Synthesis, Purification, and Characterization of an $Arg_{152} \rightarrow Glu$ Site-Directed Mutant of Recombinant Human Blood Clotting Factor VII†

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ABSTRACT: Coagulation factor VII circulates in blood as a single-chain zymogen of a serine protease and is converted to its activated two-chain form, factor VIIa, by cleavage of an internal peptide bond located at Arg_{152}-Ile_{153}. Previous studies using serine protease active-site inhibitors suggest that zymogen factor VII may possess sufficient proteolytic activity to initiate the extrinsic pathway of blood coagulation. In order to assess the putative intrinsic proteolytic activity of single-chain factor VII, we have constructed a site-specific mutant of recombinant human factor VII in which arginine-152 has been replaced with a glutamic acid residue. Mutant factor VII was purified in a single step from culture supernatants of baby hamster kidney cells transfected with a plasmid containing the sequence for $Arg_{152} \rightarrow Glu$ factor VII using a calcium-dependent, murine anti-factor VII monoclonal antibody column. Purified mutant factor VII was indistinguishable from plasma-derived or recombinant wild-type factor VII by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and migrated as a single band with an apparent molecular weight of 50 000. The average specific activity of several mutant factor VII preparations was 0.00025 unit/μg, or 0.01% of that observed for recombinant wild-type factor VII preparations. The clotting activity of mutant factor VII was, however, completely inhibited following incubation with dansyl-Glu-Gly-Arg chloromethyl ketone, suggesting that the apparent clotting activity of mutant factor VII was due to a contaminating serine protease. Immunoblots of mutant factor VII incubated with human factor IXa revealed no cleavage, whereas incubation of mutant factor VII with human factor Xa resulted in cleavage of mutant factor VII and the formation of a lower molecular weight degradation product migrating at $M_r \approx 40\,000$. Incubation of mutant factor VII with *Staphylococcus aureus* V8 protease resulted in the proteolytic activation of mutant factor VII followed by a progressive decline in activity as a result of proteolytic degradation. By comparison, incubation of recombinant wild-type factor VII with V8 protease resulted in the proteolytic degradation of factor VII and loss of coagulant activity without any apparent transient increase in activity. Our results are consistent with the proposal that zymogen factor VII possesses no intrinsic proteolytic activity toward factor X or factor IX.

Factor VII is a trace vitamin K dependent plasma glycoprotein that circulates in blood as a single-chain zymogen and plays an important role in the initiation of the extrinsic pathway of blood coagulation. Single-chain human factor VII is converted to two-chain factor VIIa by cleavage of an internal peptide bond located in the middle of the molecule at Arg_{152}-Ile_{153} (Hagen et al., 1986). Factor VIIa has no coagulant activity in the absence of a lipoprotein cofactor designated tissue factor (Nemerson & Gentry, 1986). However, in the presence of tissue factor and calcium ions, factor VIIa rapidly activates factor X or factor IX by limited proteolysis (Nemerson & Gentry, 1986).

While the proteolytic activity of the two-chain form of factor VIIa is unequivocal, there is debate over whether single-chain factor VII possesses intrinsic proteolytic activity and, furthermore, the role of this activity in the initiation of the extrinsic pathway of blood coagulation. Proponents of the intrinsic activity hypothesis argue that circulating single-chain factor VII binds to tissue factor at a site of vascular trauma and, in complex with tissue factor, converts the first molecule of factor X to factor Xa. Factor Xa, in turn, reciprocally activates tissue factor bound factor VII, resulting in amplification of factor VII proteolytic activity. Unfortunately, this hypothesis is difficult, if not impossible, to prove or disprove with our present reagents given the fact that (1) preparations of zymogen factor VII are invariably contaminated with trace amounts of two-chain factor VIIa generated during the isolation procedure and (2) the physiological substrates of factor VII, factor IX, and factor X, once activated, reciprocally activate factor VII at an extremely rapid rate. The argument that factor VII possesses intrinsic proteolytic activity is based, in part, on findings that both bovine factor VII (Zur et al., 1982) and human factor VII (Broze & Majerus, 1980), unlike other zymogen clotting factors, react with diisopropyl fluorophosphate (DFP), a potent active-site serine protease inhibitor. These findings would suggest that a factor VII–tissue factor complex is capable of directly triggering the extrinsic pathway of coagulation by slowly activating factors IX and/or X. However, recent studies from Rapaport's laboratory provide convincing evidence that single-chain factor VII is inert, and proteolytic activation of factor VII is an essential step for the expression of factor VII coagulant activity (Rao et al., 1986; Rao & Rapaport, 1988). In these experiments, a 30-s lag phase was observed in the activation of factor X by a complex of single-chain factor VII and tissue factor, but no lag phase occurred in the activation of factor X by a complex of two-chain factor VIIa and tissue factor. The addition of antithrombin III and heparin to factor VII–tissue factor mixtures markedly inhibited activation of factor X. However, incubation of antithrombin III/heparin with factor VIIa–tissue

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factor had no effect on the rate of factor X activation. In more recent studies, Williams et al. (1989) demonstrated that chloromethyl ketones specific for the active site of serine proteases react exclusively with two-chain factor VIIa and not with single-chain factor VII, thus providing strong evidence that factor VII is an inactive zymogen.

In order to investigate further the putative intrinsic proteolytic activity of single-chain human factor VII, we have constructed a site-specific mutant of recombinant human factor VII in which Arg_{152} at the cleavage site has been changed to a glutamic acid, thus effectively preventing cleavage at this site by arginine–specific serine proteases capable of activating factor VII. We report here on the coagulant activity of this mutant factor VII molecule (R152E)[1] and its activatability by a glutamic acid specific endoproteinase from *Staphylococcus aureus*.

Materials and Methods

Bovine serum albumin (fatty acid free), peroxidase-conjugated goat anti-rabbit IgG, methotrexate, and Tris (Trizma base) were products of Sigma, St. Louis, MO. Endoproteinase Glu-C (V8 protease) was obtained from ICN Biomedical, Costa Mesa, CA. Dulbecco's-modified media and fetal calf serum were obtained from Gibco, Santa Clara, CA. Dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone (DEGRck)[2] was purchased from Calbiochem, San Diego, CA. Dulbecco's cysteine-free media were obtained from Hazleton, Lenexa, KS. Vitamin K (Aquamephyton) was purchased from Merck Sharp & Dohme, Rahway, NJ. The SDS low molecular weight (low M_r) standard kit was purchased from Pharmacia, Piscataway, NJ. Protein assay dye reagent concentrate, Coomassie Brilliant Blue R-250, and sodium dodecyl sulfate (SDS) were products of Bio-Rad, Richmond, CA. Nitrocellulose was obtained from Schleicher & Schuell, Keene NH. Microtiter plates (96 well) were obtained from Dynatech Laboratories, Chantilly, VA. ^{125}I-Protein A (70–100 μCi/μg) and [^{35}S]cysteine (600 Ci/mmol) were obtained from New England Nuclear, Boston, MA. All other reagents were the best grade available from commercial sources.

Normal pooled human plasma, assumed to contain 1 unit/mL factor VII activity, was prepared by pooling citrated plasma from 20 healthy donors. Factor VII deficient plasma (<1% factor VII antigen) was obtained from a hereditary factor VII deficient patient. Human brain cephalin (mixed brain phospholipids) was prepared according to Bell and Alton (1954). Phospholipid concentrations were determined according to Chen et al. (1956). Protein concentrations were determined by the Coomassie Blue dye binding assay using bovine serum albumin as the reference protein (Bradford, 1976). Human brain thromboplastin was prepared as described (Nawroth et al., 1986).

Clotting Factor Purification. Recombinant human factors VII and VIIa, produced in BHK cells (Berkner et al., 1986; Thim et al., 1988), were generously provided by Dr. Torben-Lund Hansen, Novo-Nordisk, Copenhagen. Human factor VII, factor IX, and factor X were partially purified from therapeutic plasmapheresis plasma by a combination of barium citrate adsorption and elution and DEAE-Sepharose CL-6B chromatography as previously described (Miletich et al., 1981). Factor VII obtained from DEAE-Sepharose chromatography was purified to homogeneity by immunoaffinity chromatography and FPLC[2] chromatography as described (Wildgoose & Kisiel, 1989). The factor X pool obtained from DEAE-Sepharose chromatography was purified to homogeneity by a combination of dextran sulfate–agarose (DSA) column chromatography and Sephadex G-150 column chromatography as previously described (Kondo & Kisiel, 1987). Factor X was activated by incubation with insolubilized RVV-X (Stern et al., 1984) and separated from any residual factor X by Sephadex G-100 column chromatography (Wildgoose & Kisiel, 1989). The final product was homogeneous as assessed by SDS–PAGE and was essentially all factor X$a\beta$.

The partially purified factor IX pool obtained from DEAE-Sepharose chromatography was further purified by DSA column chromatography and immunoaffinity chromatography (Kondo & Kisiel, 1987). Human IX$a\beta$ was prepared by incubating factor IX with insolubilized RVV-X followed by gel filtration on Sephacryl S-200 as described for the preparation of bovine factors IX$a\alpha$ and IX$a\beta$ by Byrne and Castellino (1984). The factor IX$a\beta$ preparation migrated as a single band (M_r 45 000) in the absence of reducing agents, and under reducing conditions migrated as two bands exhibiting apparent molecular weights of 28 000 and 17 000. Bovine factor VII that had been purified to homogeneity as previously described (Takeya et al., 1988) was generously provided by Sadaaki Iwanaga, Kyushu University, Fukuoka, Japan.

Preparation of Antibodies. Polyclonal antibodies against human factor VII and bovine factor VII were produced in rabbits following immunization with homogeneous preparations of antigen. Antibodies were purified from crude antiserum by affinity chromatography either on an antigen–AffiGel 15 column (for antibody to human factor VII) or on a protein A–Sepharose column (for antibody to bovine factor VII). Murine anti-factor VII monoclonal antibody (AD-4) was produced in Balb/c mice essentially according to Kohler and Milstein (1975) and purified from ascites fluid by a combination of DEAE-Affi-Gel Blue column chromatography (Bruck et al., 1982) and Sephadex G-150 column chromatography. A calcium-dependent, murine anti-factor VII monoclonal antibody, coupled to Sepharose 4B, was generously provided by Dr. Torben-Lund Hansen, Novo-Nordisk, Copenhagen. The preparation of this monoclonal antibody has been described elsewhere (Thim et al., 1988). Rabbit antiserum against a novel, BHK cell-derived protease designated as PABI[2] (Tsuji & Kurachi, 1989) was a gift from Dr. Kotoku Kurachi, University of Michigan, Ann Arbor. Anti-PABI IgG was purified by protein A–Sepharose chromatography.

ELISA of Factor VII. Factor VII protein concentrations were determined by an ELISA sandwich assay using a slight modification of the procedure developed by Engvall (1980). In our assays, anti-factor VII monoclonal antibody (AD-4) was employed to catch the factor VII, and affinity-purified rabbit anti-factor VII and peroxidase-conjugated goat anti-rabbit IgG were used to quantitate it. Protein concentrations were determined from various dilutions of normal pooled plasma assuming a plasma concentration of 400 ng of factor VII/mL (Fair, 1983).

Mutagenesis of Factor VII. To generate the $Arg_{152} \rightarrow$ Glu factor VII activation cleavage site mutant, a pUC119 vector (Vieira & Messing, 1987) was first modified by using oligo-

[1] Mutant factor VII is designated according to the notation described by Shapiro and Vallee (1989) 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.

[2] Abbreviations: DSA, dextran sulfate–agarose; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; kDa, kilodalton(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS/BSA, Tris-buffered saline/0.1% bovine serum albumin; DEGRck, dansyl-Glu-Gly-Arg chloromethyl ketone; PABI, protease accumulated by inhibitors.

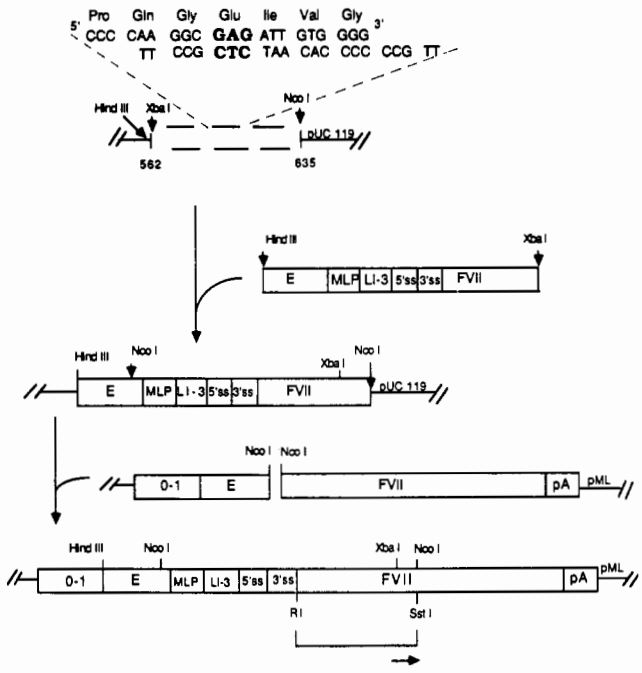

FIGURE 1: Construction of the $Arg_{152} \rightarrow$ Glu factor VII expression plasmid. The Arg to Glu mutation introduced into factor VII is indicated by the bold type within the sequence shown for two of the six oligonucleotides used to synthesize the *Xba*I–*Nco*I factor VII cDNA fragment. The numbering for the cDNA is for the 38 amino acid leader form of factor VII (Hagen et al., 1986). The abbreviations for the expression vector are as follows: 0-1 represents the left 350 bp of adenovirus 5, E the SV40 enhancer sequences, MLP and L1-3 the major late promoter and tripartite leader, respectively, of adenovirus 2, 5′ss and 3′ss asplice signal cassette, and pA the SV40 early polyadenylation signal. The 0.7-kb RI–*Sst*I fragment subcloned into M13mp19 is indicated by a bracket at the bottom of the figure. The arrow represents the actual region of factor VII that was sequenced.

nucleotide adaptors to insert an *Nco*I cleavage site between the *Xba*I and *Bam*HI sites in the polylinker sequences. This manipulation and subsequent steps described below were performed according to standard protocols (Maniatis et al., 1982). Oligonucleotides comprising the factor VII cDNA sequences between the *Xba*I site at 562 base pairs (bp) and the *Nco*I site at 635 bp were then ligated into this pUC119-derived vector (Figure 1). The oligonucleotides generated a sequence identical with that of native factor VII except for a glutamic acid for arginine at amino acid 152. The factor VII/pUC 119 plasmid was cleaved with *Hin*dIII and *Xba*I, and a 1.6-kb fragment containing part of the mammalian expression vector pDX (Foster et al., 1987) and the 5′-terminal portion of the factor VII cDNA was then inserted into this DNA. The product of this ligation was subsequently used to isolate a 1.4-kb *Nco*I fragment containing the mutated factor VII sequences and part of the expression vector. This *Nco*I fragment was ligated to a 5.2-kb *Nco*I fragment containing the remainder of the factor VII cDNA and mammalian expression vector. The DNA product, FVII(E152)/pDX, was checked for correct insert orientation by using restriction enzyme analysis. To confirm that this plasmid contained the arginine to glutamic acid substitution at amino acid 152, a 0.7-kb *Eco*RI–*Sst*I fragment was isolated from FVII(E152)/pDX DNA and subcloned into M13mp19 DNA (Messing et al., 1977). The entire sequence between the *Xba*I site and *Nco*I site of factor VII (i.e., 562–635 bp) was determined by dideoxy sequencing (Sanger et al., 1977). The expected mutation corresponding to amino acid 152 was confirmed, and no other sequences were found to differ from that of native factor VII.

Expression of R152E Factor VII. The FVII(E152)/pDX DNA (10 μg) was cotransfected into BHK cells with a plasmid (1 μg) encoding a mouse dihydrofolate reductase cDNA in pD5 (Berkner & Sharp, 1985). The BHK cells were maintained for 2 days in Dulbecco's-modified medium containing 10% fetal calf serum and penicillin–streptomycin, and were split at a 1:10 dilution into the same medium supplemented with 150 mM methotrexate. After 5 days, the cells were fluid changed, and after an additional 5 days, colonies were clearly visible. A dozen individual colonies were isolated and clonally expanded. To confirm that these BHK cell lines expressed only the R152E factor VII, genomic DNA was isolated from several of the cell lines and subjected to Southern analysis using an oligonucleotide that distinguished the mutated sequences from native factor VII DNA (data not shown). Only the mutated factor VII sequences were observed. Individual colonies were screened for levels of production and for barium citrate precipitatability. The BHK clones expressed factor VII at levels between 1 and 5 μg mL^{-1} day^{-1}, as measured by ELISA. The factor VII expressing BHK lines, as well as B4A1, a BHK line expressing native recombinant factor VII, were labeled in vivo with [^{35}S]cysteine in modified Dulbecco's cysteine-free medium containing 1% sodium pyruvate, 1% dialyzed fetal calf serum, 1% glutamine, and 5 μg/mL vitamin K. Secreted material was harvested after 6 h and subjected to barium citrate precipitation (Malhotra, 1979) followed by immunoprecipitation using an anti-factor VII monoclonal antibody. R152E factor VII quantitatively bound to barium citrate. In control experiments where vitamin K was omitted from the cell culture media, none of the factor VII was absorbed by barium citrate. When the immunoprecipitated samples were analyzed by SDS–PAGE under reducing conditions, the molecular weight of the R152E factor VII was indistinguishable from native, wild-type factor VII.

Purification of R152E Factor VII. Approximately 6.0×10^8 cells from a representative factor VII producing cell line were cultured in Dulbecco's modified medium containing 1% dialyzed fetal calf serum, penicillin–streptomycin, and 0.5 μg/mL vitamin K. The cells were cultured for up to 3 weeks and the culture supernatants harvested at approximately 5-day intervals. The culture supernatants (≈750 mL) were centrifuged to remove potential cell debris and dialyzed overnight at 4 °C against 50 L of 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. The retentate was made 10 mM in $CaCl_2$ and allowed to stand at 4 °C for 30 min. The calcified solution was centrifuged for 15 min at 30000*g* and the small, button pellet discarded. The supernatant was applied to the calcium-dependent anti-factor VII monoclonal antibody–Sepharose column equilibrated with 10 mM Tris-HCl (pH 8.0)/150 mM NaCl/10 mM $CaCl_2$ at a flow rate of 0.4–0.6 mL/min. Following sample application, the column was washed with 1.0 M NaCl/10 mM Tris-HCl (pH 8.0)/10 mM $CaCl_2$ to elute protein nonspecifically bound to the column. R152E factor VII was then eluted from the column with 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 40 mM EDTA. The EDTA-eluted factor VII fractions were pooled on the basis of factor VII antigen concentration as determined by ELISA. The pooled material was concentrated by ultrafiltration (Amicon YM-10 membrane) and dialyzed against either 0.05 M Tris-HCl/0.1 M NaCl (pH 7.5) or 0.1 M NH_4HCO_3 (pH 8.0).

Incubation of Factor VII with S. aureus V8 Protease. The effect of *S. aureus* V8 protease on the clotting activity of recombinant R152E and wild-type factor VII was measured by monitoring the change in clotting time of hereditary factor

VII deficient plasma produced by temporal aliquots of each incubation mixture. Reactions were performed in 250-μL snap-cap polypropylene tubes at 37 °C. Incubation mixtures consisted of either R152E factor VII (1 μM) or wild-type factor VII (1 μM) and 0.83 μM V8 protease in a total volume of 200 μL of 0.1 M NH_4HCO_3 (pH 8.0). The reaction was initiated by the addition of V8 protease, and at selected intervals, 10-μL aliquots were removed from the incubation mixture and diluted (25–100-fold) in 0.05 M Tris-HCl (pH 7.5)/0.1 M NaCl/0.1% bovine serum albumin (TBS/BSA). An aliquot (100 μL) of the diluted sample was then transferred to a 12 × 75 mm borosilicate glass tube followed by 100 μL of factor VII deficient plasma, 100 μL of human brain thromboplastin, and 100 μL of 25 mM $CaCl_2$. Clotting times were converted to units of factor VII activity from a standard curve constructed with 1:5 to 1:200 dilutions of normal pooled human plasma. The effect of V8 protease on the factor VII samples was also monitored by SDS–PAGE and immunoblotting (Burnette, 1981). As in the clotting assay, 10-μL aliquots were removed at selected times and subjected to SDS–PAGE following reduction with 10% β-mercaptoethanol. Following electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes and factor VII/VIIa and degradation products visualized by incubation with rabbit affinity-purified anti-factor VII IgG followed by incubation with ^{125}I-protein A and autoradiography.

Incubation of Factor VII with Human Factors Xa and IXa. Incubation of R152E factor VII and wild-type factor VII with human factors Xa and IXa was carried out essentially as described for the incubation of factor VII with V8 protease. Incubation mixtures consisted of 1.0 μM recombinant factor VII or R152E factor VII along with mixed brain phospholipids (0.5 mM final phospholipid concentration), 5 mM $CaCl_2$, and either factor IXa (21 nM) or factor Xa (20 nM) in a total volume of 200 μL of TBS/BSA. At selected intervals, 10-μL aliquots were removed for analysis of clotting activity and SDS–PAGE as described previously.

Incubation of Factor VII Preparations with Dansyl-Glu-Gly-Arg Chloromethyl Ketone. R152E factor VII (1 μM), wild-type factor VII (1 μM), and recombinant wild-type factor VIIa (1 μM) were incubated separately with 0.7 mM dansyl-Glu-Gly-Arg chloromethyl ketone at 37 °C for 1 h in a total volume of 200 μL of 0.05 M Tris-HCl (pH 7.5)/0.1 M NaCl/0.5% BSA. Following incubation, the samples were dialyzed overnight at 4 °C versus 2 L of 0.05 M Tris-HCl (pH 7.5)/0.1 M NaCl.

Inhibition of Factor VII Clotting Activity by R152E Factor VII. The effect of R152E factor VII on the clotting activity of wild-type factor VII was assessed in a one-stage clotting assay. In these experiments, various molar ratios of R152E factor VII and wild-type factor VII (0–2) were established in 100 μL of TBS/BSA in a 10 × 75 mm borosilicate glass tube. To each mixture was added in succession 100 μL of factor VII deficient plasma, 100 μL of thromboplastin, and 100 μL of 25 mM $CaCl_2$. The "apparent" factor VII activity in each mixture was corrected for the theoretical decrease in factor VII activity expected as a result of the reduction in wild-type factor VII concentration. Thus, residual factor VII activity was defined as the apparent activity of the combined R152E factor VII/wild-type factor VII mixture divided by the activity of an equal fraction containing only wild-type factor VII at that particular concentration present in the mixture.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed according to the method of Laemmli using a 2.5% polyacrylamide concentrating gel and a 10% polyacrylamide resolving gel. Gels were stained for 30 min in 0.05% Coomassie Blue R-250/50% methanol/10% acetic acid and diffusion destained in 10% methanol/10% acetic acid.

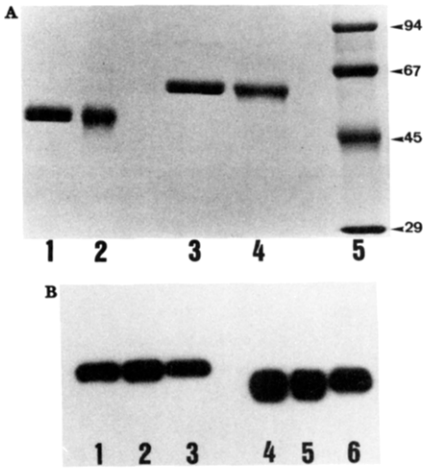

FIGURE 2: SDS–PAGE and immunoblots of purified R152E factor VII. (A) SDS–PAGE of R152E factor VII and wild-type plasma-derived factor VII. Lane 1, nonreduced plasma-derived factor VII; lane 2, nonreduced R152E factor VII; lane 3, reduced plasma-derived factor VII; lane 4, reduced R152E factor VII; lane 5, molecular weight standards (phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 45 000; carbonic anhydrase, 29 000). (B) Immunoblot analysis of R152E and wild-type factor VII. Lane 1, reduced R152E factor VII; lane 2, reduced recombinant wild-type factor VII; lane 3, reduced plasma-derived factor VII; lane 4, unreduced R152E factor VII; lane 5, unreduced recombinant wild-type factor VII; lane 6, unreduced plasma derived wild-type factor VII.

RESULTS

R152E Factor VII Purification. R152E factor VII was purified to homogeneity from the culture supernatants of BHK cells stably transfected with a plasmid containing the $Arg_{152} \rightarrow$ Glu factor VII sequence using a calcium-dependent anti-factor VII monoclonal antibody column. The purified R152E factor VII migrated as a single band in SDS gels stained with Coomassie Blue (Figure 2A). As can be seen in the SDS gels and immunoblots (Figure 2B), the molecular weight of R152E factor VII was indistinguishable from either recombinant wild-type factor VII or plasma-derived human factor VII. In a one-stage clotting assay, several preparations of purified R152E factor VII exhibited an average specific coagulant activity of 0.00025 unit/μg (range 0.0001–0.0004 unit/μg), or ≈0.01% of that observed for recombinant wild-type factor VII preparations (1.5 units/μg).

Effect of Dansyl-Glu-Gly-Arg Chloromethyl Ketone on R152E, Wild-Type, and Plasma-Derived Factor VII Activities. The very low, but nonetheless significant, clotting activity found in R152E factor VII preparations suggested that single-chain factor VII possessed intrinsic proteolytic activity. While our R152E factor VII preparation appeared homogeneous by SDS–PAGE and immunoblotting, these techniques have inherent limitations of sensitivity that may not allow detection of contaminating proteases originating either from the BHK cells or from the serum-containing media. These proteases conceivably could either contribute to or produce the decrease in clotting time in the factor VII assay due to the relatively high concentrations of R152E factor VII used in the assay system. Accordingly, as single-chain factor VII does not interact with the serine protease inhibitor DEGRck (Williams et al., 1989), we incubated our R152E factor VII preparations with a 700-fold molar excess of DEGRck and subsequently assessed its putative clotting activity following

Table I: Effect of Incubating Factor VII with Dansylglutamylglycylarginine Chloromethyl Ketone (DEGRck)

protein	specific activity (units/μg) −DEGRck	+DEGRck	% decrease
plasma FVII	2.5	0.45	82
wild-type FVII	1.5	0.35	77
wild-type FVIIa	≈50	ND[a]	100
R152E FVII	0.0004	ND	100

[a] ND, not detectable.

extensive dialysis to remove the inhibitor. Following incubation with the inhibitor, R152E factor VII preparations exhibited no detectable clotting activity, suggesting, but not proving, that the R152E factor VII preparations were contaminated with a serine protease(s) (Table I). Interestingly, treatment of both plasma-derived and recombinant wild-type single-chain factor VII preparations with DEGRck, under identical conditions, resulted in a loss of 78–82% clotting activity to a final specific activity of 0.35–0.4 unit/μg (Table I). Following incubation with the inhibitor, the residual factor VII activity was, however, still activatable 35–40-fold by factor Xa in the presence of phospholipid and calcium (data not shown). Consistent with previous findings (Williams et al., 1989), incubation of recombinant wild-type factor VIIa with DEGRck resulted in a complete loss of factor VII clotting activity (Table I). We interpret these results as a clear demonstration of the presence of factor VIIa in our plasma-derived and recombinant wild-type single-chain factor VII preparations that is undetectable by SDS–PAGE and/or immunoblotting. Assuming a specific activity of 50 units/μg for factor VIIa, we calculated that as little as 3% contaminating factor VIIa in our single-chain factor VII preparations could produce the apparent specific activity that is inhibitable 80% by DEGRck. This level of contaminating factor VIIa would not be detectable by SDS–PAGE and immunoblotting given the facts that the heavy and light chains do not stain as well as the zymogen factor VII with Coomassie blue, and the heavy and light chains of factor VIIa do not react well with the affinity-purified anti-factor VII IgG in the reduced state in comparison to the unreduced form.

In an effort to identify the putative serine protease, or proteases, that produced the trace procoagulant activity in our R152E factor VII preparations, we initially speculated that bovine factor VIIa, derived from the serum-containing media, may have copurified with R152E factor VII on our calcium-dependent, anti-human factor VII monoclonal antibody column. This was found not to be the case as prolonged incubation of R152E factor VII preparations with high concentrations of rabbit anti-bovine factor VII IgG had no measurable effect on the apparent R152E factor VII clotting activity. Likewise, incubation of R152E factor VII with rabbit anti-PABI IgG failed to neutralize the procoagulant activity of R152E factor VII preparations. Thus, while the identity of the protease remains unknown at this point, it is highly unlikely that our R152E factor VII preparations are contaminated with either bovine factor VIIa or the trypsin-like, BHK cell derived PABI.

Proteolysis of R152E and Wild-Type Factor VII by V8 Protease. Several activated clotting factors including factor Xa (Radcliffe & Nemerson, 1975; Broze & Majerus, 1980), factor IXa (Seligsohn et al., 1979), factor XIIa (Kisiel et al., 1977; Radcliffe et al., 1977), and thrombin (Radcliffe & Nemerson, 1975; Broze & Majerus, 1980) proteolytically activate wild-type factor VII by cleavage of the Arg_{152}–Ile_{153} peptide bond. It was anticipated that a similar limited proteolysis might occur at this site upon exposure of R152E factor VII to the glutamic acid specific endoproteinase V8 protease. The effects of incubating wild-type and R152E factor VII with V8 protease were monitored by SDS–PAGE/immunoblotting, as well as clotting assays (Figure 3A,B). As shown in Figure 3A, V8 protease cleaves R152E factor VII at several sites, yielding at least four cleavage products visible by immunoblots. Coincident with this cleavage is an increase in the clotting activity of the R152E factor VII. As demonstrated in Figure 3B, the starting material possessed an activity of 0.0004 unit/μg which steadily increased within 60 min of incubation to 0.065 unit/μg with V8 protease at an enzyme:substrate weight ratio of 1:5. With prolonged incubation (>60 min), R152E factor VII/VIIa was degraded by the V8 protease, resulting in a progressive decline in clotting activity. Degradation of R152E factor VII was also apparent in the immunoblots of this incubation mixture as a temporal increase in the number and intensity of low molecular weight R152E factor VII degradation fragments. Preparations of R152E factor VII, previously treated with DEGRck, were also activatable by V8 protease. In these experiments, the specific activity of DEGRck-treated R152E factor VII increased from 0 to as high as 0.15 unit/μg. In contrast to the activation of R152E factor VII by V8 protease, incubation of single-chain wild-type factor VII with V8 protease resulted in a loss of clotting activity without any apparent activation (Figure 4A,B). At an enzyme:substrate ratio identical with that used for the proteolysis of R152E factor VII by V8 protease, wild-type factor VII specific activity declined 80% during a 60-min incubation period with V8 protease. As shown in Figure 4B, recombinant factor VII was cleaved to low molecular weight degradation fragments, many of which are only faintly recognized by the polyclonal affinity-purified anti-factor VII IgG used to detect factor VII antigen. The results of the wild-type factor VII–V8 protease incubation strongly suggest that the fold-increase in R152E factor VII activity observed during its incubation with V8 protease was anomalously low due to competing activation and inactivation cleavages in the

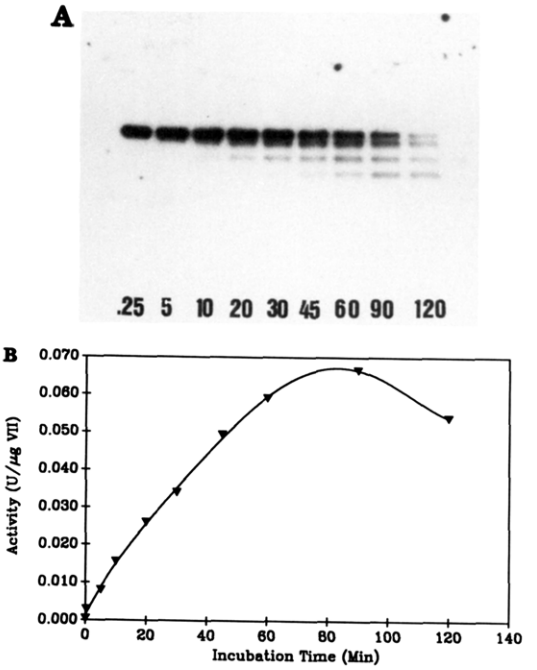

FIGURE 3: Incubation of R152E factor VII with V8 protease. (A) Immunoblots of the incubation of R152E factor VII with V8 protease. The number beneath each lane represents time of incubation in minutes. (B) Time course of R152E factor VII activity following incubation with V8 protease.

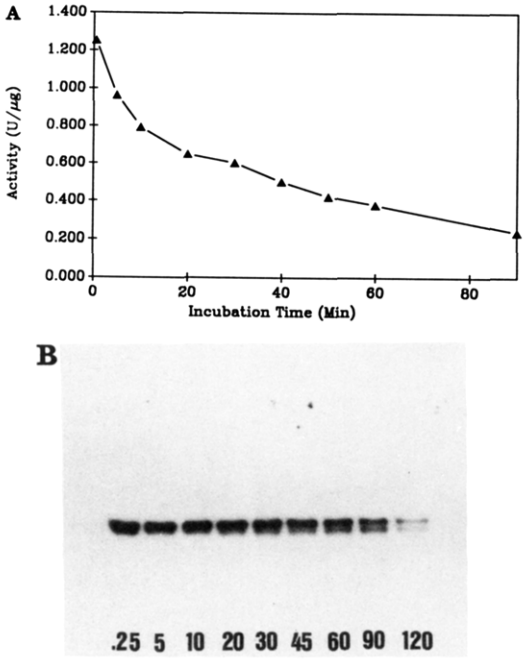

FIGURE 4: Incubation of recombinant wild-type factor VII with V8 protease. (A) Time course of recombinant factor VII activity, following incubation with V8 protease. (B) Immunoblots of temporal aliquots of the incubation mixture depicted in (A). The number beneath each lane represents time of incubation in minutes.

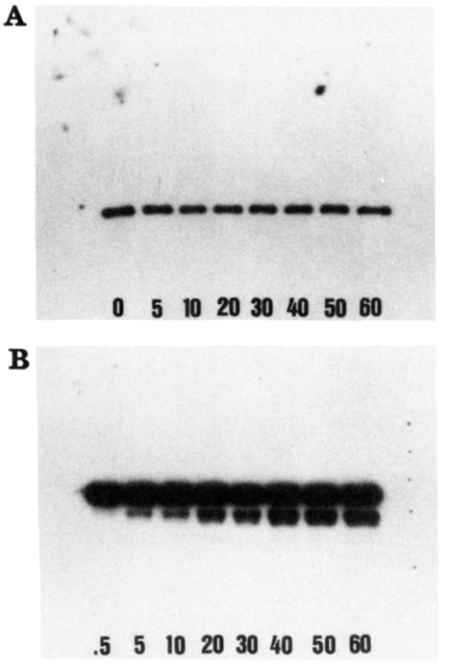

FIGURE 5: Immunoblots of the incubation of R152E factor VII with factor IXa and factor Xa. (A) Immunoblot analysis of the incubation of R152E factor VII with factor IXa. (B) Immunoblot analysis of the incubation of R152E factor VII with factor Xa. The number beneath each lane represents the time of incubation in minutes.

R152E factor VII molecule. Assuming that R152E factor VIIa possesses the same coagulant activity as wild-type factor VIIa (≈50 units/μg), one could estimate that, in the absence of inactivation cleavages, the coagulant activity of R152E factor VII would increase from 0.00025 unit/μg (average activity) to ≈50 units/μg, or roughly a 200000-fold increase. This increase in proteolytic activity is similar to other zymogen–protease activations in which rate enhancements of 10^5–10^6 were estimated through the reactivity of the zymogen and protease with DFP (Morgan et al., 1972).

Incubation of R152E and Recombinant Factor VII with Factor IXa and Factor Xa. In order to determine the effect of physiological factor VII activators on our R152E factor VII preparations, we incubated R152E factor VII with either factor Xa or factor IXa. The results of these reactions were monitored by clotting assays and immunoblotting (Figure 5). No change in the specific clotting activity (data not shown) or covalent structure (Figure 5A) of R152E factor VII was observed when it was incubated with factor IXa. However, immunoblots of the factor Xa–R152E factor VII incubation mixtures revealed that R152E factor VII was cleaved, in a time-dependent manner, to a slightly lower molecular weight form migrating at around 40K (Figure 5B). Unfortunately, measurement of clotting activity in the factor VII–factor Xa incubation mixtures could not be carried out due to interference of factor Xa in the clotting assay. The precise location of the peptide bond(s) in R152E factor VII cleaved by factor Xa is not known at this time and is currently under investigation in our laboratory. Consistent with previous results (Wildgoose & Kisiel, 1989), the incubation of recombinant factor VII with either factor IXa or factor Xa resulted in a rapid increase in specific activity paralleling the appearance of two-chain factor VIIa (data not shown).

Inhibition of Wild-Type Factor VII Clotting Activity by R152E Factor VII. In order to further demonstrate that the molecular properties of R152E factor VII and wild-type factor VII were identical, we assessed the ability of mutant factor VII to compete with wild-type factor VII for tissue factor and inhibit its clotting activity. Inasmuch as R152E factor VII possesses no clotting activity, one would expect that a 1:1 molar ratio of R152E/wild-type factor VII would exhibit a clotting activity 50% of that observed in an equivalent sample containing only wild-type factor VII. Figure 6 illustrates that R152E inhibits wild-type factor VII clotting activity in the manner expected, consistent with the notion that R152E factor VII, with the exception of the activation cleavage site, is structurally identical with wild-type factor VII.

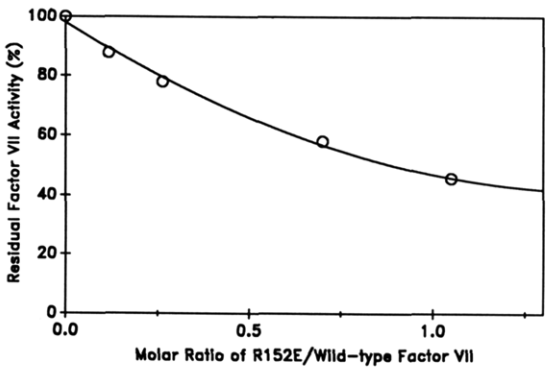

FIGURE 6: Inhibition of wild-type factor VII clotting activity by R152E factor VII. Aliquots containing various molar ratios of R152E and wild-type factor VII were assayed for clotting activity as described under Materials and Methods. The residual factor VII activity was defined as the activity of the fraction containing both wild-type and mutant factor VII divided by the activity of a fraction containing only wild-type factor VII.

Discussion

There has been considerable debate over whether single-chain factor VII possesses sufficient intrinsic proteolytic activity to initiate the extrinsic pathway of blood coagulation in the presence of tissue factor. Unfortunately, this problem is exceedingly difficult to study since the physiological substrates of factor VII, factor IX, and factor X, once activated, rapidly activate factor VII. To avoid these problems, we have

constructed, by site-specific mutagenesis, a mutant factor VII in which Arg_{152} was changed to a glutamic acid residue, thus effectively rendering the molecule incapable of cleavage by arginine-specific serine proteases. The sequence of the plasmid encoding for the R152E factor VII was confirmed by dideoxy sequencing prior to its expression in BHK cells. In order to confirm that the transfected BHK cell line expressed only mutated factor VII, genomic DNA was isolated from transfected BHK cells and subjected to Southern analysis using an oligonucleotide probe specific for the mutated sequence. As expected, only $Arg_{152} \rightarrow$ Glu factor VII was observed. The R152E factor VII was subsequently purified from the cell supernatants of the transfected BHK cells by immunoaffinity chromatography using a calcium-dependent anti-factor VII monoclonal antibody column. In order to determine the intrinsic activity of factor VII, we tested the activity of our R152E factor VII preparations in a coagulant assay using factor VII deficient plasma. We found that our $Arg_{152} \rightarrow$ Glu mutant factor VII possessed a specific clotting activity <0.01% of that observed for recombinant wild-type factor VII. This clotting activity was, however, completely inhibited following incubation with the serine protease inhibitor dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck). Inhibition of the R152E factor VII clotting activity strongly suggests that a contaminant serine protease, derived either from the BHK cells or from the serum-containing media, produced the decrease in clotting time in the factor VII assay. It is perhaps important to mention at this point that in order to assess the very weak clotting activity of R152E factor VII it was necessary to employ fairly high concentrations of the protein in the assay. It is not unreasonable to assume that at these high concentrations of R152E factor VII, contaminating serine proteases are much more likely to exert an effect in the clotting assay. While our data strongly suggest that a contaminant serine protease (BHK or serum-derived protease) is responsible for the apparent R152E factor VII activity, they did not rule out the possibility that the glutamic acid residue at position 152 increases the susceptibility of the active-site histidine residue (His-193) to become alkylated by DEGRck. This was found not to be the case inasmuch as DEGRck-treated R152E factor VII was readily activatable by digestion with *Staphylococcus aureus* V8 protease.

As the proteolytic activity of factor VII/VIIa toward its protein substrates is virtually nonexistent in the absence of tissue factor, we considered the possibility that the lack of clotting activity in our R152E factor VII preparation was due to its inability to properly interact with its cofactor, tissue factor. Recent studies have shown that a single-chain mutant tissue-type plasminogen activator ($Arg_{275} \rightarrow$ Gly) readily activates plasminogen but requires higher levels of fibrin than wild-type two-chain tissue plasminogen activator to achieve maximal activity (Boose et al., 1989). Conceivably, R152E factor VII either does not interact with tissue factor or requires higher concentrations of this cofactor for the expression of its proteolytic activity in the single-chain form. To investigate this possibility, we determined the effect of R152E factor VII on the clotting activity of wild-type factor VII. We hypothesized that if the two proteins react similarly with tissue factor, then it should be possible to demonstrate that R152E factor VII inhibits the clotting activity of wild-type factor VII. The results of these experiments indicated that a 1:1 molar ratio of wild-type/mutant factor VII possessed 50% of the clotting activity of an equivalent sample containing only wild-type factor VII. These findings strongly suggest that wild-type and R152E factor VII interact with tissue factor in an identical manner. To further demonstrate that the affinity of R152E factor VII for tissue factor was identical with wild-type factor VII, we performed preliminary experiments to assess the ability of R152E factor VII, relative to wild-type factor VII, to compete with ^{125}I-factor VII (wild-type) for cell-surface tissue factor provided by the human bladder carcinoma cell line J82 (Sakai et al., 1989). The results of these limited studies clearly indicate that unlabeled R152E factor VII is as effective an inhibitor of ^{125}I-factor VII–J82 cell binding as unlabeled wild-type factor VII (T. Sakai, unpublished results). Thus, R152E factor VII appears to interact with tissue factor in a manner indistinguishable from that observed with wild-type factor VII and greatly reduces the possibility that the absence of clotting activity in our R152E factor VII preparation was due to its inability to interact with tissue factor.

In order to determine whether R152E factor VII could be cleaved to a fully activated form, we used an approach employed previously by Tate and co-workers (Tate et al., 1987) to activate mutant $Arg_{275} \rightarrow$ Glu t-PA. Specifically, we incubated R152E factor VII with the glutamic acid specific endoproteinase from *Staphylococcus aureus*, V8 protease. Incubation of R152E factor VII with V8 protease resulted in an increase in specific clotting activity of at least 180-fold. Unlike the activation of $Arg_{275} \rightarrow$ Glu t-PA by V8 protease (Tate et al., 1987), the specific coagulant activity of the V8 protease activated R152E factor VII was only a fraction of that normally assigned to activated, wild-type factor VIIa. The reason for this incomplete activation was evident from the immunoblots of the incubation mixture which revealed extensive digestion of R152E factor VII. From these immunoblots, it was apparent that V8 protease cleavage is not restricted to a single site, and suggests that there are competing activation and degradation reactions occurring. This conclusion was supported by our observation that wild-type factor VII was cleaved at several sites resulting exclusively in the loss of coagulant activity. Thus, it is very likely that in the R152E factor VII–V8 protease mixtures only a minor portion of the R152E factor VII actually exists in the two-chain, activated form. Whatever the reason may be, it is, however, important to note that the "activated" mutant does possess coagulant activity and that the apparent 180-fold increase in clotting activity associated with V8 protease cleavage of the C-terminal side of Glu_{152} is far greater than the ≈25–50-fold increase in clotting activity that is typically observed in the conversion of wild-type factor VII to VIIa. Assuming that factor VIIa has a specific activity of 50 units/μg, and single-chain factor VII has no activity, the fold-activatability of factor VII would be an infinite number. The fact that factor VII is activatable only 25–50-fold in the test tube probably reflects two independent considerations. First, factor VII preparations are prone to be contaminated with factor VIIa, which would reduce its apparent fold-activatability. Second, the base-line value for factor VII is difficult to ascertain in the conventional clotting assay. The mere fact that single-chain factor VII has any apparent activity in the clotting assay argues for the incipient activation of factor VII during the assay. Accordingly, it is highly likely that factor VII is activated several orders of magnitude similar to the rate enhancements observed for other zymogen–protease conversions (Morgan et al., 1974).

In conclusion, our findings with the R152E factor VII molecule support the contention that zymogen factor VII possesses no intrinsic proteolytic activity and that other mechanisms are involved in the conversion of factor VII to factor VIIa and the initiation of the extrinsic pathway of blood coagulation.

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