Factor VII Central

A NOVEL MUTATION IN THE CATALYTIC DOMAIN THAT REDUCES TISSUE FACTOR BINDING, IMPAIRS ACTIVATION BY FACTOR XA, AND ABOLISHES AMIDOLYTIC AND COAGULANT ACTIVITY*

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Factor VII is a vitamin K-dependent zymogen of a serine protease that participates in the initial phase of blood coagulation. A factor VII molecular variant (factor VII Central) was identified in a 24-year-old male with severe factor VII deficiency and whose plasma factor VII antigen was 38% of normal, but expressed <1% factor VII procoagulant activity. DNA sequence analysis of the patient's factor VII gene revealed a thymidine to cytidine transition at nucleotide 10907 in exon VIII that results in a novel amino acid substitution of Phe to Ser. The patient was homozygous for this mutation, whereas each parent of the patient was heterozygous for this mutation. To investigate the molecular properties of this variant, a recombinant F328S factor VII mutant was prepared and analyzed in relation to wild-type factor VII. F328S factor VII exhibited <1% factor VII procoagulant activity and a 2-fold decreased affinity for tissue factor and failed to activate factor X or IX in the presence of tissue factor following activation by factor Xa. In addition, F328S factor VIIa exhibited no detectable amidolytic activity in the presence of tissue factor. The rate of F328S factor VII activation by factor Xa was markedly decreased relative to the rate of wild-type factor VII activation as revealed by densitometry scanning of SDS gels. Temporal analysis of this reaction by SDS-polyacrylamide gel electrophoresis also revealed the formation of two novel F328S factor VII degradation products (40 and 9 kDa) resulting from factor Xa proteolysis of the Arg-Lys peptide bond in intact F328S factor VII. Computer modeling and molecular dynamics simulations of the serine protease domain of factor VIIa suggested that the inability of F328S factor VIIa to cleave substrates may result from the apparent formation of a hydrogen bond between Tyr and Asp, a residue at the bottom of the substrate-binding pocket important for the interaction of substrate arginine side chains with the enzyme. These findings suggest that Phe, which is conserved in prothrombin, factor IX, factor X, factor VII, and trypsin, is important for factor VIIa catalysis.

Factor VII is a single-chain, vitamin K-dependent plasma glycoprotein (50 kDa) that plays a key role in the initiation of the extrinsic pathway of blood coagulation (2). The gene for factor VII is located on chromosome 13 at q34-4qter, spans 12.8 kilobase pairs, and contains nine exons (3, 4). The gene organization and protein structure for factor VII are similar to other vitamin K-dependent coagulation proteins in that each is a multidomain glycoprotein containing a Gla domain, epidermal growth factor-like or kringle domains, and a C-terminal catalytic domain homologous to trypsin. Inherited factor VII deficiency is a rare autosomal recessive disorder and is phenotypically heterogeneous. The clinical features are variable with a rather poor correlation between reported procoagulant activity and bleeding tendency (5–7). Factor VII deficiency is classified as either CRM (type 1) or CRM (type 2) based upon the absence or presence, respectively, of a disparity between the activity and antigen levels (7). In the majority of factor VII-deficient patients, plasma levels of factor VII activity diminish in parallel with immunoreactive factor VII antigen. A discrepancy between factor VII coagulant activity and factor VII antigen levels has been found in a few patients, suggesting the presence of a dysfunctional factor VII (reviewed in Ref. 8). In this report, we describe the molecular basis of a severe factor VII deficiency in a 24-year-old male whose plasma factor VII activity was <1%, while his plasma factor VII antigen was 38% of normal. We have analyzed the factor VII gene sequence of this patient, designated as factor VII Central, and demonstrate that the patient was homozygous for a single point missense mutation in exon VIII in the catalytic domain, resulting in a Phe to Ser substitution at amino acid 328. We have expressed and purified this variant from baby hamster kidney cells transfected with the cDNA coding for this mutant and demonstrated that the isolated mutant exhibits <1% procoagulant activity, no demonstrable amidolytic or proteolytic activity following activation by factor Xa, and decreased affinity for tissue factor, thus recapitulating the patient’s factor VII phenotype. In addition, the F328S factor VII mutant, as well as the isolated patient’s factor VII, undergoes proteolytic degradation by factor Xa at Arg-Lys, suggesting that the Phe to Ser substitution creates an exposed surface loop in this region readily accessible for cleavage by factor Xa, further reducing the catalytic activity of this clotting factor.

EXPERIMENTAL PROCEDURES

Materials—Recombinant Tqg DNA polymerase was obtained from Perkin-Elmer. NuSieve GTG-agarose was a product of FMC Corp. Bio-Products. Bovine serum albumin (fatty acid-free) and peroxidase-conjugated goat anti-rabbit IgG were purchased from Boehringer Mannheim. Affi-Gel 10 was obtained from Bio-Rad. Microtitration plates (96-well) were obtained from Nunc. 125I-Labeled protein A was purchased from DuPont NEN. H-1-Ile-Pro-Arg-p-nitroanilide (S-2288) was obtained from Kabi Pharmacia Hepar, Inc. Streptavidin-coated paramagnetic beads were purchased from Dynal, Inc. All other reagents were of the highest grade commercially available.
**Proteins**—Human plasma factor Xa (9), recombinant human factor VII (10), soluble recombinant human tissue factor approtein TF-1,218, (11), human brain thromboplastin (12), mixed brain phospholipids (13), and affinity-purified rabbit anti-human factor VII IgG (14) were prepared by published methods. Calcium-dependent (CaFVII2) and calcium-independent, heavy chain-specific (AD-1) murine anti-human factor VII monoclonal antibodies were produced in Balb/c mice essentially according to Kohler and Milstein (15) and purified from ascites fluid by either protein A-Sepharose or DEAE-Affi-Gel blue column chromatography. Monoclonal antibody CaFVII2 was coupled to Affi-Gel 10 according to the manufacturer's recommendation. Recombinant F328S factor VII* was expressed in stably transfected baby hamster kidney cells using mutagenesis, transfection, and culturing conditions essentially as described for S344A factor VII (16) and was purified to homogeneity in a single step from ~10 liters of serum-free baby hamster kidney conditioned medium by CaFVII2-Affi-Gel 10 immunoaffinity chromatography (18).

**DNA Sequence Analysis of the Propositus Factor VII Gene and Detection of the Mutation in the Family**—Collection of blood samples from the proband and his parents was performed following appropriate consent at a time when the proband had not been transfused with plasma for at least 2 weeks. Genomic DNA was extracted from peripheral blood samples using proteinase K lysis, phenol extraction, and ethanol precipitation (17). Based on published intron data for human factor VII (4), eight pairs of oligonucleotides (Table I) were synthesized and used to perform polymerase chain reaction amplification (18) of the seven coding regions (exons II–VIII) and exon-intron boundaries in the three DNA samples. Due to their small size, exons III and IV were amplified as a single fragment. In each reaction, one of the polymerase chain reaction primers was 5'-biotinylated to facilitate the subsequent preparation of single-stranded templates for sequence analysis. Inasmuch as exon VIII is relatively large, we used both forward and reverse 5'-biotinylated primers for separate amplifications. In the polymerase chain reaction, the biotinylated primer was incorporated into the amplification product strand complementary to the sequencing primer subsequently used. Target sequences were amplified essentially according to Chiang et al. (18) in a 100-μl volume containing 0.5 μg of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, and variable concentrations of MgCl₂ (Table I).

**General Methods**—SDS-polyacrylamide slab gel electrophoresis (PAGE) was performed according to Laemmli (19) using 10% polyacrylamide separating gels. Following electrophoresis, the proteins were visualized either by staining with Coomassie Brilliant Blue or by immunoblotting and autoradiography following electrotransfer to nitrocellulose. The coagulant activities of recombinant wild-type human factor VII and F328S factor VII were assessed in a one-stage clotting assay using immunodepleted factor VII-deficient plasma (<1% factor VII antigen) and human brain thromboplastin essentially as described (20). Factor VII antigen concentrations were determined by an enzyme-linked immunosorbent assay method (21, 22) using a factor VIIa heavy chain-specific monoclonal antibody (AD-1) as the capture antibody. The Gla content of the recombinant wild-type and F328S factor VII preparations was determined according to Kuwada and Katayama (23). Biotinylated and unbiotinylated synthetic oligonucleotides were prepared by solid-phase phosphoramidite chemistry on an automated DNA/RNA synthesizer (Applied Biosystems Model 394) in the University of New Mexico School of Medicine Protein Chemistry Laboratory. Amino-terminal amino acid sequence was determined using a Beckman Model LP3000 gas-phase sequenator.

**Tissue Factor Binding Assay**—The affinity of F328S factor VII and recombinant wild-type factor VII for immobilized soluble recombinant tissue factor approtein TF-1,218 was assessed in an enzyme-linked immunosorbent assay using a modification of the procedure described by Chiang et al. (18). In this procedure, 100 μl of TF-1,218 (10 μg/ml in 50 mM sodium carbonate (pH 9.6) was added to the wells of a 96-well microtiter plate (MaxiSorp Immunoplate) and incubated at 4°C overnight. After washing the plate with TBS and 0.1% Tween 20, each well was treated with 200 μl of 0.1% gelatin in TBS for 2 h to block nonspecific binding sites and subsequently washed with TBS/Tween 20. Aliquots (100 μl) of various concentrations of either wild-type factor VII or F328S factor VII (dissolved in TBS, 5 mM CaCl₂, 0.05% Tween 20, and 1% BSA) were added to the plate and allowed to incubate at 37°C for 2 h. The plate was then washed six times with TBS and 0.1% Tween 20 containing 5 mM CaCl₂, and 100 μl of affinity-purified rabbit anti-human factor VII IgG (5 μg/ml in TBS, 5 mM CaCl₂, and 1% BSA) was added to each well. Following a 2-h incubation at 37°C, the plate was washed six times with TBS/Tween 20/CaCl₂ and subsequently treated for 2 h at 37°C with 100 μl of peroxidase-conjugated goat anti-rabbit IgG (5 μg/ml in TBS/CaCl₂/BSA). After a final washing, 100 μl of o-phenylenediamine (1 mg/ml in 0.1 M sodium citrate (pH 4.5) and 0.5% hydrogen peroxide) was added to each well. After a 2–3-min incubation at room temperature, 100 μl of 2.5 μl H₂O₂ was added to each well, and the A₄₉₀ was measured at room temperature in a UVMAX kinetic microtiter plate reader ( Molecular Devices). The A₄₉₀ was assumed to be directly proportional to factor VII binding to immobilized TF-1,218. Factor VII specific binding (∆A₄₉₀) was obtained by subtracting the A₄₉₀ of the gelatin control from the apparent A₄₉₀. The apparent dissociation constant (Kₐ₈₀) was estimated from the slope of a plot relating ∆A₄₉₀/offset factor VII concentration versus A₄₉₀.

**Purification of the Patient's Factor VII**—The patient's factor VII was isolated by a combination of barium citrate adsorption, elution, and immunoaffinity chromatography as follows. Citrated patient plasma (~20 ml) was treated with 2.4 ml of 1 M BaCl₂ and 100 μl of 1 M benzamidine and mixed for 1 h at 4°C. The mixture was centrifuged (8000 rpm, 15 min), and the precipitate was washed twice in TBS, 0.1 M BaCl₂, and 1 M benzamidine. The washed precipitate was then dissolved in 10 ml of TBS containing 30 mM EDTA and 1 mM benzamidine, and the eluate was dialyzed overnight at 4°C against 4 litters of TBS and 1 mM benzamidine. The dialyzed sample was made 5 mM in CaCl₂ and 10 mM in benzamidine and incubated with 1 ml of CaFVII2-Affi-Gel 10 at 4°C for 90 min. After washing the CaFVII2-Affi-Gel 10 twice with 50 mM Tris-HCl (pH 8), 0.1% BSA, and 5 mM CaCl₂, the patient's factor VII was eluted from the resin with 50 mM Tris-HCl (pH 8), 0.1% BSA, and 30 mM EDTA and subsequently dialyzed against TBS.

**Analysis of Factor VII Activation by Factor Xa**—The proteolytic ac-

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*a* F, forward primer; R, reverse primer.

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**Table I**

| Exon | 5’ → 3’ oligonucleotide sequence | Nucleotides | Product size |
|------|----------------------------------|-------------|-------------|
| II   | F”CTCTCCAGGAGGCGGGGCAAATTTGATGC | 3660–3890   | 371         |
| III  | R biotin-CACAGGCGCAGCAGCAAGAGCGGGG | 4030–3999   |             |
| IV   | TCCAGGAGGAGAATGGTCTGTCCCTG      | 5718–5741   | 396         |
| V    | R biotin-CTTCTCCTGCAAAATTGGG     | 6114–6091   |             |
| VI   | F AGCTCATGACAGGCGGCA             | 6768–6791   | 303         |
| VII  | R biotin-AGGAGGTGTGTCATGGTGGACTG | 7970–7947   |             |
| VIII | F biotin-CTAGATTTTCTTCTAGTGGACCTGTC | 8829–8853   | 280         |
|      | R biotin-GTTGTCAATCTCAAGGTTTCCTG | 9109–9086   |             |
|      | F TTGGCTGACTGCTCCCATTTCCCC       | 9504–9522   | 470         |
|      | R biotin-CTAGATTTTCTTCTAGTGGACCTGTC | 10010–9987  |             |
|      | F ATCCCCATGTACGACAGGGGTTG        | 10476–10496 | 713         |
|      | R biotin-ACAGTTCGACAGGCTTCTGGTTT | 11188–11165 |             |

*1 The abbreviations used are: TF, tissue factor; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin.

*2 Mutant factor VII is designated according to the notation described by Shapiro and Vallely (1), in which the single-letter code for the original amino acid is followed by its position in the sequence and the single-letter code for the new amino acid.
rabbit anti-factor VII IgG followed by incubation with 125I-labeled pro-
atradation products were visualized by incubation with affinity-purified
transferred to nitrocellulose membranes, and factor VI/VIIa and deg-
ning products. Following electrophoresis, the proteins were electrophoretically
subjected to SDS-PAGE following reduction with 10%
final phospholipid concentration) and human factor Xa (1 nM) in a total
volume of 400 µl of TBS, 0.1% BSA, and 5 mM CaCl2. The reaction was
initiated by the addition of factor Xa, and at selected intervals, 40-µl
aliquots were removed from the incubation mixture and added to 2 µl of 0.5 mM EDTA to stop the reaction. An aliquot (20 µl) of this mixture was
used for clotting activity measurements, while the remaining 20 µl was
subjected to SDS-PAGE following reduction with 10% β-mercaptoethanol.
Following electrophoresis, the proteins were electroeluted from nitrocellulose membranes, and factor VIIa and deg-
gradation products were visualized by incubation with affinity-purified rabbit anti-factor VII IgG followed by incubation with 125I-labeled pro-
tein A and autoradiography.

Structural Modeling—The Homology module within Insight II (Bio-
sym/MSI Technologies) was used on an SGI R8000 workstation to
construct a homology-based three-dimensional model of factor VII uti-
lizing the crystal structure of factor Xa (24) as the reference template. Sets of highly conserved regions were initially identified. The noniden-
tical residues of the template were mutated to the corresponding resi-
dues of factor VII, followed by the necessary insertions and deletions in order to generate the sequence of the model. Areas that required loop
generation or in which the lengths of the model loops differed from those in the reference protein were computationally constructed by
searching the Brookhaven Data Bank for regions of proteins that meet similarity criteria. An alternative approach was taken in cases where the selected loops appeared to have steric overlaps with the newly built regions (25). To refine the final model-built structures, a few hundred
steps of steepest descent energy minimization were performed using the
Discover force field, and the resultant model was evaluated for sensible
conformations and physicochemical properties (26).

Molecular dynamics simulations were also used in order to investi-
gate possible conformational transitions in the wild-type and mutant
proteins, in particular those associated with local conformational
changes regarding the catalytic triad. Structures generated in previous
model-building exercises were used as starting conformations for the
wild-type and mutant proteins, and two sets of molecular dynamics
simulations were performed in vacuo on both proteins with a distance-
dependent dielectric constant. In all simulation runs, an integration
step of 1 fs was used, and nonbonded interaction pair lists were updated
every 20 steps. Each system was equilibrated for 10 ps followed by 50 ps
data collection, and the simulation results were analyzed visually
using interactive molecular graphics within Insight.

RESULTS

Case Report—The propositus is a 24-year-old Hispanic male
from Central, New Mexico who experienced recurrent epistaxis
as a child and joint and soft tissue hemorrhage that was usu-
ally related to trauma. He has a markedly prolonged prothrom-
bin time (85 s; control = 9–13 s), and his plasma contains <1%
factor VII coagulant activity as measured by a specific one-
stage factor VII clotting assay. The factor VII antigen in the
patient’s plasma is 190 ng/ml (normal = 400–600 ng/ml) as
measured by an enzyme-linked immunosorbent assay (21, 22).
The factor VII activity levels in plasma samples obtained from
the patient’s mother, father, and sister, all clinically asympto-
matic, are 71, 76, and 123% of normal, respectively.

DNA Sequence Analysis—All exons and exon-intron bound-
daries of the patient’s factor VII gene were enzymatically am-
plified using biotin-labeled primers and by directly sequencing the
isolated amplified biotinylated strand. When compared with the normal sequence, the sequence of the propositus factor
VII gene indicated a single mismatch in exon VIII with a
thymidine to cytidine transition at nucleotide 10907. This mutation
results in the substitution of Ser for Phe at amino acid
328. By direct sequencing of polymerase chain reaction prod-
ucts, the patient was homozygous for this mutation (Fig. 1),
whereas each of his parents was heterozygous for this mutation
(data not shown). Furthermore, this mutation neither abol-
ished nor created any restriction endonuclease site.

Characterization of Recombinant F328S Factor VII—Recom-
binant F328S factor VII was purified to homogeneity by immu-
noaffinity chromatography from the serum-free conditioned
medium of baby hamster kidney cells stably transfected with a
plasmid containing the Phe to Ser mutation. Purified F328S
factor VII migrated as a single band in SDS-PAGE with essen-
tially the same mobility as recombinant wild-type factor VII in
the presence or absence of reducing agent (Fig. 2). Analysis of
F328S factor VII for γ-carboxyglutamic acid content following
alkaline hydrolysis indicated that the preparation was fully
γ-carboxylated (data not shown). In a one-stage clotting assay,
F328S factor VII exhibited a specific clotting activity of <20
units/mg, while the specific activity of wild-type factor VII was
~2000 units/mg.

Cleavage of Factor VII Central and F328S Factor VII by
Factor Xa—Factor VII Central (−1–2 µg) was purified to ho-
mogeneity from 20 ml of the patient’s plasma by a combination of
barium citrate adsorption, elution, and immunoaffinity chrom-
atography. Incubation of recombinant wild-type factor VII
with factor Xa (1:200 enzyme/substrate molar ratio) in the
presence of calcium and mixed phospholipids resulted in the
complete conversion of wild-type single-chain factor VII to two-
chain factor VIIa within 60 min of incubation at 37 °C (Fig. 3). Under identical conditions, incubation of F328S factor VII or
factor VII Central with factor Xa-calcium-phospholipids
yields a 40-kDa intermediate in addition to the factor VIIa
heavy and light chains that migrate with apparent molecular masses of 34 and 26 kDa, respectively (Fig. 3). Although not visible by autoradiography, preparative incubation mixtures containing F328S factor VII and factor Xa-calciu-phospholipids also produced a lower molecular mass fragment in Coomassie Blue-stained gels that migrated with an apparent molecular mass of 8–9 kDa (data not shown). Subjecting these preparative incubation mixtures to reverse-phase high pressure liquid chromatography (C18 column) following reduction with 5 mM dithiothreitol resulted in the purification to homogeneity of the 40- and 9-kDa peptides. Amino-terminal sequence analyses of these peptides indicated sequences of Ala-Asn-Ala-Phe-Leu and Lys-Val-Gly-Asp-Ser for the 40- and 9-kDa peptides, respectively, which coincided with the amino terminus of the parent protein and an internal sequence located at Lys316–Ser320. Thus, substitution of Ser for Phe328 in factor VII Central appears to make the Arg315–Lys316 peptide bond more solvent-accessible, leading to its cleavage by factor Xa. Densi- tometry scans of each incubation mixture revealed that F328S factor VII, factor Xa, calcium, and phospholipids.

that while cleavage of wild-type factor VII by factor Xa resulted in an ~25–30-fold increase in factor VII clotting activity, no apparent increase in clotting activity was observed for the incubation mixtures containing F328S factor VII or factor VII Central. Furthermore, in contrast to wild-type factor VIIa (27), no tissue factor-enhanced amidolytic activity was detected in temporal aliquots of activation incubation mixtures containing F328S factor VII, factor Xa, calcium, and phospholipids.

Analysis of Factor VII Binding to Immobilized Tissue Factor Aproprotein—To determine whether the markedly reduced clotting and amidolytic activities of F328S factor VII/VIIa observed above was related to its inability to interact with its cofactor, tissue factor, we next compared the affinity of wild-type factor VII and F328S factor VII for soluble TF apoprotein. The Kd(app) was estimated from the slope of a plot relating A490offered factor VII concentration versus A490.

FIG. 4. Interaction of recombinant wild-type human factor VII and human F328S factor VII with immobilized soluble tissue factor apoprotein. A, various concentrations of recombinant wild-type factor VII (○) or F328S factor VII (●) were incubated in the presence of CaCl2 with either soluble tissue factor- or gelatin-coated microtiter plates as a control. After a 2-h incubation, bound factor VII was detected by rabbit anti-factor VII IgG and peroxidase-conjugated goat anti-rabbit IgG. Factor VII specific binding was determined by subtracting the A490 of gelatin-coated control plates from the A490 of soluble tissue factor-coated plates. B, shown is a Scatchard plot of factor VII specific binding to soluble tissue factor. Kd(app) was estimated from the slope of a plot relating A490offered factor VII concentration versus A490.
tor VII to immobilized TF$_{1-218}$. Each factor VII preparation bound to TF$_{1-218}$ in a concentration-dependent manner that approached saturation at 20–40 nM factor VII. Binding of each factor VII preparation was calcium-dependent, as little, if any, binding was observed in the presence of 10 mM EDTA (data not shown). Scatchard plots of the binding data (Fig. 4B) indicated apparent dissociation constants ($K_{d(app)}$) of 7.6 ± 0.4 and 15.9 ± 1.2 nM for wild-type factor VII and F328S factor VII, respectively. The $K_{d(app)}$ value for wild-type factor VII was consistent with that observed in previous reports (28–31). These findings indicate that F328S factor VII and presumably factor VII Central exhibit a ~2-fold decrease in affinity for TF$_{1-218}$ in relation to wild-type factor VII and suggest that substitution of Ser for Phe$^{328}$ results in a new conformation of factor VII in this region of the molecule that disrupts its local interaction with tissue factor apoprotein.

DISCUSSION

We describe the molecular basis underlying a severe factor VII deficiency designated as factor VII Central. The factor VII Central propositus was homozygous for a single point missense mutation in exon VIII in the catalytic domain of the molecule, resulting in a phenylalanine to serine substitution at residue 328. As a result of this mutation, the patient’s plasma factor VII clotting activity was <1% of normal, while his plasma factor VII antigen concentration was ~38% of normal. In an effort to functionally characterize the patient’s factor VII molecule, we constructed the F328S factor VII variant, expressed this mutant in baby hamster kidney cells, and purified it from serum-free conditioned media on a single-step, calcium-dependent immunoaffinity column. The specific clotting activity of the purified F328S factor VII preparation was 20 units/mg, in comparison with recombinant wild-type human factor VII, which exhibited a specific activity of ~2000 units/mg. Incubation of F328S factor VII with a complex of factor Xa-calcium-phospholipids resulted in the cleavage of the Arg$^{315}$–Lys$^{316}$ peptide bond, in addition to cleavage of the Arg$^{315}$–Lys$^{316}$ peptide bond, which leads to the activation of wild-type factor VII. An identical temporal cleavage pattern for F328S factor VII was observed when factor Xa was substituted with a purified preparation of a factor VII activator derived from Taipan snake venom (32), strongly suggesting that cleavage of the Arg$^{315}$–Lys$^{316}$ peptide bond was not unique to factor Xa (data not shown). As revealed by densitometry scans of temporal aliquots of the incubation mixture, the rate of F328S factor
VIIa formation was 10–20% of that observed for wild-type factor VII. At present, it is unclear as to whether this decreased rate of F328S factor VII activation by factor Xa relates to the apparent inability of factor Xa to cleave the 40-kDa fragment consisting of residues 1–315, inasmuch as no band corresponding to residues 152–315 (18 kDa) was observed in reduced samples of this incubation mixture. In spite of the formation of some two-chain F328S factor VIIa, no increase in coagulant activity was detectable in these incubation mixtures, whereas comparable incubation mixtures containing wild-type factor VII and factor Xa-calcium-phospholipids generated a 25–30-fold increase in coagulant activity that correlated with the conversion of zymogen factor VII to factor VIIa. Of additional importance, no amidolytic activity for S-2288 was observed in aliquots of F328S factor VIIa following incubation with soluble tissue factor apoprotein. Essentially identical results to those observed for F328S factor VII with respect to factor Xa-mediated cleavage and specific coagulant activity were observed using the factor VII (1–2 µg) preparation purified from small amounts of the patient’s plasma by a combination of harium citrate adsorption, EDTA elution, and immunoaffinity chromatography, providing strong evidence that our F328S factor VII preparation recapitulated the structure-function characteristics of the patient’s factor VII molecule.

The structure-function basis for the inability of F328S factor VIIa to express proteolytic and particularly amidolytic activity remains enigmatic. While direct tissue factor binding assays revealed a 2-fold decrease in affinity of F328S factor VII for tissue factor apoprotein in relation to wild-type factor VII, these differences could not account for the complete absence of proteolytic and amidolytic activity in F328S factor VIIa. Our experimental evidence is consistent with a conformational change in the vicinity of the Cys310–Cys320 loop induced by the Phe to Ser mutation, resulting in the surface expression of the Arg315–Lys316 peptide bond and a mild disturbance of the factor VII-tissue factor interaction. In the latter case, crystallographic studies by Banner et al. (35) revealed that Asp338 in the heavy chain of factor VIIa directly interacts with Tyr384 in tissue factor apoprotein through three hydrogen bonds. Thus, it is entirely conceivable that the Phe to Ser substitution and the putative surface expression of the Arg315–Lys316 peptide bond impact on this particular molecular interaction and slightly decrease the affinity of F328S factor VII for tissue factor.

To obtain insight into the complete lack of proteolytic and amidolytic activity of F328S factor VIIa, we used homology modeling, based on the published x-ray structure of human factor Xa (24), and performed dynamics simulations on both wild-type and F328S factor VII. The disposition of the critical residues (His183, Asp242, and Ser344) in the active site of factor Xa and VIIa structures appears to be virtually superimposable (data not shown). In wild-type factor VII, the catalytic triad is located immediately below a loop that separates it from the Arg315–Lys316 peptide bond. The aromatic ring of Phe328 sticks between Tyr377 and His348 while the aromatic hydroxyl of Tyr377 forms a strong hydrogen bond with Ser339. Upon inspection of the mutant, Ser328 is located immediately below a loop that separates it from the Phe328-hydroxyl. The aromatic ring of Phe328 sticks between Tyr377 and His348 while the aromatic hydroxyl of Tyr377 forms a strong hydrogen bond with Ser339. Upon inspection of the mutant, Ser328 is located immediately below a loop that separates it from the Phe328-hydroxyl.

In molecular simulation runs, the catalytic triad domain of wild-type factor VIIa appeared quite stable. The hydrogen bond between Ser344 and His183 weakened or broke occasionally, but was re-formed with favorable distances and acceptable dihedral angles. The hydrogen bond distances increased up to 5 Å, but the bond breakage did not last for more than 1 ps. However, this was not observed for the hydrogen bond between Ser344 and Asp422 as this interaction was retained throughout the simulations with very little deviation from the original distance and geometry. In addition, Tyr377 appeared to be rotating away from Ser339 in wild-type factor VII, thus resulting in a breakage of the hydrogen bond interaction with Ser339 (Fig. 5A). This motion of Tyr377, however, strengthens its stacking interaction with Phe328, for the phenol chromophore is oriented roughly perpendicular to the aromatic Phe328, which is typical for interactions of this type. The mutant enzyme displayed a similar behavior throughout the simulations, with transient weakening or breakage followed by reformation. However, examination of the environment surrounding Ser328 provided some clues as to the local motions related to the single mutation. In this regard, Tyr377 no longer formed a hydrogen bond with Ser339, but rather interacted with Asp338, forming a strong linear hydrogen bond, while Ser328 rotated toward His348 (Fig. 5B). Thus, based upon these types of analyses, we speculate that the Phe to Ser substitution at residue 328 in factor VII central results in the formation of a new conformation in the molecule such that Tyr377 interacts with Asp338, a critical residue at the bottom of the substrate-binding pocket of the enzyme (34), and thereby precludes substrate binding. Whether the interaction of F328S factor VII with tissue factor induces other changes in this conformation that reduce substrate-enzyme interactions further is unknown and will require additional studies.

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