substrate S2222 (benzoyl-L-Ile-L-Glu-Gly-L-Arg-p-nitroanilide; Kabi-Vitrum, Uxbridge, UK). The inserts were sequenced by the dideoxy chain termination method.14
Table 1. Oligonucleotides and Conditions Used to Amplify the Human Factor VII Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotides</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
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<tr>
<td>2</td>
<td>5'GCGGGACAGGGGTCG3'</td>
<td>94°C</td>
<td>—</td>
<td>72°C</td>
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<tr>
<td></td>
<td>5'CGCCGCGGTGACGTCG3'</td>
<td>45 s</td>
<td>—</td>
<td>135 s + 3 s</td>
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<tr>
<td>3+4</td>
<td>5'GGGGCTCTGAGAAGGTG3'</td>
<td>94°C</td>
<td>—</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>5'ACGGCTCCAGGGTC3'</td>
<td>30 s</td>
<td>15 s</td>
<td>120 s</td>
</tr>
<tr>
<td>5</td>
<td>5'CTGACCCCCAGGGGTC3'</td>
<td>94°C</td>
<td>—</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>5'TATGAGGCTCAGCTC3'</td>
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<tr>
<td></td>
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<td>60 s</td>
<td>60 s</td>
<td>180 s</td>
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<tr>
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<td>—</td>
<td>70°C</td>
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<td></td>
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<td>45 s</td>
<td>60 s</td>
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<tr>
<td>8</td>
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<td>—</td>
<td>70°C</td>
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<tr>
<td></td>
<td>5'CCACAGGCCAGGGTGC3'</td>
<td>45 s</td>
<td>60 s</td>
<td>128 s</td>
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</table>

ity and that the propositus is homozygous. Different results were obtained when human TF was used in the same assay. Significant FVII:C levels of 0.3 U/mL were consistently recorded for the CRM+ individual. Identical results were obtained using relipidated recombinant human TF and TF from human brain. These data indicate that the CRM+ molecule is able to catalyze the conversion of FX and/or FIX to the active enzymes only in the presence of human TF and not with rabbit TF, in contrast to the wild-type molecule.

**Kinetics of FX activation by wild-type and CRM+ FVII.** Experiments were conducted using a coupled amidolytic assay to derive steady-state kinetic parameters for wild-type or CRM+ FVII in the presence of human, bovine, or rabbit TF. In this assay the substrate, purified human FX, was varied in an initial incubation mixture in which the other reactants, FVII and TF, were held constant. After an initial incubation period the FXa generated was then added to a chromogenic substrate. The data are presented as Lineweaver-Burk plots where the reciprocal of the initial rate of generation of FXa (reciprocal of the initial △Abs/min⁻¹ of the chromogenic substrate) is plotted against the reciprocal of the substrate concentration (1/[FX] · μM⁻¹). In experiments using human recombinant TF and normal FVII the Km for FX activation was 220 nmol/L, Vmax 0.052 △Abs/min⁻¹ (Fig 1). In the same experiment results using the CRM+ FVII were Km 2.6 μmol/L, Vmax 0.062 △Abs/min⁻¹, representing a 12-fold increase in the Km for FX activation compared with the normal enzyme. Similar results were obtained using saturating concentrations of bovine TF in the same assay: wild-type FVII Km 250 nmol/L, Vmax 0.066 △Abs/min⁻¹, CRM+ FVII Km 2.2 μmol/L, Vmax 0.057 △Abs/min⁻¹. Consistent with the results obtained with the one-stage FVII:C assays the CRM+ FVII molecule was unable to catalyze the conversion of FX to FXa in this assay in the presence of rabbit TF.

To determine if the CRM+ FVII competed with normal FVII for rabbit TF binding sites the following experiments were performed: The concentration of rabbit TF was titrated in the amidolytic assay to a rate-limiting concentration and the steady-state kinetic parameters derived for normal FVII, Km 135 nmol/L, Vmax 0.107 △Abs/min⁻¹ (Fig 2A) The apparent decrease in the Km in this system reflects the reduced concentration of competing phospholipid in the reaction mixture as a consequence of the lower TF

Fig 1. \( \text{Lineweaver-Burk plot for the activation of FX by CRM+ and wild-type FVII in the presence of human TF. FVII, (----); CRM+ FVII, (-----).} \)

<table>
<thead>
<tr>
<th>Subject</th>
<th>FVII:C U/mL</th>
<th>TRF</th>
<th>hTF</th>
<th>FVII:Ag U/mL</th>
</tr>
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<tbody>
<tr>
<td>Father</td>
<td>0.42</td>
<td>0.51</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>0.42</td>
<td>0.56</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Propositus</td>
<td>0.00</td>
<td>0.3</td>
<td>1.04</td>
<td></td>
</tr>
</tbody>
</table>
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characterization

Fivefold molar excess of CRM+ FVII.

were unaltered in the presence of a twofold and a fivefold molar excess of CRM+ FVII; (C) normal FVII with a fivefold molar excess of CRM+ FVII.

similar recoveries of FVII:Ag at each step. Thus, the CRM+ molecule was not grossly different in size, charge, or posttranslational modification, and the epitope for the MoAb was present. Wild-type FVII purified in this manner or posttranslational modification, and the epitope for the MoAb was present. Wild-type FVII purified in this manner had a specific activity of 1,500 U FVII:C activity/mg by one-stage FVII:C assay and coupled amidolytic assay, indicating that the molecule does not undergo activation as a consequence of the purification procedure. Western blot analysis under reducing conditions using a second MoAb as the probe (231/7) showed that the relative mobility was identical to the wild-type molecule (Fig 3). This result confirmed that there was no gross difference in relative molecular mass between the wild-type and the CRM+ molecule.

Activation of purified FIX by CRM+ and normal FVII. Kinetic analysis demonstrated that the CRM+ FVII was capable of catalyzing the conversion of FX to FXa at a reduced rate in the presence of human, but not rabbit, TF. In addition, the CRM+ molecule was unable to compete with normal FVII for limited rabbit TF binding sites in the coupled amidolytic assay. To investigate the ability of the CRM+ molecule to proteolyze FIX, purified biotinylated human FIX was incubated at 37°C with either human or rabbit TF, calcium, CRM+ or normal FVII and a trace amount of cold FIXa to fully activate the FVII. Aliquots were removed at various time points and subjected to SDS-PAGE under nonreducing conditions. Gels were subsequently Western blotted onto nitrocellulose membranes, probed with streptavidin-HRP, and detected with a chemiluminescent substrate. Figure 4A shows the time-course activation of biotinylated human FIX in the presence of human TF and normal FVII (lanes 1 through 6) and CRM+ FVII (lanes 7 through 12). Rapid activation of the single-chain FIX, as evidenced by the appearance of the 43-kD two-chain polypeptide, was seen in the reaction mixture containing wild-type FVII. Some proteolysis of the zymogen FIX was detected in the reaction mixture containing CRM+ FVII and human TF, but at a much slower rate and to a lesser extent than that seen with the wild-type molecule. In contrast, the same experiment performed using rabbit TF showed a rapid conversion of FIX to FIXa by the wild-type FVII but little or no conversion in the presence of the CRM+ enzyme (Fig 4B). After a 16-hour incubation of the FIX with the rabbit TF and the dysfunctional FVII, minimal activation was detected. These data confirm that while the CRM+ FVII was able to catalyze the conversion of FIX to FIXa in the presence of human TF at a reduced rate, no significant proteolytic activity could be detected in the presence of the rabbit cofactor.

Ligand blot analysis. To investigate further the interaction between normal and CRM+ FVII and TF, a ligand blot procedure was developed. In this system purified FVII was subjected to nondenaturing polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane. The membranes were then blocked with a gelatin/CaCl₂/Triton X-100 containing buffer (NET buffer) and probed with nonrelipidated, biotinylated recombinant human TF apoprotein, in the presence of 0.1% Triton X-100. Binding of the biotinylated TF was detected using HRP-conjugated streptavidin and a chemiluminescent detection system (ECL). In control experiments no binding of TF to purified FX, FVIII, FIX, or prothrombin could be detected and binding of TF to FVII was detected in the presence of concentration added. At higher TF concentrations the increased phospholipid competes with the substrate, FX. Increasing amounts of the CRM+ FVII antigen were then added to the reaction mixture containing the normal FVII, rabbit TF, and FX. Km and Vmax for FVII activation of FX were unaltered in the presence of a twofold and a fivefold molar excess of the CRM+ FVII over the normal enzyme (Fig 2, B and C, respectively). These data indicate that the CRM+ molecule did not compete with the wild-type for the available TF binding sites in the reaction mixture.

Purification and Western blot analysis of wild-type and CRM+ FVII. Table 3 shows the results of the purification of CRM+ FVII from 190 mL of frozen citrated plasma. The protocol involved adsorption to barium chloride, affinity chromatography using the MoAb RFFVII 2, and Superose 12 HPLC gel permeation chromatography. Overall recoveries of normal FVII using this protocol were routinely 10% to 20%. The CRM+ and the wild-type molecules gave similar recoveries of FVII:Ag at each step. Thus, the CRM+ molecule was not grossly different in size, charge, or posttranslational modification, and the epitope for the MoAb was present. Wild-type FVII purified in this manner has a specific activity of 1,500 U FVII:C activity/mg by one-stage FVII:C assay and coupled amidolytic assay, indicating that the molecule does not undergo activation as a consequence of the purification procedure. Western blot analysis under reducing conditions using a second MoAb as the probe (231/7) showed that the relative mobility was identical to the wild-type molecule (Fig 3). This result confirmed that there was no gross difference in relative molecular mass between the wild-type and the CRM+ molecule.

Table 3. Purification of CRM+ FVII From Plasma

<table>
<thead>
<tr>
<th>No.</th>
<th>Vol (mL)</th>
<th>FVII:Ag (U/mL)</th>
<th>% Recovery</th>
</tr>
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<tr>
<td>Plasma</td>
<td>190</td>
<td>&lt;1</td>
<td>1.04</td>
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<tr>
<td>BaC12 eluate</td>
<td>190</td>
<td>&lt;1</td>
<td>2.5</td>
</tr>
<tr>
<td>RFFVII eluate</td>
<td>15</td>
<td>&lt;1</td>
<td>5.3</td>
</tr>
<tr>
<td>Superose 12</td>
<td>1</td>
<td>&lt;1</td>
<td>33.3</td>
</tr>
</tbody>
</table>

FVII: C assays were performed using rabbit TF.

Fig 2. Lineweaver-Burk plot for the activation of FX by normal FVII in the presence of rabbit TF. (A) Normal FVII alone; (B) normal FVII with twofold molar excess of CRM+ FVII; (C) normal FVII with a fivefold molar excess of CRM+ FVII.
Fig 3. Western blot analysis of normal (lane 1) and CRM+ (lane 2) FVII purified from plasma. Samples were electrophoresed in reduced SDS-PAGE 10% gels and Western blotted onto nitrocellulose and probed with MoAb 231/7 a FVII antibody labeled with ¹²⁵I. Relative molecular mass x 10^3 are shown on the left of the figure.

Fig 4. SDS-PAGE/Western blot analysis of the time course of the activation of labeled FIX by CRM+ and normal FVII. (A) Activation of FIX by normal FVII in the presence of human TF (lanes 1 through 6) and CRM+ FVII (lanes 7 through 12). Time points were 0, 5 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours. (B) Activation of FIX by normal FVII in the presence of rabbit TF (lanes 1 through 8) and CRM+ FVII (lanes 9 through 16). Time points were 0, 5 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, and 16 hours after the addition of FVII. See Materials and Methods for full details.
calcium-containing buffers, but not in the presence of chelating agents such as EDTA (data not shown).

Figure 5 shows the result of a ligand blot of normal and CRM+ FVII probed with recombinant biotinylated TF. An equal amount of FVII:Ag, as determined by ELISA, was loaded in each case. Significantly reduced binding of the TF to the CRM+ molecule was detected (Fig 5A, lane 1) with respect to the wild-type molecule (Fig 5A, lane 2). Two lanes representing the same antigen loadings were run concurrently and subjected to Western blot analysis using an HRP-conjugated polyclonal antihuman FVII antibody as the probe (Fig 5B). This demonstrated that the amounts of FVII and CRM+ FVII loaded were similar. The upper band represents a small amount of residual FVII which remained in the top of the gel. This phenomenon was not always seen. In a separate experiment 1, 0.5, 0.25, and 0.125 U of normal or CRM+ FVII were electrophoresed and first probed with biotin-TF (Fig 6A) and reprobed with the HRP-conjugated polyclonal antibody to FVII (Fig 6B). Because the chemi-luminescent reaction inactivates HRP-conjugated streptavidin it was possible to reprobe TF ligand blots with the polyclonal antibody to FVII to ensure that the same amount of FVII had been electrophoresed in each case. The results of the ligand blot analysis clearly demonstrate that the CRM+ FVII molecule exhibited reduced TF binding. Densitometric scans showed that the binding of recombinant human TF was approximately 10- to 15-fold lower that the binding to normal FVII.

**FVII gene analysis.** To identify the mutation causing the observed phenotype, exons 2 through 8 were amplified by PCR. The amplified fragments were completely sequenced after cloning into plasmid vectors. The sequence of the cloned PCR products did not differ from the previously published sequence for the human FVII gene, except at positions 10828 and 9778. The single-base change at 9778 is in intron 8 and therefore cannot be responsible for the altered phenotype of the FVII protein. The single-base change at position 10828 results in a substitution of arginine by glutamine at position 304 (Fig 7).

**DISCUSSION**

In this study we have undertaken the biochemical analysis of a dysfunctional FVII molecule, isolated from the plasma of a clinically unaffected individual. A differential response to human and rabbit brain TF was detected in one-stage and amidolytic FVII:C functional assays. Significant but reduced FVII:C activity was detected in the presence of human TF, but no activity was detected in the presence of rabbit TF. Even at high concentrations of FVII:Ag the CRM+ FVII was unable to catalyze the conversion of FX to FXa in the presence of the rabbit cofactor. In a coupled amidolytic assay using human TF and purified FX a 12-fold increase in the Km for FX activation was recorded for the CRM+ over the wild-type enzyme, with no difference in the Vmax. These results may reflect the requirement of TF binding to FVII for substrate recognition, as described by Nemerson and Gentry. These investigators have proposed an ordered addition, essential activation model of the TF pathway of coagulation, in which the enzyme (FVIIa)-activator (TF) complex picks up sub-
strate (FX or FIX) to form a ternary complex; a model that precludes the formation of binary enzyme-substrate complexes. Thus, in this model the affinity of the enzyme for its substrate is dependent on prior TF/FVII/FVIIa binding. It is also possible that, in addition, defects of substrate recognition or zymogen activation may result from the point mutation detected in this study. It has been shown that FVII bound to TF is more readily activated by FXa. Defective TF binding could conceivably result in reduced enzyme activation in the assays. The dysfunctional molecule did not compete with wild-type FVII for limited rabbit TF binding sites, because the addition of a fivefold molar excess of the CRM+ molecule to a reaction mixture containing wild-type FVII, a rate-limiting concentration of rabbit TF, and FX did not alter the kinetic parameters derived for the normal enzyme. This result indicated that the CRM+ molecule was unable to act as a competitive inhibitor of the wild-type FVII.

To expand these studies we have developed a purification protocol that effects the purification of FVII from plasma donations of approximately 200 mL. FVII purified in this manner is isolated in zymogen form, as evidenced by a 1:1 correlation between the results of the one-stage coagulation and amidolytic assays for FVII:C.8 Yields of FVII over the multistep process are routinely 10% to 20%. Purified CRM+ and normal FVII isolated from plasma by this protocol had identical relative mobilities on SDS-PAGE/Western blot analysis, indicating that the defect did not arise from a major deletion or rearrangement of the gene. Using the purified CRM+ FVII we were able to demonstrate that the dysfunctional enzyme was able to proteolyze purified human FIX at a reduced rate in the presence of human, but not rabbit, TF. These data indicated that the defect in this molecule may involve the interaction with TF.

To confirm this hypothesis a ligand blot assay was developed to assess the binding of biotinylated recombinant human TF to membrane-bound normal and CRM+ FVII. This assay is specific for the TF/FVII interaction because, in control experiments, there was no evidence of binding of biotinylated TF to related vitamin K-dependent coagulation factors. Biotinylated TF bound specifically to FVII in calcium-containing buffers but not in EDTA-containing buffers. The binding of TF-apoprotein to immobilized FVII is known to be calcium dependent. Therefore, the assay was used to investigate the binding of biotinylated recombinant human TF to purified wild-type and CRM+ FVII. With identical antigen loading the binding to the CRM+ molecule was repeatedly 10- to 15-fold less than that to the wild-type FVII.

To characterize the genetic defect, exonic and flanking intronic sequences of the propositus’ FVII gene were amplified, cloned, and sequenced by the dideoxy chain termination method. The only discrepancy with the previously published sequence of the human FVII gene detected within the coding sequence of the CRM+ gene was a G to A substitution in codon 304, leading to the substitution of a glutamine for an arginine residue. This most probably is the result of a C to T transversion on the noncoding strand in a CpG dinucleotide. There is now a considerable body of evidence in the literature that CpG dinucleotides are mutation hot spots in the human genome. This mutation gives rise to a radical substitution because arginine side chains are positively charged at physiologic pH, whereas glutamine is a polar but uncharged residue.

Little is known about the structural requirements of the FVII molecule for interaction with TF. The data presented in this study suggest that the arginine residue at position 304 (Arg-304), which is located in the catalytic domain of
the protease, may be important in this interaction. The substitution of Arg-304 by Gln-304 could prevent the association of the FVII/rabbit TF complex and allow only weak binding of the variant molecule to human TF. Presumably, differences in the primary structure of rabbit and human TF account for the differential response of the CRM+ FVII to the human and rabbit cofactors. Replacement of Arg-304 by Gln-304 might cause a conformational change in the FVII molecule resulting in reduced TF binding affinity, through a propagat effect on a distal binding site for TF. Alternatively, the mutation may occur directly in the TF binding site. It is also possible that the mutation impacts more than one functional domain of the enzyme. The present study cannot distinguish between these possibilities.

By comparison with the primary structures of the other vitamin K-dependent coagulation factors, it is evident that Arg-304 is located in a structurally conserved region of FVII. Furie et al.10 have proposed an alignment of the bovine vitamin K-dependent coagulation factors based on the crystallographic coordinates of bovine trypsin and chymotrypsin. We have superimposed the sequences of the human zymogens on this alignment. Using this model FVII, Arg-304 is homologous to FIX Arg-333, FX Arg-298, prothrombin Arg-490, and protein C His-326 (Fig 8). This residue is not conserved in elastase, chymotrypsin, or trypsinogen. However, there is an insertion of three residues unique to FIX before a conserved cysteine residue immediately C-terminal to Arg-304, and a second insertion of five residues from positions 311 to 315 not found in the other proteases. Thus, while FVII Arg-304 is located in a region that is highly conserved in FIX, FX, prothrombin, and protein C, the insertions in FVII reduce the homology between it and the other vitamin K-dependent coagulation factors around this position. Furie et al.10 identified six variable regions and seven conserved regions in the bovine vitamin K-dependent coagulation factors. Using the same model FVII Arg-304 would form part of conserved region 5, which is not in or near the active site of the enzyme. Sequences N-terminal to conserved region 5 form part of variable region 4 which, it was speculated, may define a recognition domain for the binding of proteins to FIX, FX prothrombin, protein C, and therefore, by inference FVII.

Interestingly, Arg to Gln-333 of FIX has been identified as the mutation in patients with severe hemophilia B (in whom there is synthesis and expression of CRM+ FIX).21 FVII Arg-333 would be homologous to FVII Arg-304 according to the alignment proposed by Furie et al. Because one consequence of the substitution of FVII Arg-304 to Gln is defective cofactor interaction it is possible that substitution of FIX Arg-333 to Gln results in an FIX molecule with defective FVIII binding.

There are several lines of evidence to suggest that the cofactor binding sites of the vitamin K-dependent group of serine proteases reside in the catalytic domains of these molecules. An MoAb has been mapped to residues 180 to 310 of human FIX by Frazier et al.12 and this antibody has been shown to interfere with the binding of FVII to FIX.23 This region of FIX is homologous to the variable region of FVII N-terminal to the arginine at position 304. This would place the FVIII binding site on FIX close to the analogous region of FVII where the mutation described in this study leads to defective TF binding. The defect in FX Friuli, a CRM+ molecule with normal activation by RVVX but defective activation by the intrinsic and extrinsic activators, has been attributed to abnormal cofactor (TF and FVIII) binding.22 The precise location of the presumed point mutation in this variant FX gene has not been identified but the defect has been mapped to the catalytic domain of the molecule.

Two recent reports also support the hypothesis that the FVII TF binding site resides in the catalytic domain of the molecule. Kumar et al.25 reported that a peptide spanning residues 206 to 218 of FVII inhibited FVII/TF interaction. In contrast, Wildgoose et al.27 were unable to demonstrate inhibition of FVII/TF interaction with this peptide but reported that three catalytic domain peptides, 195 to 206, 263 to 274, and 314 to 326 were inhibitory, with 195 to 206 inhibiting binding of human FVII to TF expressed on the surface of human bladder carcinoma (J82) cells. In this report, using a combined epidemiologic and biochemical approach, another catalytic domain residue, Arg-304, has been shown to be important in FVII function.

ACKNOWLEDGMENT
We thank David Howarth for performing one-stage factor VII assays and Dr Yoshikai Ohkubo for the purification of FIX.

REFERENCES
Purification and characterization of factor VII 304-Gln: a variant molecule with reduced activity isolated from a clinically unaffected male

DP O'Brien, KM Gale, JS Anderson, JH McVey, GJ Miller, TW Meade and EG Tuddenham