Surface Plasmon Resonance Studies of the Interaction between Factor VII and Tissue Factor. Demonstration of Defective Tissue Factor Binding in a Variant FVII Molecule (FVII-R79Q)

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ABSTRACT: The blood coagulation cascade is initiated when vessel injury allows factor VII (FVII) to form a complex with tissue factor (TF). Complete deficiency of FVII causes a lethal bleeding diathesis, but individuals with moderately reduced FVII levels are often asymptomatic. Some of these individuals have circulating partially functional FVII, as a result of point missense mutations in critical parts of the molecule. One such mutation has been reported at position 79 in the first epidermal growth factor-like (EGF) domain of FVII, where an arginine residue has been replaced by glutamine. There is controversy as to whether or not this mutation reduces the affinity of the FVII/TF interaction compared to wild-type FVII. To address this problem, we have expressed recombinant FVII-R79Q and subjected it to detailed biochemical analysis. One-stage FVII:C assays show the variant FVII to have reduced activity with respect to the wild type. Rates of autoactivation and activation by FXa to the two-chain molecule were identical for wild-type and variant FVII. The V_{max} for FX activation was lower for the mutant as measured using an amidolytic assay for FX activity. In contrast, the K_m for FX was lower for the variant than the wild-type molecule. Peptidyl substrate hydrolysis was virtually identical for both variant and normal FVIIa in the presence and absence of TF. The variant has reduced affinity for TF as measured by surface plasmon resonance. FVII-R79Q has an association rate constant (k_{assoc}) one-fifth of that of normal FVII, but a similar k_{diss}, resulting in a decrease in the affinity of the enzyme for its cofactor. We conclude that the first EGF domain of FVII plays a key role in its complex formation with TF and that a decrease in the affinity of FVII for TF may secondarily affect macromolecular substrate binding and catalysis.

Factor VII (FVII) is a single-chain glycoprotein that circulates in blood as a zymogen of the protease activated FVII (FVIIa). FVII is converted to FVIIa by limited proteolysis in complex with its cell-surface receptor and cofactor tissue factor (TF). The complex of TF and FVIIa initiates blood coagulation by catalyzing the conversion of coagulation factors IX and X to the respective serine proteases, FIXa and FXa. FVII is a member of the vitamin K-dependent group of blood coagulation factors, which share considerable sequence identity and a common domain structure (Furie & Furie, 1988). The N-terminal γ-carboxyglutamic acid domain of FVII, which contains 10 posttranslationally carboxylated glutamic acid (Gla) residues, is connected by a short amphipathic helix to 2 epidermal growth factor-like domains (EGF). The heavy chain of FVIIa is disulfide-bridged to the N-terminal light chain of the molecule, and contains the serine protease domain, with the His, Asp, and Ser catalytic triad common to the proteases of the trypsin superfamily (Furie et al., 1982).

A variant FVII molecule has been identified in a patient with a severe bleeding diathesis (Chaing & High, 1990). This patient was homozygous for two missense mutations: one Arg→Gln at position 79 in the first EGF-like domain and the other Arg→Gln at position 152, the residue immediately N-terminal to the scissile bond that is cleaved in wild-type (wt) FVII to generate FVIIa. Unlike normal FVII, this variant was resistant to activation by FXa and, in addition, defective in binding to TF in a solid phase immunoadsorbent assay (Clarke et al., 1992). The authors postulated that the substitution of Arg 152 by Gln prevented the proteolytic activation of this molecule, and proposed that the radical substitution of Arg by Gln at position 79 was responsible for reduced TF binding. In contrast, Kazama and colleagues expressed the recombinant molecule FVII-R79Q, and reported that the procoagulant activity and interaction of the molecule with TF were not impaired to any measurable extent (Kazama et al., 1992). We have recently characterized the molecular defect in an individual with abnormal in vitro FVII activity and demonstrated that the molecular lesion is associated with a homozygous Arg to Gln substitution at position 79 (Takamiya et al., 1993). We now report the synthesis and characterization of recombinant FVII-R79Q and demonstrate that in comparison to wtFVII, the molecule binds to TF with reduced affinity, using the technique of biospecific interaction analysis (BIA) as quantified by surface plasmon resonance (SPR).

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EXPERIMENTAL PROCEDURES

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting were performed as described elsewhere (O'Brien et al., 1991). Prestained molecular weight standards for SDS−PAGE were from Bio-Rad Laboratories (Richmond, CA). Enhanced chemiluminescence (ECL) reagent was from Amersham International plc. (Amersham, U.K.). Purified FX and FXa were homogeneous by SDS−PAGE and Coomassie blue staining, and were obtained from ERL (Swansea, U.K.). FVII antigen was measured using a commercial enzyme-linked immunoadsorbent assay (Asserachrom-Diagnostica Stago, Asnieres sur Seine, France). Protein estimations were performed by the method of Bradford (1976) using bovine serum albumin as standard. Q-Sepharose fast flow and Mono Q HR 5/5 columns were from Pharmacia (St. Albans, U.K.). Monoclonal antibody (MoAb) RFF VII-2 was the gift of Dr. A. H. Goodall, Royal Free Hospital, London, U.K. TF1-219 and TF1-243 were the gift of Dr. D. L. Eaton, Genentech Inc., South San Francisco, CA. Sulfo-NHS biotin and horseradish peroxidase were from Pierce Europe, Luton, U.K. Restriction endonucleases and DNA polymerases were obtained from commercial suppliers and used according to the manufacturers' instructions (Promega Biotec, Southampton, U.K.). Geneticin (G418) was obtained from Gibco BRL, Paisley, U.K. Methotrexate (MTX) was from Sigma, Poole, Dorset, U.K. All other chemicals were from Sigma and were reagent grade or better.

Expression of Recombinant Wild-Type and Variant FVII. A cDNA for wtFVII was provided by Dr. E. Davie (Hagen et al., 1986) and was cloned into the novel expression vector pNeoIG502 (Kemball-Cook et al., 1993). Briefly, this vector contains the neomycin resistance-encoding gene as a selectable marker and the dihydrofolate reductase gene to enable amplification of the transfected DNA. The Rous sarcoma virus long terminal repeat (RSV LTR) promoter ensured efficient transcription of FVII cDNA, and in addition elements of the murine *c-mos* (UMS) and human β-globin genes facilitated efficient transcriptional termination, polyadenylation, and splicing of the primary transcript. A single base change at codon 79 was introduced into the wild-type FVII cDNA sequence by oligonucleotide *in vitro* site-directed mutagenesis using a kit according to the manufacturers' protocol (Amersham International). The sequence of the variant cDNA was verified by nucleotide sequence analysis using chain termination methods (Sanger et al., 1977). This variant FVII cDNA was also inserted into the expression vector pNeoIG502. CHO K1 cells (ECACC no. 85050302; Porton Down, U.K.) were transfected by electroporation and stably transfected cells isolated by G418 selection and then successively treated with increasing levels of MTX (Kemball-Cook et al., 1993). Cells were expanded in roller bottles, grown to confluence, and subsequently transferred into Hybrimax protein-free medium (Sigma U.K.) containing 5 μg/mL menadione sodium bisulfite (vitamin K_3), for the recovery of FVII.

Purification of Wild-Type and Variant FVII. Factor VII was expressed in serum-free medium as about 1% of total protein. Culture supernatants were filtered through 0.22 μm membranes, and benzamidine hydrochloride was added to 5 mM. The medium was made 0.38% trisodium citrate, and 1 M $BaCl_2$ was then added dropwise to a final concentration of 50 mM. The mixture was stirred for 30 min at 4 °C and then centrifuged at 5000*g* for 1 h. The pellet was resuspended in 0.05 M Tris·HCl/0.15 M NaCl, pH 7.4 (TBS), containing 5 mM benzamidine and centrifuged as before. The pellet was collected and resuspended in TBS containing 0.2 M Na_2EDTA and 5 mM benzamidine. On dissolution of the pellet, the supernatant was dialyzed against TBS/5 mM benzamidine at 4 °C. The dialysate was applied to MoAb RFFVII-2 Sepharose; the column was washed and eluted as described previously (O'Brien et al., 1991). The eluate was concentrated in Centricon-30 microconcentrators (Amicon, Danvers, MA) and applied to a Superose 12 PC 3.2/30 column installed in a microscale HPLC (SMART system; Pharmacia LKB, Milton Keynes, U.K.). The column was developed isocratically with TBS at 40 μL/min, and 40 μL fractions were collected and subjected to SDS−PAGE analysis and one-stage FVII:C assay. FVII-containing fractions were pooled and stored at −70 °C in 50 μL aliquots.

Determination of γ-Carboxyglutamic Acid Content of Recombinant Protein. The extent of γ-carboxylation of individual glutamic acid residues in the Gla domain of recombinant FVII preparations was determined by direct sequencing of methylated proteins (Cairns et al., 1991). In brief, a sample of the dry protein (approximately 5 μg) was treated with 200 μL of 1 M HCl in dry methanol for 2 h at room temperature, dried under a stream of nitrogen, and redissolved in 40 μL of 0.1% trifluoroacetic acid. The solution was applied in two parts to a polybrene-coated glass fiber disk for sequencing in an Applied Biosystems 473A protein sequencer. In the standard chromatographic conditions used for analysis in this instrument, the phenylthiohydantoin (PTH) derivative of the dimethyl ester of γ-carboxyglutamic acid emerged just prior to that of proline; PTH-monomethyl esters of aspartic acid and glutamic acid eluted after PTH-alanine and PTH-tyrosine, respectively. Quantification of peaks was carried out using the 610A software supplied by Applied Biosystems (Warrington, U.K.); 1 M HCl in methanol was prepared by reacting 8 mL of acetyl chloride with 95 mL of dry methanol at 0 °C. This reagent also replaced the 25% trifluoroacetic acid normally used in the sequencer for the conversion of anilinothiazolinone-amino acid to PTH-amino acid, in order to prevent hydrolysis of the methyl esters.

Activation of FVII. For Western blot analysis of the formation of two-chain FVIIa, FVII was activated by FXa. One hundred micrograms of wtFVII and FVII-R79Q was diluted to 1 mL in TBS containing 10 mM $CaCl_2$ and 10% v/v rabbit brain cephalin, reconstituted according to the manufacturers' instructions (RBC, Sigma, U.K.) as a source of phospholipid. Two micrograms of human FXa was then added and the sample incubated at 37 °C. Aliquots were removed from the reaction mixture at various time points, boiled, and then subjected to SDS−PAGE and Western blot analysis using a rabbit anti-human FVII polyclonal antibody. FVII/antibody complexes were detected using a goat anti-rabbit IgG coupled to horseradish peroxidase and enhanced chemiluminescence substrate (ECL Amersham International U.K.). For activity assays, FVII (1 μM) in TBS, pH 7.4, was incubated at ambient temperature with 5 mM $CaCl_2$, and aliquots were removed at various time points for assay in a one-stage FVII:C assay using human placental TF or rabbit brain thromboplastin.

One-Stage FVII Coagulation Assays. One-stage FVII coagulation assays were performed with rabbit brain thromboplastin (Sigma, Poole, Dorset, U.K.), human placental thromboplastin (Behringwerke AG, Marburg, Germany), or relipidated recombinant human TF 1-243 (the gift of Dr. D. L. Eaton, Genentech Inc., San Francisco, CA). Substrate was immunodepleted FVII-deficient plasma prepared as described previously (Takase et al., 1988).

Determination of K_m for FX Activation by TF/FVIIa. TF1-243 (final concentration 21.7 nM), relipidated as described previously, was incubated with FVIIa (final concentration 50 pM) and various concentrations of FX in a total reaction volume of 100 μL: samples were diluted in TBSA. At these concentrations of reagent, greater than 95% of both FVIIa-R79Q and wtFVIIa was in complex with TF. The reaction was started by the addition of 25 mM $CaCl_2$ to a final concentration of 5 mM. Chromogenic substrate S2222 was added (25 μL, 2 mg/mL) at 2 or 4 min. Color generation was halted by the addition of 25 μL of glacial acetic acid and absorbance measured at 405 nm. FXa activity was determined as the change in rate of color generation ($\Delta\Delta A_{405nm}$·time^{-2}). K_m and V_{max} were determined by iterative fitting of the data to the Michaelis–Menten equation using the curve-fitting program P.Fit (Biosoft, Cambridge, U.K.).

K_m Determination for Hydrolysis of Chromogenic Substrate S2288 by FVIIa. Four microliters of wild-type or variant FVIIa (1 μM), 25 μL of S2288, and 25 μL of either relipidated TF1-243 (10 μM) or TBSA were added to 125 μL of TBSA in a 96-well microtiter plate. Twenty-five microliters of 25 mM $CaCl_2$ was added to start the reaction, and the absorbance at 405 nm was monitored continuously. At these concentrations, greater than 90% of the TF (where present) is in complex with FVIIa or FVIIa-R79Q. Activity was determined as ΔA_{405nm}·time^{-1}. K_m and V_{max} were determined by iterative fitting of the data to the Michaelis–Menten equation using the curve-fitting program P. Fit (Biosoft).

Surface Plasmon Resonance Studies. Surface plasmon resonance studies were carried out using a BIAcore instrument (Pharmacia Biosensor AB, Uppsala, Sweden). The instrument allows real-time molecular interaction analysis from which association and dissociation rate constants and equilibrium constants can be derived. The carboxylated dextran surface of the sensor chip was activated by 30 μL of an equimolar mixture of 0.1 M *N*-hydroxysuccinimide/*N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide hydrochloride at a flow rate of 5 μL/min. Soluble TF1-219 was immobilized onto the sensor chip at a concentration of 30 μg/mL in 10 mM NaOAc, pH 4.0. This buffer was selected on the basis of the calculated p*I* of TF1-219 (pH 5.1) which facilitated concentration of the ligand on the carboxylated dextran surface by an ion-exchange effect, followed by covalent coupling of the ligand to the matrix via primary amino groups. The flow cell was equilibrated with 15 mM Tris·HCl, pH 7.3, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.05% P20 (equilibration buffer) at a flow rate of 5 μL/min. Several cycles of equilibration buffer followed by regeneration buffer (10 mM Na_2EDTA, pH 9.3) were performed prior to the collection of experimental data. For kinetic runs, FVII or FVIIa, at concentrations ranging from 25 to 250 nM in equilibration buffer, was allowed to interact with the sensor surface for 4 min at a flow rate of 2 μL/min. Injections were carried out in random order and in triplicate. Results were verified using several different FVII samples purified from different batches of CHO cell culture medium. Comparative kinetic analyses of wild-type and variant FVII and FVIIa were performed sequentially on the same sensor chip.

Binding of the ligand to the coupled moiety is measured by the BIAcore instrument as response (R) per unit time in resonance units (RU s^{-1}). The analyte (FVII/FVIIa) concentration is kept constant through the continuous flow of fresh analyte solution past the sensor surface. Binding curves are collected for several concentrations of analyte expressed as response in RU s^{-1} over time. dR/dt against R is then plotted for each analyte concentration, and the slopes of these lines are plotted against analyte concentration (C). The slope of this plot gives the association rate constant (k_{assoc}) and the intercept on the abscissa the dissociation rate constant (k_{diss}) according to the equation:

$$\text{slope}(dR/dt \text{ vs } R) = k_{assoc}C + k_{diss}$$

Following the sample pulse, equilibration buffer is passed over the sensor chip, the free analyte concentration falls to zero, and the derivative of the response curve reflects the dissociation rate constant k_{diss}. The dissociation rate constant can therefore be obtained by plotting the log of the change in response against time interval according to the equation:

$$\ln(R_{t1}/R_{tn}) = k_{diss}(t_n - t_1)$$

Affinity constants (K_A) were also calculated from the response at equilibrium (R_{eq}) using the equation:

$$R_{eq}/C = K_A R_{max} - K_A R_{eq}$$

where R_{eq} is the response at equilibrium, C is the analyte concentration, K_A is the affinity constant, and R_{max} is the response corresponding to saturation of the immobilized ligand.

RESULTS

Expression and Purification of Recombinant Wild-Type and Variant FVII. Culture medium conditioned by cells transfected with constructs containing wild-type FVII cDNA and FVII-R79Q cDNA contained between 5 and 8 units of FVII antigen/mL by ELISA. Identical purification profiles were obtained from culture medium conditioned by cells transfected with either construct. Following purification, SDS–PAGE and Coomassie blue staining demonstrated that the two proteins were homogeneous with identical relative mobilities (Figure 1). Under reducing conditions, no light- or heavy-chain species were detected, indicating that the material was isolated in zymogen form. The zymogens were allowed to autoactivate in the presence of 5 mM $CaCl_2$ at 4 °C for 36 h (Pedersen et al., 1989), and SDS–PAGE resolved heavy and light chains of wtFVIIa and FVIIa-R79Q of identical relative mobilities (Figure 1, lanes 2 and 4).

Determination of γ-Carboxyglutamic Acid Content of Recombinant Protein. Gla content was determined by direct sequencing of methylated proteins. All 9 glutamic acid residues in the first 30 amino acids of factor VII were greater than 80% γ-carboxylated; in many cases, no uncarboxylated glutamic acid could be detected. The unconserved glutamic acid at residue 35 was approximately one-fourth to one-third carboxylated only. This residue is probably unimportant in the calcium-dependent conformation of functional FVII. The

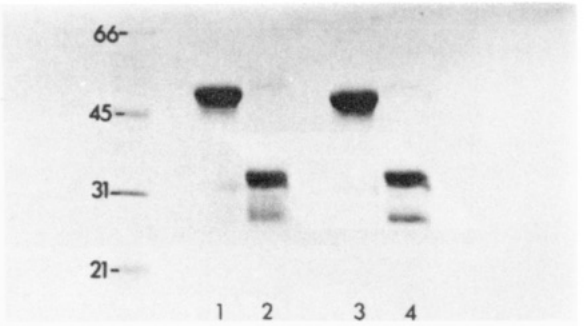

FIGURE 1: SDS−PAGE analysis of wtFVII and FVII-R79Q and the activated forms under reducing conditions. 10% homogeneous PAGE gels were electrophoresed and stained with Coomassie blue R250 as described under Experimental Procedures. Relative molecular mass standards are given on the left-hand side of the gel (kDa). Lane 1, wtFVII. Lane 2, wtFVIIa. Lane 3, FVII-R79Q. Lane 4, FVIIa-R79Q.

Table 1: γ-Carboxylation of the 10 Most N-Terminal Glutamic Acid Residues of FVII

	% γ-carboxylation	
residue	wild type	R79Q
6	87.6	94.0
7	92.8	100
14	100	97.2
16	87.5	82.0
19	100	91.4
20	82.9	100
25	89.5	89.0
26	83.1	100
29	75.4	100
35	24.8	30.8

Table 2: One-Stage FVII Activity of Wild-Type and Variant FVII and FVIIa Using Rabbit TF (RTF) and Human TF (HTF)

FVII sample,	specific activity	
concn 1 μM	units/mg of RTF	units/mg of HTF
wtFVII	2320	2060
FVII-R79Q	216	624
wtFVIIa	25000	21000
FVIIa-R79Q	2126	6376

sequence data are summarized in Table 1. Percent carboxylation was determined from the molar proportion of carboxylated species to all glutamic acid species in that cycle.

Assay of wtFVII and FVII-R79Q Activity. Activity of FVII/FVIIa was measured in a one-stage FVII:C assay using human (HTF) or rabbit (RTF) tissue factor (Table 2). Measured against a normal plasma standard, the specific activity of wtFVII was approximately 2000 units/mg in one-stage assays with both rabbit and human placental TF (similar results were obtained using recombinant human TF; data not shown). These data indicate that the recombinant FVII was predominantly in zymogen form and was fully functional. In contrast, the specific activity of FVII-R79Q was 624 units/mg by one-stage assay with human thromboplastin and 216 units/mg (10% of wild type) using the rabbit cofactor. Following complete conversion to two-chain FVIIa by autoactivation in the presence of calcium, as assessed by SDS−PAGE, the specific activity of the wild-type FVIIa increased 10−11-fold, similar to that observed for plasma-derived FVIIa autoactivated in an identical manner. Higher fold activation is achieved when plasma-derived FVII is activated by FXa as has been reported by other groups (e.g., Kazama et al., 1992), but we consistently observe 10−15-fold activation of plasma derived FVII autoactivated in this way, which may reflect interassay variations. Approximately 10-fold increases in the specific activity of activated FVIIa-R79Q over the zymogen were also recorded.

Rates of Activation of FVII and FVII-R79Q. Rates of autoactivation of FVII-R79Q and the wild-type molecule were assessed by SDS−PAGE and Coomassie blue staining on incubation of zymogen FVII with 5 mM $CaCl_2$ at room temperature. Identical rates of autoactivation were seen in both cases (Figure 2A). Activation of wtFVII and R79Q following the addition of FXa was also assessed by Western blotting. Blots were scanned densitometrically, and the percentage of FVII converted to two-chain FVIIa was plotted as % FVIIa against incubation time (Figure 2B). No significant difference between the activation of wtFVII and FVII-R79Q was detected. These data confirm the results of Kazama et al. (1992), who showed identical rates of activation of wtFVII and FVII-R79Q by FXa in a one-stage clotting assay.

Determination of K_m for FX. Macromolecular substrate affinity and catalysis are altered in the variant molecule. The observed K_m for FX for the wild-type molecule (34.6 ± 3.8 nM) was approximately 2-fold higher than that for FVIIa-R79Q (17.9 ± 2.6 nM) (Figure 3). It is not possible from these data to determine whether the dimunition of V_{max} in the case of the mutant protein (70.61 ± 3.09 mAU/min compared to 141.97 ± 5.69 mAU/min for wtFVIIa) is the result of a defect in the catalytic activity of the enzyme or the consequence of a smaller proportion of FVIIa being complexed with TF. If a K_D of 200 pM is assumed (Waxman et al., 1992), then greater than 95% of both wtFVIIa and FVIIa-R79Q are in complex with TF at the concentrations used. The substitution of this residue may, through an indirect structural interaction, impact the conformation of the FVII Gla domain, recently shown to be involved in a direct interaction with FX (Martin et al., 1993). This observation does not preclude a TF binding defect in the variant molecule since FX hydrolysis is dependent on the interaction of FVII and its cofactor. Single-point mutations in FVII have previously been shown to lower the affinity for TF and for macromolecular substrates (O'Brien et al., 1991).

Determination of K_m for S2288. Assays performed with wild-type and variant FVIIa in the presence of relipidated TF1-243 produced the following kinetic parameters: wt-FVIIa, V_{max} 100 ± 5.5 mAU/min, K_m(S2288) 1.35 ± 0.15 mM; FVIIa-R79Q, V_{max} 100 ± 4.8 mAU/min, K_m 1.24 ± 0.13 mM (Figure 4A). TF1-243 is a variant molecule comprising the extracellular and transmembrane domains, and while it lacks the intracellular domain, it is identical to the wild type with respect to *in vitro* function. In the absence of TF and phospholipid, the following kinetic parameters were obtained: wtFVIIa, V_{max} 12.2 ± 0.89 mAU/min, K_m-(S2288) 3.64 ± 0.54 mM; FVIIa-R79Q, V_{max} 12.1 ± 1.17 mAU/min, K_m 4.47 ± 0.81 mM (Figure 4B).

The amidolytic assays demonstrate little difference between the two FVIIa molecules, with identical maximal velocities of S2288 hydrolysis, and half-maximal rates being achieved at very similar substrate concentrations. These data demonstrate that the functional defect found in FVII-R79Q is not directly associated with peptidyl substrate recognition or catalysis.

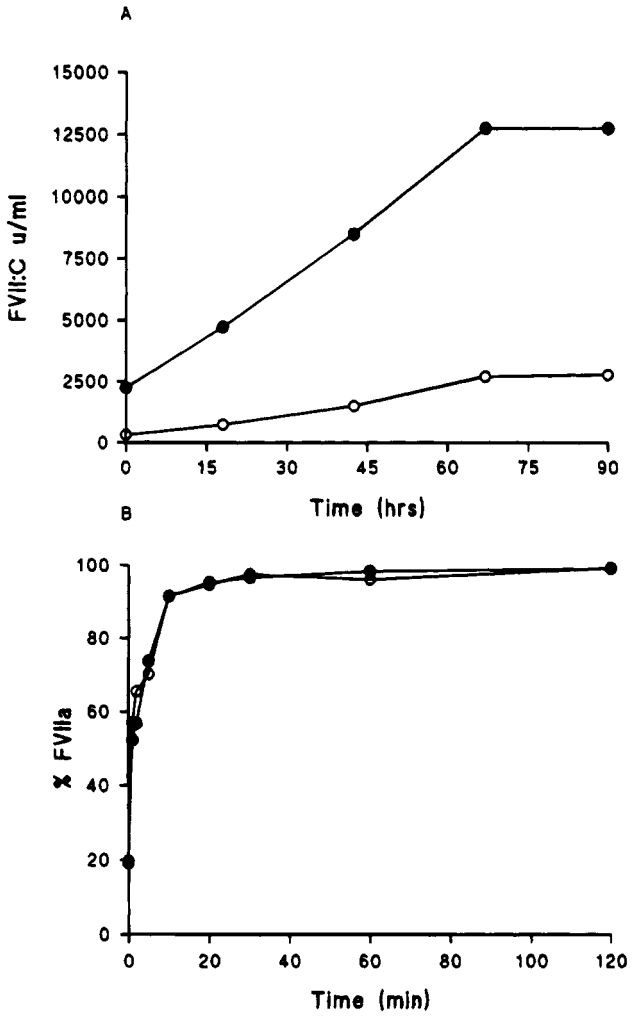

FIGURE 2: Panel A: Autoactivation of wtFVII (●) and FVII-R79Q (○) in the presence of 5 mM $CaCl_2$ and phospholipid. FVII activity was assayed in a one-stage FVII:C assay. Panel B: Activation of wtFVII (●) and FVII-R79Q (○) by FXa. FVII was activated by FXa as described under Experimental Procedures and assayed by densitometry following Western blot analysis of two-chain FVII.

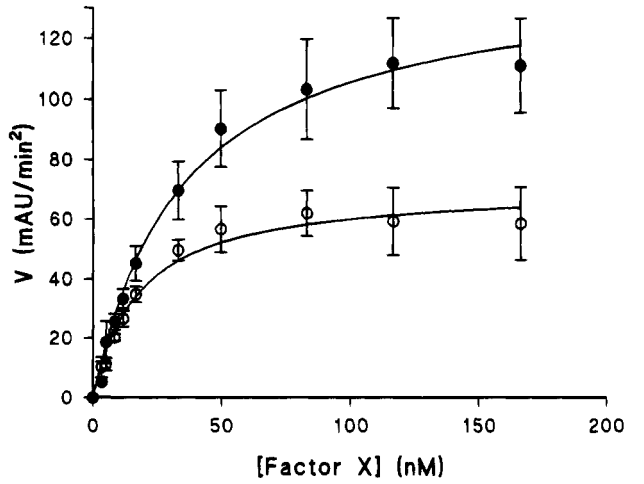

FIGURE 3: FX activation by activated wtFVII (●) and FVII-R79Q (○) in complex with relipidated TF1-243. The rate of FX activation was determined by observing the change in the rate of FXa-mediated hydrolysis of the chromogenic substrate S2222.

Surface Plasmon Resonance Studies. Soluble TF1-219 covalently coupled to the dextran surface of the BIAcore sensor chip readily bound FVII from solution in a calcium-dependent manner. The calcium-dependence of this reaction has been well documented (Broze et al., 1985; Yamamoto et al., 1993), and therefore control injections were performed in the presence of 10 mM EDTA. No binding of wild-type FVII was detected under these conditions, and thus regeneration of the chip was achieved by elution of FVII from TF by 10 mM EDTA between injection cycles. It is noted that the TF used in these experiments was covalently coupled to a dextran surface as opposed to being inserted into a phospholipid bilayer.

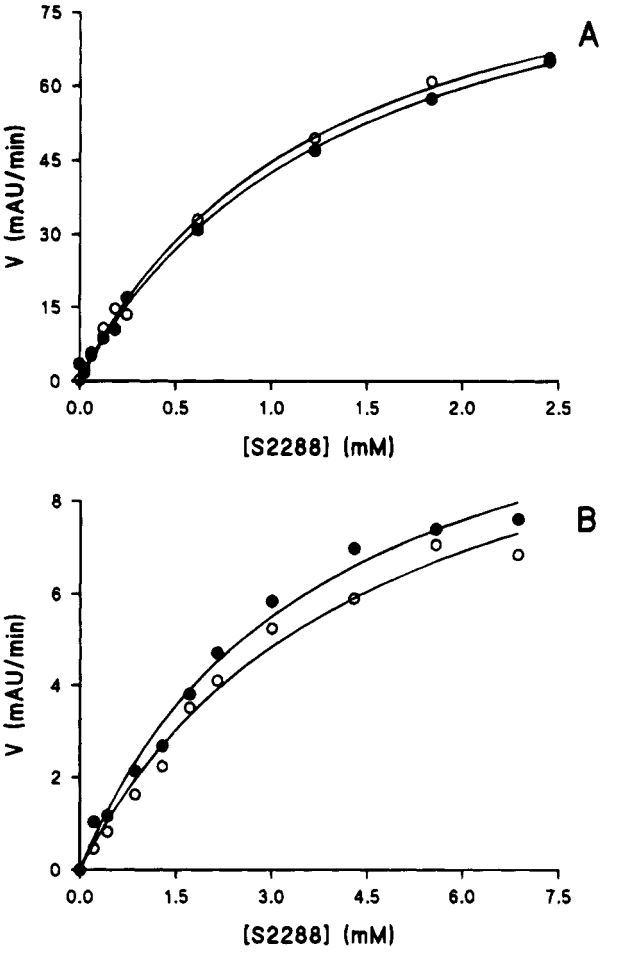

FIGURE 4: Hydrolysis of the chromogenic substrate S2288 by activated wtFVII (●) and FVII-R79Q (○) in complex with (A) relipidated TF1-243 or (B) no cofactor.

Control injections were also performed with 250 nM FIXa, this being equivalent to the maximal FVII concentration used in kinetic experiments; no binding of FIXa was detected in the presence or absence of ionic calcium. FIXa does not interact with TF but is highly homologous to FVII and was therefore an appropriate control. Kinetic runs for wtFVII and FVII-R79Q are depicted in Figure 5. Concentrations of both molecules of between 25 and 250 nM were injected in random order to obviate potential drift effects. Binding curves were collected (Figure 5A), and the rate of change of FVII binding was plotted as dR/dt vs R. The slopes of these curves were then plotted against the concentration of analyte (Figure 5B), and dissociation phases were plotted as $\ln(R_1/R_n)$ vs time. Association and dissociation rate constants (k_{assoc} and k_{diss}) and affinity constants (K_D) were calculated from these data (Table 3). FVII-R79Q was found to have a K_D 7.5-fold higher than that of wtFVII for TF (1.51×10^{-8} compared with 2×10^{-9}): this resulted from a 5-fold

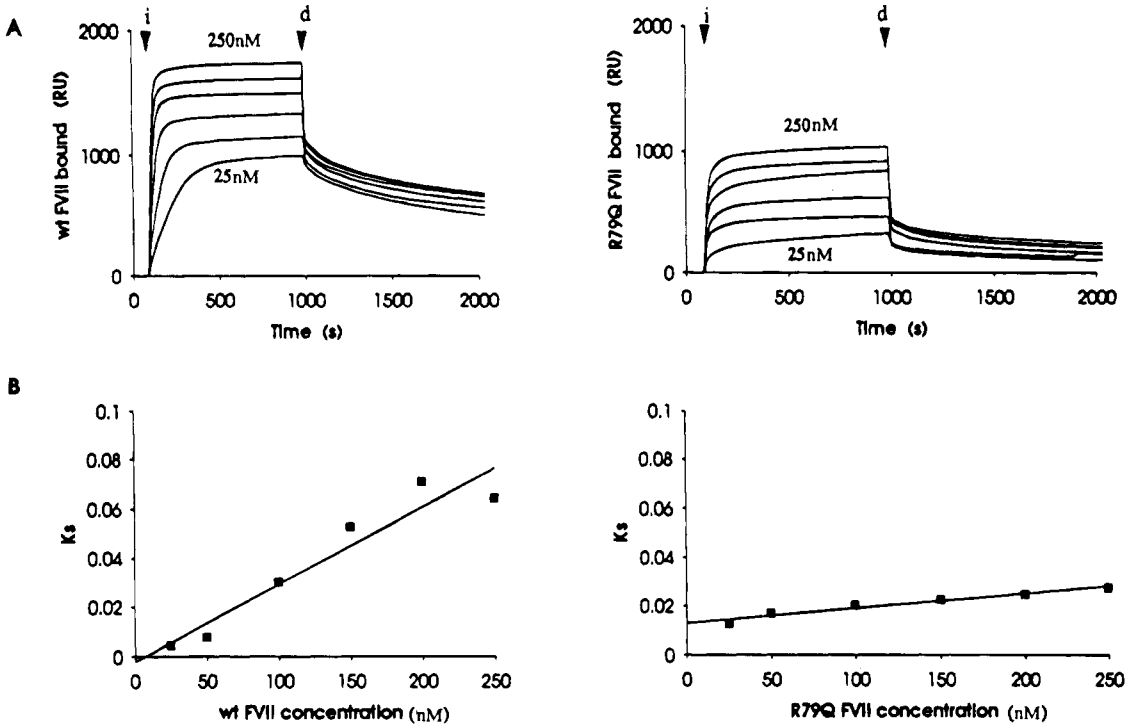

FIGURE 5: Surface plasmon resonance studies of the interaction between wtFVII and FVII-R79Q and TF1-219. **Panel A**: Binding curves of wt and variant FVII, 25−250 nM, interacting with immobilized TF1-219 as a function of time. Injection of FVII is indicated by **i**, and the return to flow in equilibration buffer (start of dissociation) by **d**. **Panel B**: Plot of K_s versus FVII concentration, derived from the rate of change of binding shown in panel A. k_{assoc} is calculated from the slope of this plot; k_{diss} is calculated from the dissociation phase of the same binding curves.

Table 3: Kinetic Parameters for the FVII/FVIIa Interaction with TF Using SPR

	wtFVII	FVII-R79Q	wtFVIIa	FVIIa-R79Q
K_D (M^{-1})	2.00×10^{-9}	1.51×10^{-8}	5.47×10^{-9}	5.24×10^{-8}
k_{assoc} (M^{-1} s^{-1})	3.14×10^{5}	6.05×10^{4}	1.60×10^{5}	7.51×10^{4}
k_{diss} (s^{-1})	6.29×10^{-4}	9.15×10^{-4}	8.76×10^{-4}	3.94×10^{-3}

decrease in the association rate constant for the FVII-R79Q/TF interaction compared to wtFVII. FVII was converted to FVIIa, and kinetic parameters were reassessed on the BIAcore instrument. The wild-type FVIIa K_D was slightly increased with respect to the zymogen (K_D 5.4 nM), and the K_D for variant FVIIa was increased by a similar ratio compared to the single-chain form of the molecule (K_D 52 nM; Table 3). Since a change in k_{assoc} but not in k_{diss} for the variant was recorded, the arginine residue at position 79 may play a role in the initial complex formation between TF and FVII, but once the complex has formed, other residues in FVII and TF contribute to the noncovalent bonds which maintain the complex. It is possible, therefore, to posulate that arginine 79 in the first EGF domain of FVII plays a role in cofactor recognition and initial complex formation between FVII and TF but that extended regions of the FVII molecule are responsible for the interaction with its cofactor.

DISCUSSION

Coagulation factor deficiencies resulting from point missense mutations are useful probes of structure/function relationships in wild-type proteins. We recently identified an individual with CRM+ factor VII deficiency (FVII:C 0.2 unit/mL) who is homozygous for substitution of arginine by glutamine at position 79 (Takamiya et al., 1993). We have now transfected CHO cells with an expression vector containing FVII cDNA with the appropriate mutation in order to characterize the defective factor VII using surface plasmon resonance (SPR) and other methods. Purified recombinant FVII-R79Q was homogeneous by SDS−PAGE and had identical relative mobility and the same N-terminus as the wild-type molecule. In one-stage FVII:C assays, the specific activity of this molecule was only 30% compared to the wild type. This is in contrast to the results of Kazama (1992) but in agreement with the figure quoted by Clarke et al. (1992) and Sridhara and colleagues (Sridhara et al., 1993). Reduced activity was consistently detected in different batches of the variant recombinant molecule, both in culture medium and as purified protein. One-stage FVII:C assays performed with rabbit TF resulted in even lower specific activities. These values correlate with the FVII:C levels measured in the propositus' plasma. Purified FVII-R79Q underwent autoactivation to the two-chain molecule at the same rate as the wild type. One-stage FVII:C assays using human and heterologous TF revealed that fully activated FVIIa-R79Q also had reduced specific activity when compared to wild-type FVIIa, indicating that the defect was not the result of reduced rates of FVII activation. Kinetic analysis was performed using a chromogenic assay for wtFVIIa and FVIIa-R79Q activity and revealed a similar V_{max} and K_m for S2288 hydrolysis by wtFVII and FVII-R79Q both in the presence and in the absence of excess relipidated tissue factor. In contrast, the V_{max} for FX activation was lower in the mutant while the K_m was also 2-fold lower than the wild-type molecule. These data are consistent with an alteration in cofactor interaction and in macromolecular substrate recognition and catalysis. Similar multiple functional abnormalities were seen in another variant, FVII-R304Q, which bound TF with reduced affinity in ligand blot analyses and had a higher K_m for FX in functional assays (O'Brien et al.,

1991). These results suggest that the affinity of FVII for its macromolecular substrate is dependent on the TF/FVIIa interaction.

We used SPR to analyze directly the interaction of FVII and of FVII-R79Q with TF. The BIAcore instrument allows real-time molecular interaction analysis such that association and dissociation rate constants can be readily measured. The technique has been used to study a variety of molecular interactions in real time including antigen/antibody (Altschuh et al., 1992), receptor/hormone (Wang et al., 1993), and protein/DNA interactions (Bondeson et al., 1993). SPR has not previously been used to determine kinetic parameters for the interaction of a coagulation factor with its cofactor. The TF/FVII and TF/FVIIa interactions are ideally suited to the technique in that stable, high-affinity complexes of factor VII with immobilized TF readily form on the sensor surface with substantial mass increments, and factor VII can be eluted by sequestration of divalent metal ions. Wild-type FVII bound to immobilized TF1-219 with high affinity (K_D 2 nM) as determined by SPR. Repeated measurements with different batches of FVII gave highly consistent results.

Dissociation constants for the full-length TF/FVII/FVIIa interaction have been measured by several groups using binding studies or kinetic analyses with results which range from 1 to 20 nM as follows: 1.1−2.1 nM using the synthetic substrate 6-(mes-D-Leu-Gly-Arg)-amino-1-naphthalene diethyl sulfide (Lawson et al., 1992), 13.8 nM in cell-surface TF binding studies (Ruf et al., 1992), 9.2 nM with detergent-solubilized TF (Ruf et al., 1991), and 21 nM using TF bound to Immobilon-P membranes (Toomey et al., 1991). FVII and FVIIa have been reported to have a similar K_D for TF (9.2 ± 3.5 and 4.2 ± 2.6 nM, respectively; Ruf et al., 1992). Two other reports gave markedly lower dissociation constants: 250 pM inferred from the enhanced rate of S2238 hydrolysis (Krishnaswamy, 1992) and 7.3 pM using pressure-induced dissociation of an active-site-modified fluorescent derivative of FVIIa (Waxman et al., 1992). Ruf and coworkers (Ruf et al., 1991) reported that the soluble TF1-219 extracellular domain bound FVII and FVIIa with lower affinity (K_D 120.4 ± 41.8 nM for FVII and 90.3 ± 26.5 nM for FVIIa). Soluble TF1-217 expressed in yeast cells also had lower affinity for FVIIa, K_D 47.4 nM (Shigematsu et al., 1992). We used soluble TF1-219 in SPR analyses to facilitate binding of nonmicellar TF to the carboxylated dextran on the sensor chip. Interestingly, soluble TF immobilized in this way binds FVII with similar affinity (K_D 2 nM) to the full-length relipidated molecule measured by a variety of differing assays. Ruf et al. (1991) speculated that the lower affinity binding of FVII/FVIIa to soluble TF1-219 may be related to the lack of the transmembrane domain, resulting in conformational destabilization of the molecule, with the concomitant loss of some protein/protein interactions. It seems likely, therefore, that soluble TF1-219 adopts a more favorable conformation for FVII binding on covalent attachment to dextran on the BIAcore sensor chip. In support of this hypothesis, we have found that FVII is readily activated to FVIIa by TF1-219 immobilized on agarose columns, but not by solution-phase TF1-219 (unpublished observation, D. P. O'Brien). The nature of the surface may not be critical for this effect, a notion which is supported by the similar K_D values observed for TF inserted into Triton micelles and phospholipid-associated TF (Ruf et al., 1991).

Notwithstanding variations between dissociation constants measured using a variety of proteins and assays, the direct comparison of wtFVII and FVII-R79Q in real-time SPR analysis revealed a profound difference in the association rate constants for the two molecules. The variant FVII molecule bound TF1-219 with reduced affinity compared to the wild type, as a consequence of a slower on rate (k_{assoc}) and near-identical off rates (k_{diss}). These results are consistent with the kinetic parameters determined using other functional assays. The observed K_D is consistent with a reduction in FVII:C activity in one-stage assays to about 30% of the wtFVII. The functional abnormality in this variant FVII molecule is only detected under conditions where the concentrations of both free TF and FVII are not negligible with respect to the concentration of the TF/FVII complex. Assays performed at high TF or high FVII concentrations therefore show little difference between the two molecules. The conflicting conclusions of Sridhara et al. (1993) and Kazama et al. (1992) may therefore be explained simply by differing assay conditions.

Position 79 in FVII is in the first epidermal growth factor-like domain (EGF). Many proteins with EGF-like domains are powerful mitogens of a number of cell types in culture and are found in a variety of functionally unrelated proteins, including the serine proteases of blood coagulation, receptors such as thrombomodulin, transforming growth factors, and cell matrix proteins (Stenflo et al., 1991). The EGF-like domains are grouped together on the basis of sequence similarity and disulfide bond pairing. Most comprise approximately 50 amino acids and contain 3 conserved disulfide bonds. The solution structures of human EGF (Cooke et al., 1987), the FIX EGF domain (Baron et al., 1992), and the FX EGF domains (Selander et al., 1990, 1992; Ullner et al., 1992) have been determined by ^{1}H NMR. The prototypical EGF structure comprises an N-terminal domain with an antiparallel β-sheet and a C-terminal domain containing one disulfide bond and a smaller β-sheet structure, linked by a β-turn. Alignment of the FVII EGF sequence with human FIX EGF1 places Arg 79 in this C-terminal β-turn as a solvent-exposed, and accessible, residue. The loss of charge associated with the nonconservative substitution of arginine by glutamine at this site may directly impact a TF binding site in this part of FVII EGF1 or, alternatively, may impact global folding and, in consequence, perturb protein/protein interactions elsewhere in the EGF module. Several lines of evidence suggest that FVII EGF domains are important in TF/FVII interaction. Chimeric FIX/FVII molecules only bind to TF when the FVII-EGF1-EGF2 modules are present (Toomey et al., 1991), and recently a proteolytic digest fragment of FVII comprising the Gla-EGF1-EGF2 modules was shown to bind TF (Kazama et al., 1993). It is evident that the serine protease domain of FVII also plays a role in TF/VII interaction (Wildgoose et al., 1990; O'Brien et al., 1991), but SPR analysis of FVII-R79Q shows clearly that the first EGF domain of FVII plays a key role in the initial formation of the TF/FVII complex but that other, possibly extended, FVII sequences then contribute to the overall binding energy. This may have important implications for potential anticoagulant development targeting the TF/FVII complex. SPR is a biophysical method which should facilitate further investigation of the structural requirements for this physiologically important interaction.

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