Factor VIIa Residue Arg^{290} Is Required for Efficient Activation of the Macromolecular Substrate Factor X†

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Received June 2, 1994; Revised Manuscript Received August 1, 1994®

ABSTRACT: The serine protease factor VIIa (VIIa) in complex with tissue factor is responsible for initiating proteolytic events in the coagulation pathways. Efficient proteolysis by the extrinsic activation complex appears to depend on structural determinants in the cofactor as well as the light and heavy chain domains of VIIa. This study characterizes the functional defect resulting from alanine replacement for R^{290} in the VIIa protease domain. VIIa R^{290}→A bound both full-length and soluble tissue factor with affinities indistinguishable from wild-type VIIa, consistent with overall unaltered folding of the mutant protein. The catalytic function of VIIa R^{290}→A was further demonstrated to be unperturbed when analyzed with three different peptidyl *p*-nitroanilide substrates, indicating that the function of the catalytic triad is not affected by the mutation. However, proteolytic activation of factor X was diminished due to a 4–5-fold decreased k_{cat} in the presence and a >10-fold decreased rate in the absence of a negatively charged phospholipid surface. The functional defect resulting from the R^{290}→A replacement was observed in the presence and absence of cofactor. Within the structural framework of serine protease domains, R^{290} is predicted to be localized in a surface-exposed loop suggested to contribute to substrate selectivity in other serine proteases, consistent with the proposed functional role of R^{290} in the proteolytic activation of the natural substrate factor X.

Catalytic and proteolytic activity of serine protease VIIa is regulated by the assembly of VIIa with its macromolecular cofactor tissue factor (TF),[1] a transmembrane protein structurally related to the class II cytokine receptors (Ruf & Edgington, 1994). Both the light chain domains (Ruf et al., 1991a; Toomey et al., 1991; Clarke et al., 1992; Kazama et al., 1993) and the protease domain (O'Brien et al., 1991; Kumar & Fair, 1993; Matsushita et al., 1994) of VIIa appear to contribute to binding of TF. The interaction involves predominantly protein–protein contacts with only minor contributions provided by the insertion of TF into a phospholipid environment (Ruf et al., 1991a; Schullek et al., 1994). Assembly of VIIa with the isolated TF extracellular domain is sufficient to enhance the catalytic activity toward small peptidyl substrates (Ruf et al., 1991b), indicating changes of the catalytic function mediated by cofactor interaction. However, full amidolytic activity of VIIa is observed under various conditions which preclude efficient proteolytic activation of macromolecular substrates.

These observations lead to the identification of sites specifically contributing to macromolecular assembly of substrate factor X. Whereas amidolytic activity is not dependent on the presence of a negatively charged phospholipid surface, phospholipid-bound factor X is the preferred substrate for the TF·VIIa complex (Ruf et al., 1991b; Krishnaswamy et al., 1992). In the TF sequence 157–167, alanine replacement of residues Y157, K165, and K166 caused a selective reduction of the activation of factor X, indicating discrete interactions of cofactor with macromolecular substrate (Ruf et al., 1992a,b). The deletion of the amino-terminal γ-carboxyglutamic acid rich domain in VIIa resulted in only minor reduction of amidolytic function, whereas the rate of activation of factor X was reduced approximately 100-fold (Ruf et al., 1991a). These data indicate that the assembly and cleavage of macromolecular substrate are dependent on multiple interactions involving a supporting phospholipid surface as well as structural determinants in the cofactor TF and the light and heavy (Kumar & Fair, 1993; Kumar et al., 1991) chains of VIIa. Specific residues which form the recognition structure in VIIa remain to be identified. In search for functionally important residues in VIIa, this study identifies one residue in the protease domain of VIIa which selectively contributes to the activation of substrate factor X.

EXPERIMENTAL PROCEDURES

Proteins. Recombinant human TF (Ruf & Edgington, 1991b) was reconstituted into mixed phospholipid (30% phosphatidylserine/70% phosphatidylcholine, w/w) as previously described (Ruf & Edgington, 1991a). The concentration of nonrelipidated TF was determined using the BCA assay (Pierce Chemical Co.) which had been calibrated by amino acid analysis. Concentrations of relipidated TF were calculated from the amount of TF introduced into the relipidations reaction followed by correction for 50% surface availability (Bach et al., 1986) which was confirmed by functional titrations (Schullek et al., 1994). Recombinant wild-type VIIa was obtained from Novo Nordisk. Factor X was purified from citrated plasma, and protein concentration was based on $E^{1\%}$ = 11.6 at 280 nm, as described by Fair et al. (1979). Monoclonal antibody F4–2.1B was obtained from ascites and purified on protein A as described (Ruf et al., 1991a).

Mutagenesis and Expression of VII. The VII coding sequence (Hagen et al., 1986) was obtained from ATCC and

† This work was supported by National Institutes of Health Grant RO1 HL48752.

® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: VII/VIIa, factor VII/VIIa; TF, tissue factor; Gla, γ-carboxyglutamic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

0006-2960/94/0433-11631$04.50/0

subcloned into cDM8 for oligonucleotide-directed mutagenesis as described in detail (Ruf et al., 1993). To establish stably transfected clones for production, the coding sequence was subcloned into pED4 (Kaufman et al., 1991), kindly provided by Dr. R. J. Kaufman. The introduced mutation in the VII/pED4 construct was confirmed by DNA sequencing (Ruf et al., 1993). The Chinese hamster ovary (CHO) cell line DG44 which is deficient in dehydrofolate reductase (Pittman & Kaufman, 1993), was transfected using the calcium phosphate precipitation method (Ruf et al., 1993), and stably transfected cells were selected for the incorporation of the dehydrofolate reductase gene encoded for by pED4. The colonies were grown in increasing concentrations of methotrexate in order to increase the levels of protein expression. Amplified colonies were seeded onto Cytodex 1 microcarrier beads (Pharmacia Biotech, Piscataway, NJ) for growth in spinner flasks followed by adaptation to serum-free medium (Excel 301, JRH Biosciences, Lenexa, KS) supplemented with vitamin K_3. Serum-free culture supernatant was adjusted to 5 mM Ca^{2+} for immunoaffinity purification on the calcium-dependent immobilized MAb F4–2.1B. After adsorption, the affinity support was extensively washed with 1 M NaCl/5 mM $CaCl_2$ followed by elution with 5 mM EDTA, 20 mM NaCl, and 20 mM Tris-HCl, pH 8.5. The eluted protein was applied to an anion-exchange resin (MonoQ) and eluted with a linear $CaCl_2$ gradient (0–100 mM) in Tris-HCl, pH 8.5, in order to further select for γ-carboxylated protein, as described for the homologous protein C (Yan et al., 1990). The peak fractions which eluted at 22–24 mM $CaCl_2$ were pooled and concentrated on a microconcentrator followed by buffer exchange to 130 mM NaCl/Tris-HCl, pH 7.4 (TBS), for storage at −70 °C. Protein concentrations of wild-type and mutant VIIa were determined by the BCA assay (Pierce Chemical Co.) and the calculated molecular weight of 45 500 (Hagen et al., 1986). The mutant protein converted completely to the active enzyme VIIa during the purification, based on SDS–polyacrylamide gel electrophoresis and staining with Coomassie Blue which demonstrated homogeneous protein. The degree of γ-carboxylation of the recombinant protein was assessed by amino acid analysis after alkaline hydrolysis (Kuwada & Katayama, 1981). This analysis was calibrated by a reference standard mixture of glutamic acid and γ-carboxyglutamic acid (Gla) which was used to calculate the ratio of Gla to Glx in the hydrolysate.

Functional K_{Dapp} Determination. Binding of VIIa or VIIa $R^{290}\rightarrow A$ to full-length TF reconstituted into mixed phospholipid was characterized by a linked functional assay using factor Xa generation, as previously described in detail (Schullek et al., 1994; Ruf et al., 1994). Briefly, a fixed concentration of TF (2 pM) was assembled with varying concentrations of VIIa at 37 °C for 10 min. Factor X (100 nM) was added for a fixed time followed by determination of factor Xa formation with chromogenic substrate. Rates of factor Xa generation were converted to concentrations of bound VIIa, assuming that the maximal rate observed at saturation corresponded to the activity of TF·VIIa complexes at the concentration of the fixed ligand TF. These data were used for Scatchard analysis which yielded linear Scatchard plots for both wild-type VIIa and VIIa $R^{290}\rightarrow A$. Binding to soluble TF_{1-218} was characterized with a fixed concentration of VIIa (5 nM) and varying concentrations of TF_{1-218}, as described (Schullek et al., 1994). To exclude effects of the substrate on K_{Dapp} (Butenas et al., 1994), different peptidyl *p*-nitroanilide substrates were used to monitor complex formation: S2366 (Kabi Pharmacia Hepar, Franklin, OH), Spectrozyme FXa (American Diagnostica, Greenwich, CT), and Pefachrome VIIa (Pentapharm, Basel, Switzerland).

Analysis of Amidolytic and Proteolytic Function. The amidolytic function of wild-type or mutant VIIa (25–100 nM) was analyzed in TBS, 5 mM $CaCl_2$, and 0.025% bovine serum albumin at ambient temperature (22–23 °C). The rate of chromogenic substrate (1.25 mM) hydrolysis in the 200 μL reaction volume was recorded in a kinetic 96-well microtiterplate reader. The same experimental conditions were employed in the presence of varying concentrations of soluble TF_{1-218}. Proteolytic function was analyzed in TBS, 0.025% bovine serum albumin, and 5 mM $CaCl_2$ at 37 °C. Phospholipid-reconstituted TF (50 pM) was assembled with excess VIIa (2 nM) for 10 min at 37 °C followed by addition of factor X at a concentration from 7 to 1000 nM. Initial rates of factor Xa generation and kinetic parameters were determined as described in detail (Ruf et al., 1991b). Activation of factor X in the absence of phospholipid was studied under the same reaction conditions with non-relipidated TF (20 nM) solubilized by 50 μM CHAPS ((3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), VIIa at 5 nM, and factor X at concentrations ranging from 30 nM to 3 μM. The proteolytic function of the commercial VIIa preparation used in this study was indistinguishable from wild-type VIIa produced in CHO cells, when analyzed in the presence as well as absence of phospholipid. Activation of factor X (1 μM) in the absence of TF was evaluated in the presence of 5 mM $CaCl_2$ and 100 μM phospholipid (70% phosphatidylcholine/30% phosphatidylserine, w/w) at a VIIa concentration of 100 nM. Competition of VIIa $R^{290}\rightarrow A$ (0–100 nM) with a fixed concentration of wild-type VIIa (5 nM) was studied with TF (2.5 nM) solubilized by CHAPS and factor X at 3 μM.

RESULTS

Expression and Purification of VIIa $R^{290}\rightarrow A$. Based on preliminary screening using transient transfection experiments, a loss of proteolytic function resulted from exchange of R^{290} with alanine. To further characterize the mutant protein, stably transfected cell lines were established which secreted the mutant VII $R^{290}\rightarrow A$. The mutant protein was purified from culture supernatant by a two-step procedure of monoclonal antibody affinity and anion-exchange chromatography. The monoclonal antibody reacts with a Ca^{2+}-dependent epitope in the Gla domain (Ruf et al., 1991a), selecting for protein which adopts a normal Ca^{2+}-saturated conformation. The protein obtained from the affinity chromatography step was applied to an ion-exchange resin from which it eluted at a Ca^{2+} concentration of 22–24 mM. This concentration is sufficient to elute wild-type VIIa under identical conditions. Under both reducing and nonreducing conditions, the purified VIIa $R^{290}\rightarrow A$ demonstrated an electrophoretic mobility which was identical to the commercial wild-type VIIa used as the control in this study. Binding to the Ca^{2+}-dependent antibody, normal elution from the ion-exchange resin, and electrophoretic mobility identical to wild-type VIIa indicated that the mutant was properly processed during synthesis, including the post-translational modifications of glycosylation and γ-carboxylation. The ratio of Gla to Glx after alkaline hydrolysis was found to be 1:3.8, which is consistent with the expected ratio of 10:37 for protein in which all 10 Gla residues are γ-carboxylated (Thim et al., 1988). In agreement with the analysis of crude culture supernatant from transient transfection experiments, the purified mutant enzyme was found to be defective in the activation of factor X. Because the majority of VII found in the culture supernatant was in the

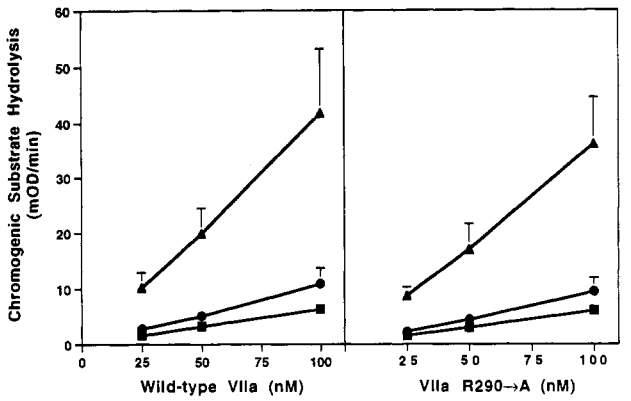

FIGURE 1: Amidolytic activity of VIIa R290→A in comparison to wild-type VIIa. The catalytic activities of the indicated concentrations of enzyme were determined at ambient temperature with a 1.25 mM aliquot of the peptidyl *p*-nitroanilide substrate Spectrozyme FXa (■), S2366 (●) or Pefachrome VIIa (▲).

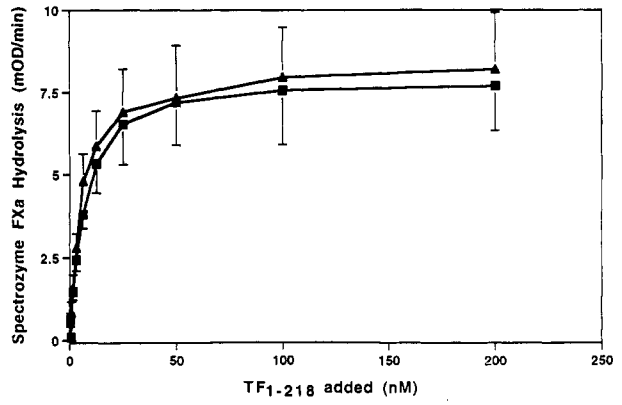

FIGURE 2: TF-enhanced catalytic activity of wild-type VIIa (■) or VIIa R290→A (▲). The amidolytic activity of a fixed concentration of VIIa (5 nM) in the presence of the indicated concentrations of TF_{1-218} was determined with Spectrozyme FXa (1.25 mM) at ambient temperature.

zymogen form, this finding indicated that the initially identified functional defect was not solely caused by a reduction in the conversion of zymogen VII to the active enzyme. The complete conversion of the mutant to the active enzyme during anion-exchange chromatography further provides evidence that the mutant is qualitatively capable to autoactivate, as observed for wild-type VII (Pedersen et al., 1989). However, quantitative alterations in the activation rate for the VIIa R290→A mutant cannot be excluded, because of the nonlinear activation kinetics under these conditions (Pedersen et al., 1989).

Amidolytic Activity of VIIa R290→A. The catalytic function of VIIa R290→A was tested in the presence of calcium ions and a series of peptidyl *p*-nitroanilide substrates (Figure 1). The mutant enzyme in the absence of TF had a catalytic activity similar to wild-type VIIa, when tested with the chromogenic substrates S2366, Spectrozyme FXa, and Pefachrome VIIa. The catalytic activity in the presence of TF was further analyzed in order to assess whether the mutant acquires enhanced catalytic activity upon assembly with the cofactor, as observed with the wild-type enzyme. Figure 2 shows the enhanced catalytic activity of a fixed concentration of wild-type or mutant VIIa in the presence of increasing concentrations of soluble TF_{1-218}. At saturation, the rate of chromogenic substrate Spectrozyme FXa hydrolysis by VIIa R290→A was indistinguishable from the rate of wild-type VIIa. Identical results were obtained with a limiting concentration of full-length TF and excess VIIa, when tested with all three chromogenic substrates used in this study (data not shown).

These data demonstrate that critical structures in the VIIa R290→A active site are properly conformed and capable of activating small pseudosubstrates with normal rates, both in the presence and in the absence of cofactor.

Binding Function of VIIa R290→A. The binding of VIIa R290→A to soluble TF_{1-218} was analyzed by a direct functional assay using chromogenic substrates to monitor TF·VIIa complex formation (Schullek et al., 1994). Because of potential effects of the substrate on the functionally determined K_{Dapp} (Butenas et al., 1994), the binding constants were determined with three different substrates. With each substrate used, the apparent dissociation constant for binding of VIIa R290→A to soluble TF was similar to the values for wild-type VIIa (Table 1). The extrapolated number of sites (B_{max}) obtained from the fit of the data to the single site binding equation was in good agreement with the concentration of the fixed ligand (5 nM), indicating reliability of the fit. No significant difference in K_{Dapp} was observed when the three substrates were compared, providing evidence that the binding constant for the interaction of wild-type and mutant VIIa with soluble TF_{1-218} is not influenced by the choice of substrate used to monitor TF·VIIa complex formation. Binding of VIIa R290→A to full-length TF reconstituted into negatively charged phospholipid vesicles was studied in a linked functional assay with factor Xa generation as a measure for TF·VIIa complex formation. The calculated B_{max} for this analysis was consistent with the concentration of the fixed ligand TF (2 pM). The dissociation constants for mutant and wild-type VIIa were similar (Table 1) and in agreement with previous studies demonstrating tight binding with a K_{Dapp} of 3–4 pM (Ruf et al., 1994; Schullek et al., 1994). These data demonstrate a normal binding function of VIIa R290→A to TF in the absence and presence of phospholipid. This excludes that the basic residue R290 contributes to functionally significant interactions with TF or phospholipid during TF·VIIa complex formation.

Proteolytic Function of VIIa R290→A. The proteolytic function of VIIa R290→A was evaluated with the macromolecular substrate factor X. In the presence of phospholipid, an excess of VIIa R290→A with a limited concentration of TF failed to activate factor X at the same rate as the control wild-type VIIa·TF complex (Figure 3A). Kinetic parameters for the activation of factor X demonstrated that the mutation did not affect the apparent K_M which was similar for VIIa R290→A (62 ± 27 nM) and wild-type VIIa (102 ± 37 nM). The functional defect could be attributed to a 4–5-fold decrease of the k_{cat} of the mutant TF·VIIa complex (1.6 ± 0.2 s^{-1}) in comparison to wild-type TF·VIIa (7.3 ± 0.7 s^{-1}). The functional defect was further apparent when mutant VIIa and wild-type VIIa were analyzed in the absence of cofactor but in the presence of 100 μM charged phospholipid. Activation of factor X at 1 μM by wild-type VIIa [(2.4 ± 0.4) × 10^{-5} mol of factor Xa (mol of VIIa)$^{-1}$ s^{-1}] was approximately 4-fold faster compared to the rate of activation by VIIa R290→A [(8.0 ± 2.0) × 10^{-6} mol of factor Xa (mol of VIIa)$^{-1}$ s^{-1}]. In the absence of phospholipid (Figure 3B), the functional defect appeared to be more pronounced with an at least 10-fold difference in the rate of factor Xa generation comparing the mutant and wild-type VIIa·TF complexes. Kinetic analysis for the solution phase reaction was not attempted, because of previously shown limitations originating from the high K_M under these conditions (Ruf et al., 1991b).

These analyses suggest a selective defect of VIIa R290→A in the activation of macromolecular substrate, whereas amidolytic and TF binding functions are unaltered. To test this hypothesis, competition between VIIa R290→A and wild-

Table 1: Affinity of Binding of Wild-Type VIIa or VIIa $R^{290}\rightarrow A$ to Soluble TF_{1-218} and Full-Length, Phospholipid-Reconstituted TF

	K_{Dapp}		B_{max}	
	wild-type VIIa	VIIa $R^{290}\rightarrow A$	wild-type VIIa	VIIa $R^{290}\rightarrow A$
TF_{1-218}[a]				
S2366	2.4 ± 0.5 nM	1.9 ± 0.7 nM	4.7 ± 0.1 nM	4.7 ± 0.2 nM
Pefachrome VIIa	2.5 ± 1.9 nM	1.9 ± 0.2 nM	4.9 ± 0.2 nM	4.9 ± 0.1 nM
Spectrozyme FXa	2.7 ± 1.0 nM	2.3 ± 1.0 nM	4.8 ± 0.1 nM	4.8 ± 0.1 nM
full-length TF[b]	4.5 ± 1.3 pM	5.3 ± 2.1 pM	1.9 ± 0.1 pM	1.9 ± 0.1 pM

[a] Determined by direct chromogenic assay using a fixed concentration of VIIa (5 nM) and the indicated chromogenic substrate, $n = 3$. [b] Determined by linked functional assay with factor Xa generation and a fixed concentration of phospholipid-reconstituted TF (2 pM), $n = 3$.

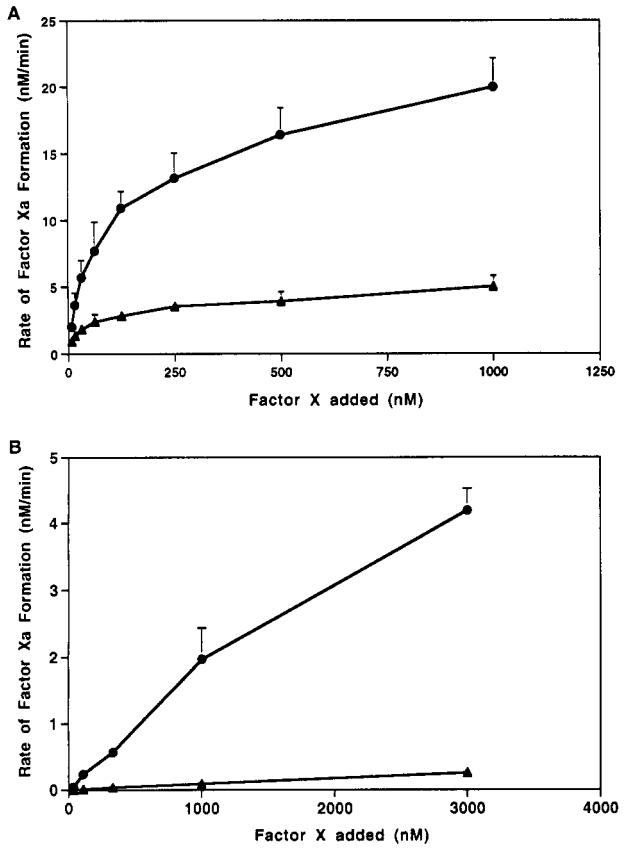

FIGURE 3: Proteolytic activity of wild-type VIIa (●) or VIIa $R^{290}\rightarrow A$ (▲) in the presence (A) or absence (B) of phospholipid. (A) Rate of factor Xa formation by excess enzyme (2 nM) in the presence of 50 pM phospholipid-reconstituted TF and the indicated concentrations of substrate. (B) Rate of factor Xa formation by 5 nM enzyme and an excess (20 nM) of full-length TF solubilized with CHAPS dependent on the concentration of substrate factor X.

type VIIa was analyzed. VIIa $R^{290}\rightarrow A$ efficiently competed with wild-type VIIa for limited TF, as demonstrated by a progressive reduction in factor Xa generation with increasing concentrations of the mutant (Figure 4). A 50% reduction in the rate of factor X activation was observed at approximately equimolar concentrations of mutant and wild-type VIIa, demonstrating that the mutant indeed binds with high affinity and that the functional defect is independent of the TF–VIIa interaction.

DISCUSSION

The assembly of the extrinsic activation complex involves multiple interactions between the macromolecular substrate and enzyme VIIa as well as cofactor TF. This study characterizes an alanine replacement mutant in the protease domain of VIIa which exhibited a defect in the activation of the macromolecular substrate factor X. The VIIa $R^{290}\rightarrow A$ mutant was expressed in mammalian cells and purified to

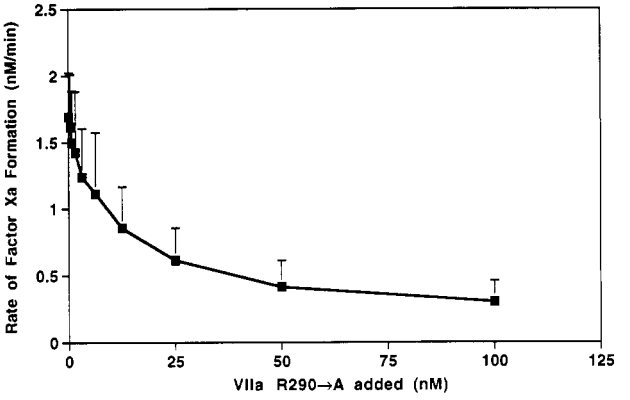

FIGURE 4: Efficient competition of VIIa $R^{290}\rightarrow A$ with wild-type VIIa. The proteolytic activity of 5 nM wild-type VIIa in the presence of 2.5 nM full-length TF solubilized with CHAPS is reduced by increasing concentrations of VIIa $R^{290}\rightarrow A$.

homogeneity by a combination of affinity for the Ca^{2+}-dependent Mab F4–2.1B and ion-exchange chromatography. The purified protein was found to be activated enzyme VIIa and γ-carboxylated at the 10 glutamic residues in the Gla domain. Efficient cellular secretion and apparently normal posttranslational modification indicated proper overall folding of the mutant protein. Consistent with this notion was the unaltered binding function of the mutant both to full-length TF reconstituted into phospholipid as well as to soluble TF_{1-218}. Apparent dissociation constants for binding to soluble TF were determined with three peptidyl *p*-nitroanilide substrates which differed in their P_1–P_3 structures. The apparent dissociation constants determined for both wild-type and mutant VIIa were not influenced by the different chromogenic substrates which were used to monitor TF·VIIa complex formation. These data substantiate the unaltered binding function of the mutant enzyme independent of the presence of a particular substrate interacting with the TF·VIIa complex.

The catalytic activity of the mutant enzyme toward the three chromogenic substrates was indistinguishable from wild-type VIIa, both in the absence and in the presence of TF. The function of the catalytic triad of VIIa thus appears to be unaffected by the R^{290} exchange to alanine. Because the rate of hydrolysis by wild-type VIIa differed for each of the three substrates, one can assume some contribution of the P_2 and P_3 structures to the interaction with the enzyme. Normal catalysis of these substrates by the mutant enzyme thus provides evidence that the residue replacement most likely does not affect the S_1–S_3 structures in the enzyme. The proteolytic activation of the macromolecular substrate factor X by VIIa $R^{290}\rightarrow A$ was severely reduced as a consequence of a 4–5-fold reduction in the k_{cat} when analyzed in the presence of a negatively charged phospholipid surface. Rates of activation of factor X bound to phospholipid were found to be reduced similarly whether the cofactor TF was present or absent, suggesting that cofactor interaction is not required to

induce the defect in macromolecular substrate activation. In the absence of phospholipid, the rate of hydrolysis was further decreased by at least 2-fold, indicating that the supporting phospholipid surface may attenuate the functional defect. The selective defect of VIIa R^{290}→A in proteolytic function is further demonstrated by efficient competition of the mutant with the wild-type enzyme for limiting cofactor TF. These data exclude any defect in the cofactor interaction as the cause for the loss of function.

The identification of R^{290} as a residue specifically contributing to the activation of macromolecular substrate is consistent with the characterization of inhibitory peptides corresponding to regions of the VIIa sequence. These peptidyl mimicry studies suggested that the sequence 285–305 is a substrate interactive region (Kumar & Fair, 1993; Kumar et al., 1991). However, a different role of residues in this sequence span has been suggested from elucidation of the molecular defect of several hereditary VII deficient patients characterized by point mutations at R^{304}. The exchange of this residue to glutamine or tryptophan resulted in significant loss of TF binding function (O'Brien et al., 1991; Matsushita et al., 1994). This could indicate that R^{304} and R^{290} contribute to distinct functional sites in the VIIa protease domain. In a comparative alignment of serine protease domains, these two residues are indeed found in different structural loci (Greer, 1990). Whereas R^{304} is located adjacent to a highly conserved proline residue (James et al., 1993) with possible importance to maintain the structural integrity, R^{290} is found in a loop of nonconserved sequence. Surface loops which are not conserved between homologous coagulation serine proteases have been hypothesized by Furie and co-workers (Furie et al., 1982) to contribute significantly to the substrate specificity by providing extended recognition in addition to recognition at the active site. The loop in which R^{290} is localized is surface-exposed as judged from the structure of the homologous serine protease domain of factor Xa (Padmanabhan et al., 1993) and thrombin (Bode et al., 1992). This stretch of sequence is susceptible to proteolysis in factor Xa (Padmanabhan et al., 1993) and thrombin (Berliner, 1984) and appears to exhibit a significant degree of conformational flexibility (Bode et al., 1992). Proteolytic cleavage of this loop sequence in thrombin, however, appears to only slightly affect the proteolytic function (Hofsteenge et al., 1988) and the interaction with the macromolecular inhibitor hirudin (Stone et al., 1987). Residue Y151 in trypsin and residue L143 in chymotrypsin are found in this surface loop, and these residues have been suggested to contribute to the S'_2 specificity pocket, based on structures of these enzymes with protease inhibitors (Schellenberger et al., 1994). These findings raise the possibility that R^{290} in VIIa may constitute or conformationally stabilize the S'_2 subsite in VIIa.

The present study identified a basic residue in the VIIa protease domain which was selectively required for the proteolytic activation of factor X. The functional defect of VIIa R^{290}→A is similar to the effect resulting from the replacement of the TF residues K165 and K166 by alanine (Ruf et al., 1992a). The mutations in TF also affected predominantly the k_{cat}, and the absence of a negatively charged phospholipid surface increased the functional defect. These data argue that the residue replacements in both enzyme and cofactor affected protein–protein interactions between substrate and the TF·VIIa complex. Competition experiments with synthetic peptides further suggested a possible contribution of R^9 in the VIIa Gla domain to proteolytic function of the TF·VIIa complex (Martin et al., 1993). Although our structural knowledge is presently insufficient to evaluate the hypothesis, these data might indicate that basic residues from both cofactor and enzyme may align to form an extended charge field for the assembly of macromolecular substrate.

ACKNOWLEDGMENT

I thank Pablito Tejada, Cindi Biazak, and David Revak for excellent technical assistance and Barbara Parker for preparation of the illustrations.

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