A frequent human coagulation Factor VII mutation (A294V, c152) in loop 140s affects the interaction with activators, tissue factor and substrates

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Activated Factor VII (FVIIa) is a vitamin-K-dependent serine protease that initiates blood clotting after interacting with its cofactor tissue factor (TF). The complex FVIIa–TF is responsible for the activation of Factor IX (FIX) and Factor X (FX), leading ultimately to the formation of a stable fibrin clot. Activated FX (FXa), a product of FVIIa enzymic activity, is also the most efficient activator of zymogen FVII. Interactions of FVII/FVIIa with its activators, cofactor and substrates have been investigated extensively to define contact regions and residues involved in the formation of the complexes. Site-directed mutagenesis and inhibition assays led to the identification of sites removed from the FVIIa active site that influence binding specificity and affinity of the enzyme. In this study we report the characterization of a frequent naturally occurring human FVII mutant, A294V (resi-due 152 in the chymotrypsin numbering system), located in loop 140s. This region undergoes major rearrangements after FVII activation and is relevant to the development of substrate specificity. FVII A294V shows delayed activation by FXa as well as reduced activity towards peptidyl and macromolecular substrates without impairing the catalytic efficiency of the triad. Also, the interaction of this FVII variant with TF was altered, suggesting that this residue, and more likely loop 140s, plays a pivotal role not only in the recognition of FX by the FVIIa–TF complex, but also in the interaction of FVII with both its activators and cofactor TF.

Key words: exosite, recombinant protein, serine protease.

INTRODUCTION

Factor VII (FVII) is a liver-derived vitamin-K-dependent protein that circulates in the blood at trace concentrations almost exclusively in the inactive zymogen conformation. Several plasma enzymes, including activated Factor IX (FIXa) [1], activated Factor X (FXa) in the presence of phospholipids [2], activated Factor XII (FXIIa) [3], thrombin [4] and the activated FVII (FVIIa)–tissue factor (TF) complex itself [5], can convert FVII into a two-chain form after cleavage of the peptide bond between Arg^{152} and Ile^{153}. Activation results in insertion of the nascent N-terminus into the protein to form a salt bridge between the amino group of Ile^{153} and the carboxyl group of Asp^{343}, an interaction that participates in the transition of the inactive zymogen to the active enzyme (FVIIa). Upon vascular damage, the active form of FVII interacts with the newly exposed TF to form the complex FVIIa–TF, which then activates FIX and FX, thus triggering the coagulation process [6]. Comparison of the recent crystal structure of zymogen FVII [7] with those of FVIIa [8–10] indicates that conversion from the zymogen to the active state involves four peptide segments, residues 16–19, 142–152 (loop 140s), 184–194 (loop 1) and 216–223 (loop 2), forming the so-called 'activation domain' [11] (note that residue numbers of the proteinase domain of FVIIa are denoted using the homologous numbering system based on chymotrypsin [12]). Conformational changes in the activation domain of FVIIa include creation of the S1 binding site [11] (nomenclature of Schechter and Berger [13]).

Productive molecular docking between enzyme and substrates requires the recognition of regions distinct from the active site (exosites). As already demonstrated for the prothrombinase complex [14], the FVIIa–TF complex presents a defined area of interaction with its macromolecular substrate FX [15]. *In vitro* mutagenesis of amino acid residues in both FVII and TF, as well as the use of short peptide sequence inhibitors, has led to the identification of residues in the complex involved in recognition [16–18]. Recent studies have also shown areas of contact between the FVIIa–TF complex and its physiological substrates FIX and FX. It has been demonstrated that FIX and FX interact with the same TF region, located proximal to the putative phospholipid surface [19]. The same TF residues also seem to play an interface role in the interaction of FVII–TF with its activator FXa, thus providing evidence for the use of a common TF surface for the divergent functions of zymogen FVII activation by FXa and zymogen FX activation by FVIIa [20].

In this study we define the biochemical properties of the FVII mutant A294V (c152; meaning residue 152 in the chymotrypsin numbering system), a frequent naturally occurring variant in Caucasians, responsible for asymptomatic FVII deficiency [21–23]. FVII levels associated with the A294V mutation in patients were 6–18% (FVII coagulant activity) and 25–61% (FVII antigen) [21,23] of pooled normal plasma. The reduced antigen levels could be imputable to the linkage of the A294V mutation with the Gln^{353} polymorphic allele, known to affect FVII secretion [24]. However, transient expression of the A294V mutation in the presence of arginine at the 353 position also resulted in reduced protein secretion [25].

Ala^{294}, located in loop 140s of the activation domain, precedes the β-strand B2 region that undergoes a three-amino acid shift, determining a re-registration of FVII after its cleavage [7]. This partially active FVII mutant provides a molecular tool to investigate the role of this evolutionarily important region in the interaction of FVII/FVIIa with other macromolecules.

EXPERIMENTAL

Materials

Hepes, poly(ethylene glycol) with an average molecular mass of 8000 Da (PEG 8000) and Nunc cell factories were from Fisher Scientific (Pittsburgh, PA, U.S.A.). L-α-Phosphatidylcholine, L-α-phosphatidylserine and benzamidine were from Sigma (St. Louis, MO, U.S.A.). Q Sepharose Fast Flow was from Pharmacia (Piscastaway, NJ, U.S.A.).

The chromogenic substrates methoxycarbonyl-D-cyclohexyl-glycyl-glycyl-arginine-p-nitroanilide acetate (Spectrozyme fXa) and methane sulphonyl-D-cyclohexylalanyl-butyl-arginine-p-nitroniline monoacetate salt (Spectrozyme fVIIa) were from American Diagnostica (Greenwich, CT, U.S.A.). Insulin/transferrin/sodium selenite supplement was purchased from Roche Diagnostics (Indianapolis, IN, U.S.A.). Dade Innovin was from Dade (Miami, FL, U.S.A.), whereas Simplastin® Excel and FVII-deficient plasma were from Organon Teknika (Durham, NC, U.S.A.). The buffer, designated as reaction buffer, was 20 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$ and 0.1 % (w/v) PEG 8000, pH 7.4.

Phospholipid vesicles composed of 75 % (w/w) L-α-phosphatidylcholine and 25 % (w/w) L-α-phosphatidylserine (PCPS) were prepared as described previously [26].

Proteins

Plasma-derived human FVII, FIX, FX and human FIXa were from Haematologic Technologies (Essex Junction, VT, U.S.A.). Plasma-derived FXa and FVa were provided generously by Dr Sai Kumar Buddai (Children's Hospital of Philadelphia, Philadelphia, PA, U.S.A.). Recombinant FVIIa was from Novo-Nordisk (Gentofte, Denmark), while human FXIIa, corn trypsin inhibitor and the ELISA kit to detect FVII were from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Soluble human TF (amino acid residues 1–219) and tick anticoagulant peptide (TAP), expressed in *Pichia pastoris*, were generous gifts from Dr Sriram Krishnaswamy (Children's Hospital of Philadelphia). Ca^{2+}-dependent anti-FVII monoclonal antibody was a generous gift from Dr Ulla Hedner of Novo-Nordisk.

The γ-carboxyglutamic acid content of purified protein determined by alkaline hydrolysis performed at Commonwealth Biotechnologies (Richmond, VA, U.S.A.) verified full carboxylation of recombinant proteins.

In vitro expression and purification of FVII recombinant proteins

The mutation A294V was introduced into FVII cDNA cloned into the pCDNA3.1 vector (Invitrogen, San Diego, CA, U.S.A.) by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the instructions of the manufacturer. The presence of the point mutation was confirmed by DNA sequence analysis of the entire FVII cDNA.

The HEK-293 cell line was transfected using the FuGene™ 6 transfection reagent (Roche Diagnostics) following the manufacturer's instructions. Cells were grown in Dulbecco's modified Eagle's medium/F12 in the presence of 10 % fetal bovine serum, 5 μg/ml vitamin K, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Selection was performed as described previously [27]. The highest producers (1.5–2 μg/ml/10^6 cells per 24 h) were selected by ELISA, following the manufacturer's instructions, and expanded further to a cell-factory system using medium containing 10 μg/ml of insulin/transferrin/sodium selenite supplement. Starting after 72 h, conditioned medium was collected at 24 h intervals. Benzamidine (10 mM)

was added after filtering medium through a 0.45 μm cellulose nitrate filter, and the filtrate was frozen at −20 °C.

Frozen medium thawed at 37 °C was diluted with 3 vol. of 20 mM Tris (pH 7.4)/5 mM EDTA/5 mM benzamidine and was loaded on to a Q Sepharose Fast Flow column, equilibrated previously in 20 mM Tris/50 mM NaCl, pH 7.4. Following washing with the same buffer, protein was eluted with 20 mM Tris/750 mM NaCl, pH 7.4. Benzamidine (5 mM) was added to eluted fractions before dialysing them into 20 mM Tris/100 mM NaCl, pH 7.4. Following dialysis, $CaCl_2$ (10 mM) was added and the pH adjusted to 7.4 before immunopurification of the concentrated medium using the Ca^{2+}-dependent monoclonal antibody. Unbound protein was eluted from the column by successive washes with 20 mM Tris/100 mM NaCl/10 mM $CaCl_2$/0.05 % Tween 20, pH 7.4, followed by 20 mM Tris/1 M NaCl/10 mM $CaCl_2$, pH 7.4, and finally 20 mM Tris/100 mM NaCl/10 mM $CaCl_2$, pH 7.4. FVII was eluted with 20 mM Tris/100 mM NaCl/20 mM EDTA, pH 7.4. Fractions were dialysed into 20 mM Hepes/150 mM NaCl, pH 7.4, concentrated by ultrafiltration (Centripep-30; Amicon, Beverly, MA, U.S.A.), and frozen and stored at −80 °C. The FVII concentration was determined using a molecular mass of 50000 Da and a molar absorption coefficient, ϵ, of 69500 $M^{-1} \cdot cm^{-1}$. For the purification of wild-type FVII, 50 mM benzamidine was present in all the buffers to minimize the rate of autoactivation [28].

FVII cleavage

Recombinant FVII proteins (2 μM) were incubated with 400 μM PCPS in reaction buffer at 37 °C with 20 nM human FXa in the absence or presence of 100 nM FVa for 30 and 15 min respectively; 2 μM TAP was used to specifically inhibit FXa. The same experiment was also performed in the presence of 2 μM soluble TF to investigate a possible role of TF in FVII activability. FVII (2 μM) was incubated with 400 μM PCPS in reaction buffer at 37 °C with 40 nM human FIXa for 2 h or with 100 nM soluble TF–FVIIa complex for 4 h. Human FXIIa (200 nM) was incubated for 6 h at 37 °C to activate FVII in 20 mM Hepes/150 mM NaCl/0.1 % (w/v) PEG 8000, pH 7.4. Corn trypsin inhibitor (5 μM) was used to inactivate FXIIa in the control reaction. The formation of double-chain FVII was evaluated on SDS/PAGE after Coomassie Brilliant Blue staining. The rate of activation over 30 min of incubation with FXa was estimated by densitometric analysis on three different gels and the average value is reported.

FVIIa activity towards a peptidyl substrate

FVIIa (10 nM) was incubated in reaction buffer at ambient temperature in the presence or absence of 50 nM soluble TF and 100 μM PCPS. Spectrozyme fVIIa was added at concentrations ranging from 0 to 1 mM immediately before monitoring absorbance at 405 nm, as a function of time, using a V_{max} plate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Due to its reduced activity, K_m values of FVII(a) A294V [where FVII(a) is FVII not fully converted into the two-chain form] for the peptidyl substrate were evaluated using 20 nM FVII(a) in the presence or absence of 100 nM soluble TF.

FVIIa activity towards macromolecules

FX activation

FVIIa (1 nM) was incubated at 37 °C with 10 nM soluble TF, 100 μM PCPS and 100 nM human FX in reaction buffer. Aliquots of the reaction mixture were quenched at several time points in

20 mM Hepes/150 mM NaCl/50 mM EDTA/0.1 % PEG 8000, pH 7.4, and the FXa peptidyl substrate Spectrozyme fXa was added at a final concentration of 100 μM. FXa activity was monitored at 405 nm, for 3.5 min at ambient temperature. The concentration of FXa formed as a function of time was determined by interpolation from the linear dependence of the initial rate of Spectrozyme fXa hydrolysis on known concentrations of FXa, determined separately. The initial steady-state rate of FXa formation was determined from the slope of plots documenting the linear appearance of FXa with time.

FIX activation

FVIIa, at a concentration of 60 nM, was used to activate 5.4 μM FIX in the presence of 400 μM PCPS/300 nM soluble TF/1 mM CaCl$_2$ in 20 mM Hepes/150 mM NaCl/0.1 % (w/v) PEG 8000, pH 7.4. After incubation at 37 °C for 2 h, FIXa formation was determined by SDS/PAGE analysis and visualization of separated proteins was by Coomassie Brilliant Blue staining.

Coagulation assay with recombinant proteins

Prothrombin clotting time was measured using a one-stage clotting assay. Innovin or Simplastin® were used as a source of TF. Recombinant FVII or FVIIa were initially diluted to 10 nM (500 ng/ml) in FVII-deficient plasma. Several dilutions of this stock were made in 10 mM Tris/150 mM NaCl/0.01 % BSA (pH 7.5). An aliquot (50 μl) of each diluted sample was added to 50 μl of FVII-deficient plasma; 200 μl of thromboplastin was added and the clotting time was recorded immediately using a fibrometer. Standard curves with plasma-derived FVII or Novoseven™ (human recombinant FVIIa produced by Novo-Nordisk) were prepared in parallel.

TF dependency of FVIIa amidolytic activity

FVIIa (10 nM) was incubated in reaction buffer in the presence of 100 μM PCPS with various concentrations of soluble TF, ranging from 0 to 50 nM. The activity of the soluble TF–FVIIa complex was measured by adding the peptidyl substrate for FVIIa (400 μM). Due to its reduced amidolytic activity, FVII(a) A294V was used at two different concentrations: at 10 nM in the presence of soluble TF ranging from 0 to 500 nM, and at 40 nM in the presence of soluble TF ranging from 0 to 160 nM.

Data analysis

Steady-state kinetic constants for synthetic substrate hydrolysis were determined from duplicate measurements of the initial velocity obtained using different substrate concentrations. Initial velocity data were fit to the Michaelis–Menten equation [29] by non-linear least-squares regression analysis using the Marquardt algorithm [30].

Soluble TF concentrations that gave 50 % of FVIIa saturation, and therefore 50 % of FVIIa activity towards peptidyl substrate, were calculated by non-linear least squares regression analysis using the Marquardt algorithm [30].

RESULTS

Expression and purification of wild-type and mutant FVII

Although previous plasma and *in vitro* data suggested reduced secretion of FVII A294V, in our stably transfected cells, wild-type and mutant FVII expression levels were comparable (1.5–2 μg/ml/10^6 cells per 24 h).

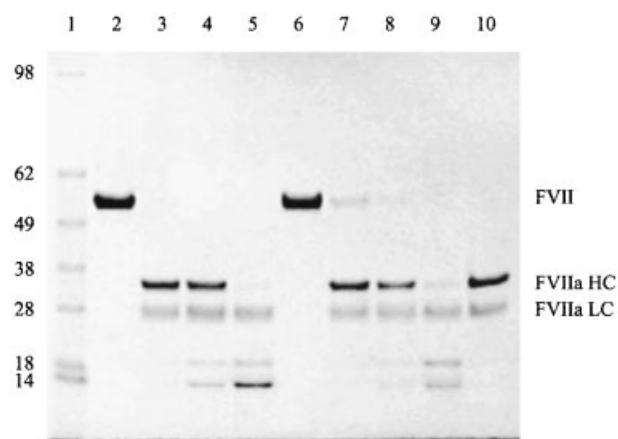Recombinant proteins were purified from conditioned medium

Figure 1   FVII activation by FXa

Recombinant FVII was activated by FXa and separated on reducing SDS/PAGE. FVII (2 μM) was incubated at 37 °C in reaction buffer in the presence of 20 nM FXa and 400 μM PCPS. Aliquots were withdrawn at several time points and the reaction was quenched with 2 μM TAP. HC, FVIIa heavy chain of 30 kDa; LC, FVIIa light chain of 20 kDa. Lane 1, molecular-mass markers (kDa); lanes 2–5, wild-type FVII at 0 min, 30 min, 60 min and 16 h incubation times; lanes 6–9, FVII A294V at 0 min, 30 min, 60 min and 16 h incubation times; lane 10, Novoseven™.

Table 1   Kinetic constants of FVIIa towards the peptidyl substrate Spectrozyme fVIIa

The experiment with wild-type FVIIa was performed in reaction buffer in the presence of 100 μM PCPS using 10 nM wild-type FVIIa with or without 50 nM soluble TF. The experiment with FVII(a) A294V was performed in reaction buffer in the presence of 100 μM PCPS using 20 nM FVII(a) A294V with or without 100 nM soluble TF. N.M., not measurable, even at 100 nM FVII(a) A294V.

	K_m (mM; $\pm$ S.D.)	k_{cat} (s^{-1})	k_{cat}/K_m (M$^{-1}\cdot$s^{-1})
+ Soluble TF			
Wild-type FVIIa	0.67 $\pm$ 0.05	17	2.5 × 10^4
FVII(a) A294V	> 2.5	16	< 6 × 10^3
− Soluble TF			
Wild-type FVIIa	> 3	5	< 1 × 10^3
FVII(a) A294V	N.M.	N.M.	N.M.

and appeared as single-band zymogen forms on SDS/PAGE (Figure 1). The lack of autoactivation during FVII A294V purification carried out in the absence of benzamidine was indicative of the impaired activity of this mutant FVII molecule.

Migration of plasma-derived, wild-type and mutated FVII was indistinguishable, excluding major differences in post-translational modifications.

Proteolytic activation

Proteolytic activation of purified recombinant proteins was tested using human FIXa, FXa, prothrombinase complex, FXIIa and soluble TF–FVIIa complex. The cleavage patterns of wild-type and plasma-derived FVII were indistinguishable (results not shown), whereas FVII A294V was not completely cleaved by either FXa (Figure 1) or any of the above-mentioned enzymes, even in the presence of soluble TF. Only an incubation of 4 h with FXa in the presence of phospholipids led to complete processing to the two-chain form. However, this prolonged incubation also led to protein degradation, as shown by the
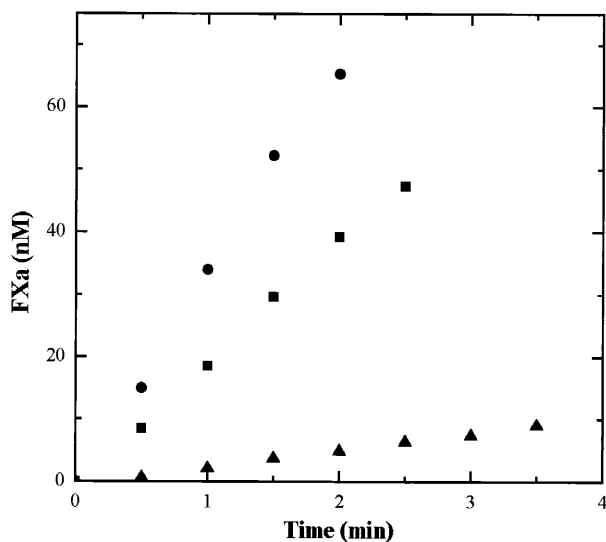
Figure 2   FX activation by soluble TF–FVIIa complex

FXa generation was assayed in reaction buffer using 100 nM FX, 10 nM soluble TF, 100 μM PCPS and 1 nM FVIIa. The FVIIa proteins included wild-type FVIIa (●), Novoseven[TM] (■), FVII(a) A294V (▲). Aliquots of the reaction were quenched at time points between 0 and 3.5 min using 20 mM Hepes/150 mM NaCl/50 mM EDTA/0.1 % PEG 8000, pH 7.4. Spectrozyme fXa (100 μM) was used to determine FXa generation.
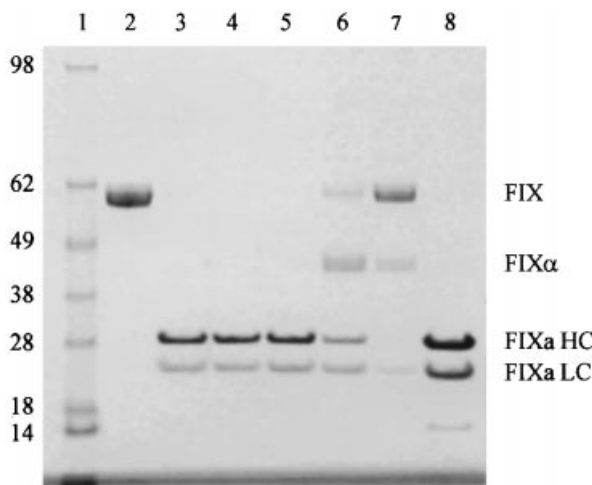

Figure 3   FIX activation by soluble TF–FVIIa complex

Plasma-derived FIX as visualized by reducing SDS/PAGE after activation with soluble TF–FVIIa complex. FIX (5.4 μM) was incubated at 37 °C for 2 h in 20 mM Hepes/150 mM NaCl/1 mM CaCl$_2$/0.1 % PEG 8000, pH 7.4, in the presence of 400 μM PCPS, 60 nM FVIIa and 300 nM soluble TF. The TF–FVIIa complex catalyses cleavage at Arg[145]–Ala[146] and Arg[180]–Val[181] (FIX numbering). Cleavage at the Arg[145]–Ala[146] peptide bond generates FIXα (heavy chain, 39 kDa; light chain, 18 kDa), cleavage between Arg[180] and Val[181] generates FIXaα (heavy chain, 29 kDa; light chain, 28 kDa), whereas cleavage at Arg[145] and Arg[180] gives rise to FIXa (heavy chain, 28 kDa; light chain, 18 kDa). Lane 1, molecular-mass markers (kDa); lane 2, zymogen FIX; lanes 3–7 include FIX after incubation with (lane 3) wild-type FVIIa, (lane 4) plasma-derived FVIIa, (lane 5) Novoseven[TM], (lane 6) FVII(a) A294V and (lane 7) negative control, without FVIIa; lane 8, FIXa.

Figure 4   TF-dependent increase in FVIIa amydolytic activity

Top panel: wild-type FVIIa (10 nM) was incubated with increasing concentrations of soluble TF (0–50 nM), in the presence of 100 μM PCPS in reaction buffer. The activity of the soluble TF–FVIIa complex was measured adding the peptidyl substrate for FVIIa, Spectrozyme fVIIa (400 μM). Bottom panel: due to its reduced activity, 40 nM FVII(a) A294V was incubated with soluble TF (0–160 nM).

appearance of degradation bands at the bottom of the gel after 60 min of activation (Figure 1) [31]. After a 30 min incubation of zymogen FVII wi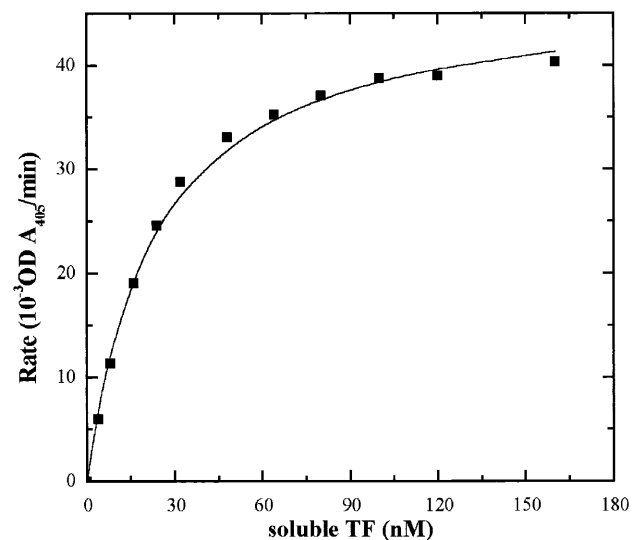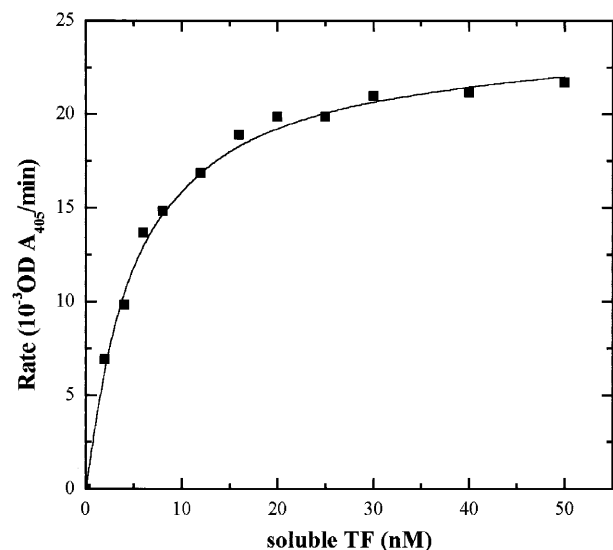th FXa, densitometric analysis of the electro-phoretic pattern showed FVII activation of approx. 80 % for FVII A294V as compared with 100 % for wild-type FVII.

FVIIa activity

Peptidyl substrate cleavage

After a 30 min activation period, wild-type and mutated FVIIa were used in chromogenic assays to test soluble TF–FVIIa complex activity towards a synthetic substrate (Spectrozyme fVIIa). K_m values calculated for wild-type FVIIa and mutated FVII(a) are shown in Table 1. The presence in this experiment of a residual FVII A294V zymogen form cannot be avoided. However, it cannot explain the detected increase in K_m and should not substantially affect the k_{cat} value.

Macromolecular substrate cleavage

FVIIa activity towards macromolecules was tested using FX and FIX as substrates of the soluble TF–FVIIa complex. The initial rates of FXa generation were 33.8 and 19.6 nM FXa/min for wild-type and Novoseven™, respectively, and 2.7 nM FXa/min for FVII(a) A294V (Figure 2). Whereas recombinant wild-type FVIIa showed similar activity to Novoseven™, FVII(a) A294V was characterized by reduced activity towards FIX (Figure 3).

Coagulation assay

Recombinant zymogens were added to FVII-deficient plasma and prothrombin times were determined. The activity of FVII A294V was 10–25 % compared with wild-type FVII. Comparison between activated recombinant proteins in a similar assay indicated that FVII(a) A294V retained 25–28 % of the activity of wild-type FVIIa. Considering the normal protein levels we used for these assays, the specific activity values of the recombinant FVII A294V agree with the FVII coagulant activity found in patients.

TF dependency of FVIIa amidolytic activity

Soluble TF concentrations of 5.0 ± 0.3 and 22.4 ± 1.3 nM for wild-type FVIIa and FVII(a) A294V, respectively, yielded a FVIIa activity of 50 % towards the peptidyl substrate Spectrozyme fVIIa (Figure 4). A similar difference in TF dependency of FVIIa activity was observed using 10 nM wild-type FVIIa or FVII(a) A294V. The presence in this experiment of a residual FVII A294V zymogen form, characterized by a lower affinity for soluble TF compared with FVIIa [32], should not have affected the assay appreciably.

DISCUSSION

Studies on macromolecular interactions of the coagulation complexes prothrombinase and extrinsic Xase have led to the identification of a common mechanism for substrate recognition that takes advantage of an extended area of contact away from the active site [14,15]. These exosites, responsible for substrate affinity and specificity, include residues that play a pivotal role in determining the nature and strength of contacts, and thus the subsequent productive interactions.

In this study we report the characterization of the FVII substitution A294V in the putative FVIIa exosite for FX [16,18, 33]. This modification affects not only the interaction of FVII/FVIIa with substrates but also with activators and the cofactor. The A294V mutation, a frequent cause of asymptomatic FVII deficiency in Caucasians [23], involves a residue conserved in all known FVII molecules, with the notable exception of a serine residue in the zebrafish, but which is extremely variable in other serine proteases [34–36].

Substitution of Ala[294] in FVII impairs the activation of this variant by several known FVII activators. In the crystallographic structure of bovine chymotrypsinogen this residue is close to the cleavage site of the zymogen form [37]. A similar activation defect was demonstrated or suspected for other FVII mutants in loops 140s and 170s (amino acid residues c168–c182), which suggests a possible influence of residues of both loops in the spatial orientation of the cleavage site [38,39]. However, the lack of resolution in the FVII crystallographic structure of amino acids between 143 and 152 of the light chain and at the cleavage site makes it impossible to model this region reliably [7].

The substitution A294V also has a measurable effect on the TF-dependent increase in FVIIa amidolytic activity, very likely by decreasing the affinity of FVII(a) A294V for its cofactor. Although Ala[294] is not located at the canonical interface between TF and FVIIa, the mutation of this surface residue affects TF interaction to the same extent as β-strand B2 mutations, known to directly influence FVIIa affinity for TF [40,41].

The markedly reduced activity of FVII(a) A294V is caused by neither the modestly reduced activability nor alteration of the catalytic triad, as suggested by the normal k_{cat} value calculated for this FVII mutant. Instead, the increased K_m value of FVII(a) A294V for Spectrozyme fVIIa points towards a reduced affinity for substrates. As a matter of fact, loop 140s, together with loops 1 and 2, and the newly-formed N-terminus, belongs to the activation domain responsible for shaping the specificity pocket. Moreover, recent studies have identified some of these loops as regions involved in transmitting the TF-mediated effect from the FVIIa–TF interface to the catalytic domain [42]. Alterations of these regions appeared to destabilize the N-terminal insertion with a concomitant effect on the S1 specificity pocket and substrate recognition [42].

Taken together, these findings and the reduced activity of FVII(a) A294V towards FIX and FX, suggest that the mutation influences the interaction of this FVII variant with all macromolecules relevant for coagulation initiation. Although loop 140s undergoes a three-amino acid shift after FVII activation, the Ala[294] residue would maintain a key role by either recognizing different macromolecules directly or transmitting conformational changes responsible for specific interaction with macromolecules.

The first hypothesis, which supposes the presence of a unique area of interaction of FVII/FVIIa with activators and substrates, may find further support in studies identifying TF regions contacting FX, FIX and FXa [19,20]. The same few TF residues involved in the interaction of the FVII–TF complex with the activator FXa would play a major role also in FX recognition by the FVIIa–TF complex. The evidence that wild-type and mutant TF bind both FVII and FVIIa with comparable affinity, together with the similar K_m values of the FVIIa–TF complex for zymogen FX and inactivated FXa, would further sustain the hypothesis of high structural homology between the ternary complexes FVII–TF–FXa and FVIIa–TF–FX [15,20].

In accordance with the second hypothesis, activators and substrates may interact with distinct regions of FVII and FVIIa, as proposed by Jin et al. [43], and Ala[294] would participate with different roles to various macromolecular interactions.

The A294V mutation determines the synthesis of a dysfunctional FVII with partially impaired interaction with its activators, cofactor and substrates, without affecting the basic serine protease features of the catalytic triad. These findings would explain the asymptomatic defect observed in humans and the associated coagulation phenotype in plasma [21–23]. *In vitro*, a similar change in a spectrum of functions was achieved recently by removing five amino acids in loop 170s, which, however, resulted in a reduced catalytic efficiency [39]. The unique features of FVII A294V make this mutant a powerful tool to shed light on FVII site(s) of interaction with other coagulation proteins and, possibly, an example of natural parsimony in searching for new protein affinities and properties [44,45].

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