Influence of mutations in tissue factor on the fine specificity of macromolecular substrate activation

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The C-terminal fibronectin-type-III-like module of the tissue factor (TF) extracellular domain plays a requisite role in the activation of macromolecular substrates by factor VIIa (VIIa) in complex with TF. Unlike the mutations Lys¹⁶⁵ → Ala, Lys¹⁶⁶ → Ala in TF, which prevent efficient proteolysis of factor X, we found that the coagulant defect of a site-specific Trp¹⁵⁸ → Arg, Ser¹⁶⁰ → Gly replacement mutant of TF is largely attributable to the inability of TF to efficiently support the activation of the bound zymogen VII to the active protease VIIa. Binding studies demonstrated comparable affinity of binding of VIIa or VII by wild-type TF and TF_{R158G160}. In comparison with wild-type TF, the catalytic efficiency of factor X activation was

reduced 56-fold with TF_{A165A166} as the cofactor, but only 3.5-fold with TF_{R158G160}. The activation of VII bound to TF by factor Xa or VIIa was reduced 2-fold in the presence of TF_{R158G160} and 7–8-fold with TF_{A165A166}. This suggests that the molecular recognition of VII in complex with TF by the enzymes TF–VIIa and factor Xa are similar. Generation of factor IXa by TF_{R158G160}–VIIa was unaltered, but reduced 2-fold with TF_{A165A166}. In addition, the mutations affected the cleavage of the two scissile bonds of factor IX differently, providing further support for the idea that the cofactor, TF, influences the fine specificity of activation of macromolecular substrates by the TF–VIIa complex.

INTRODUCTION

Tissue factor (TF) is an integral transmembrane glycoprotein that serves as the high-affinity receptor for coagulation factor VII (VII) and its activated form, factor VIIa (VIIa) [1], forming the initiating cell surface complex of the coagulation protease cascade. Assembly of VIIa with TF enhances the catalytic efficiency of VIIa towards small peptide substrates [2–4] and towards the macromolecular substrates, factor X [5] and factor IX [6]. VII can be proteolytically activated by factors Xa, VIIa, IXa and XIIa [7,8], and susceptibility to activation appears to be greatly enhanced when VII is bound to TF [9–12].

Mutagenesis studies [13] and the recently solved molecular structure of the TF–VIIa complex [14] have provided detailed insight into the interactions between TF and VIIa. The structural modules of VIIa bind in an extended conformation with the TF extracellular domain through contacts of the protease domain with the N-terminal module of TF, of the first epidermal-growth-factor-like (EGF) domain with a groove formed by the two modules of TF, and of the γ -carboxyglutamic acid-rich (Gla) domain with the C-terminal module of TF. Certain residues in the C-terminal module of TF, which do not contact VIIa, have been identified to be important for the function of the TF–VIIa complex [15–17]. Whereas the functional defect was originally attributed to defective phospholipid interaction of TF [15], our data indicated that the mutations of Lys¹⁶⁵ and Lys¹⁶⁶ in TF selectively affected protein–protein interactions with macromolecular substrate [16].

In this study, we analyse the role of cofactor residues in recognition of the different macromolecular substrates by the TF–VIIa complex. We focus on two mutants with severe coagulant defects [16,18]. In the first mutant, the replacement of

Lys¹⁶⁵ and Lys¹⁶⁶ with Ala eliminated two basic side chains which are well exposed to solvent on the protein surface [14]. The second mutant exchanged the partially solvent-exposed Trp¹⁵⁸ side chain with Arg, which provides the most conservative substitution with regard to size but changes the side-chain properties with the addition of the basic guanidino groups. Because of the partially buried position of Trp¹⁵⁸, subtle packing-dependent local conformational effects may be introduced by this mutation. Ser¹⁶⁰ was also replaced by Gly in this mutant, but we conclude from the unaltered function of the Ser¹⁶⁰ → Ala mutant [18] that the functional defect is largely attributable to the Trp¹⁵⁸ replacement. Trp¹⁵⁸ is part of a hydrophobic interface in contact with the VIIa Gla domain [14], which plays a critical role in the interaction of TF–VIIa with the macromolecular substrate factor X [19,20]. We here demonstrate that the two mutants, Trp¹⁵⁸ → Arg/Ser¹⁶⁰ → Gly and Lys¹⁶⁵/Lys¹⁶⁶ → Ala, exhibit different functional defects with respect to the recognition of macromolecular substrate.

MATERIALS AND METHODS

Proteins

Recombinant full-length (residues 1–263) TF was purified from insect cells [21]. VIIa was purchased from Novo Nordisk (Gentofte, Denmark). Human factor X was purified from plasma and factor Xa was obtained by activating factor X with Russell's viper venom [22]. Factor IX was purified from human plasma as described by Lawson and Mann [23]. To remove trace amounts of VII and factor X, the factor IX preparation was passed over immobilized monoclonal antibody (mAb) F1-3G12 (against VII) and mAb F21.4.2C (against factor X), which were generated in the laboratory against purified protein using standard hybridoma

technology with screening for reactivity of the respective protein and for discrimination of the homologous proteins, factor X, factor VII, factor IX and prothrombin. Recombinant tick anticoagulant peptide (rTAP) [24] was kindly provided by Dr. George Vlasuk (Corvas International, San Diego, CA, U.S.A.). Normal pooled human plasma was immunodepleted of VII and VIIa by passage over immobilized mAb F1-3G12. Residual VII levels in this plasma were less than 0.1 pM VII/VIIa.

To obtain VII devoid of catalytic activity and the propensity to autoactivate during purification [8], the catalytic triad Ser residue was mutated to Ala, i.e. VII_{A344}. Mutagenesis, stable expression and purification of recombinant VII from serum-free culture supernatant have been described in detail [25]. The generation of stably transfected Chinese hamster ovary cell lines which express full-length wild-type TF, TF_{R158G160} or TF_{A165A166} has been previously described [16,18]. Mutants of TF were isolated from detergent lysates of cells which were grown in suspension culture by immunoaffinity chromatography as described [26]. TF was reconstituted into mixed phosphatidylcholine/phosphatidylserine vesicles (70:30, w/w) using detergent solubilization followed by dialysis, as previously described [26]. The efficiency of relipidation and relative inside-out distribution of TF in the vesicles were analysed by TF–VIIa-dependent peptide and fluorogenic substrate hydrolysis in the presence and absence of the detergent CHAPS, as described by Krishnaswamy [4]. The concentrations given are the effective concentrations on the outside of the phospholipid vesicle. Soluble TF (sTF), corresponding to residues 1–218, with wild-type or mutant Arg¹⁵⁸, Gly¹⁶⁰ extracellular domain sequence were expressed in *Escherichia coli*, refolded and purified as described [27]. Protein concentrations were determined by the BCA assay (Pierce Chemical Co.) and the homogeneity of the preparations was judged by SDS/PAGE.

Effect of varying VIIa concentrations on the procoagulant activity of mutant TF

TF, TF_{R158G160} and TF_{A165A166} expressed in Chinese hamster ovary cells were assayed for specific procoagulant activity in normal pooled human plasma. To obtain uniformly dispersed TF, cell pellets were lysed by adding 15 mM octyl β -D-glucopyranoside (Calbiochem, San Diego, CA, U.S.A.) in HBS (20 mM Hepes, 150 mM NaCl, pH 7.4) followed by an incubation at 37 °C for 15 min and a final 1:3 dilution with HBS [28]. Using a manual one-stage clotting assay, diluted cell lysates of TF-, TF_{R158G160}- or TF_{A165A166}-expressing cell lines (0.1 ml/tube) were placed into polystyrene test tubes containing 0.1 ml of normal plasma, supplemented with various VIIa concentrations (10⁻⁴–10 nM), followed by an incubation at 37 °C for 2 min. The reaction was started with the addition of 0.1 ml of 0.1 M CaCl₂ (prewarmed to 37 °C) and the clotting time was determined by manual tilting in triplicates. The specific activity of mutant or wild-type TF at each of the concentrations of exogenously added VIIa was derived from a double-reciprocal calibration curve established with serial dilutions of wild-type TF [29]. The activity of TF that yielded a 50 s clotting time was arbitrarily defined as 1 U. Experiments with phospholipid-reconstituted mutant or wild-type TF in normal or VII-depleted plasma were performed under the same conditions.

Functional K_D(app) determinations

The K_D(app) for binding of VIIa (2 nM) to wild-type or mutant sTF (0.15–9 nM) was determined using an amidolytic assay with the chromogenic substrate Spectrozyme FXa (American Diagnostica, Greenwich, CT, U.S.A.), as previously described in

detail [30]. Binding of VIIa to full-length TF or TF_{R158G160} reconstituted in mixed phospholipid vesicles was monitored with the fluorogenic substrate methanesulphonyl (Mes)-D-Leu-Gly-Arg-6-amino-1-naphthalenesulphonamide(C₂H₅)₂ [Mes-D-L-G-R-ANSN(C₂H₅)₂] (Haematologic Technologies Inc., Essex Junction, VT, U.S.A.) which is efficiently hydrolysed by VIIa bound to TF [31]. A fixed concentration of full-length wild-type TF or TF_{R158G160} (50 pM) was incubated with increasing concentrations of VIIa (1 pM to 5 nM) in HBS, containing 5 mM CaCl₂, at 37 °C. Mes-D-L-G-R-ANSN(C₂H₅)₂ at 20 μ M was added and the change in fluorescence at 470 nm was monitored in a Perkin–Elmer fluorescence spectrophotometer with excitation at 352 nm. The increase in fluorescence was linear over a 4 h period. The rate of substrate hydrolysis was corrected for background hydrolysis by free VIIa, and the concentration of fluorophore generated per minute was calculated on the basis of a calibration with the fluorescent reporter group ANSN(C₂H₅)₂. The concentration of bound and free VIIa was calculated and the apparent dissociation constant evaluated by Scatchard analysis as described above for chromogenic substrates. The binding of VIIa to full-length TF, TF_{R158G160} and TF_{A165A166} reconstituted in mixed phospholipid vesicles, was also analysed in a linked functional assay that measures factor X activation as a readout to assess TF–VIIa complex formation, as described in detail [30]. Concentrations in this assay were as follows: TF (1 pM, 5 pM), VIIa (0.2 pM–2 nM) and factor X (100 nM).

Determination of binding constants using surface plasmon resonance

Kinetics of the interaction between sTF and VII or VIIa were determined using a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden) at ambient temperature. The non-perturbing mAb (TF9-10H10) against TF was immobilized by amine coupling on to the preactivated sensor chip surface (50 μ g/ml in 10 mM sodium acetate, pH 5.0) to capture wild-type or mutant sTF (30 μ g/ml) in equilibration buffer (HBS containing 5 mM CaCl₂ and 0.05 % surfactant P20). VII_{A344} or wild-type VIIa diluted in equilibration buffer to concentrations ranging from 12 nM to 1 μ M were injected at a flow rate of 10 μ l/min. The kinetic binding constants (k_a , k_d) were determined by non-linear regression analysis as described for analysis of the TF–VIIa interaction [32,33]. Briefly, binding of ligand (VII, VIIa) to wild-type sTF or sTF_{R158G160} was monitored by recording the increase in resonance units over time. Plotting the rate of binding (dR/dt) versus R yielded a slope (k_s) which was plotted against the respective ligand concentration (C). The slope of this plot yielded the association rate constant (k_a). The dissociation rate constant (k_d) was calculated from the initial dissociation phase of the binding curves. The equilibrium dissociation constant (K_D) is the calculated ratio of k_d over k_a .

Determination of kinetic parameters for the activation of factor X

The kinetics of factor X activation were studied by incubating fixed concentrations of phospholipid-reconstituted wild-type or mutant TF (50 pM) with an excess of VIIa (2 nM). We chose a high concentration of VIIa to overcome potentially decreased affinities of the mutant TF. In previous studies, we did not detect a change in kinetic parameters for factor X activation when reactions with stoichiometric amounts of TF and VIIa were compared with reactions with a 20-fold molar excess of VIIa [2]. The kinetic analysis at high concentrations of VIIa is therefore considered valid. After formation of TF–VIIa complexes by incubation in Tris-buffered saline (10 mM Tris/HCl, 150 mM NaCl, pH 7.4; TBS)/0.1 % BSA/5 mM CaCl₂ for 10 min at

37 °C, substrate factor X was added at concentrations ranging from 0.05 to 3 μ M. Aliquots were removed from the ongoing reactions at 37 °C every 60 s and quenched in 0.1 M EDTA; factor Xa concentration was determined in each aliquot with chromogenic substrate. The initial rates of factor Xa generation at the various substrate concentrations were fitted to the Michaelis–Menten equation to obtain $V_{\max}$ and K_m .

Analysis of activation of VII

Single-chain recombinant factor VII_{A344} was radiolabelled with Na¹²⁵I using the lodogen method [34], yielding a specific radioactivity of 2 μ Ci/ μ g of protein. The activation of ¹²⁵I-VII_{A344} (10 nM) by 1 nM VIIa in the presence of phospholipid-reconstituted full-length wild-type TF, TF_{R158G160} or TF_{A165A166} (10 nM) was analysed in TBS/0.1 % BSA/5 mM CaCl₂ at 37 °C. At selected times, aliquots from the reaction mixture were quenched in equal amounts of reducing SDS/PAGE sample buffer and kept on ice until separation by SDS/PAGE. The activation of ¹²⁵I-VII was monitored by autoradiography (Kodak X-Omat film) which was quantified by scanning densitometry (ImageQuant program, Molecular Devices). The ratio of the integrated volume of (VIIa_{heavy chain} + VIIa_{light chain}) over the sum of (VII + VIIa_{heavy chain} + VIIa_{light chain}) was calculated for each time as the percentage of VII activation. The activation of ¹²⁵I-VII_{A344} bound to a stoichiometric amount of wild-type or mutant TF, by factor Xa (100 pM, 1 nM) was analysed under the same experimental conditions.

Analysis of the activation of factor IX

Factor IX activation was monitored by coagulation assay, as described by Komiyama et al. [35]. Briefly, VIIa (50 pM) and stoichiometric amounts of phospholipid-reconstituted full-length TF, TF_{R158G160} or TF_{A165A166} were incubated in TBS/5 mM CaCl₂ for 10 min at 37 °C, and this was followed by the addition of 20 nM factor IX. After being quenched with 25 mM EDTA at designated time points (0–120 min), the samples were kept on ice until the factor IXa coagulant activity was determined as follows: 75 μ l of sample (diluted 1:10 or 1:20 in TBS), 75 μ l of factor IX-deficient plasma (George King Biomedical Inc, Overland Park, KS, U.S.A.) and 75 μ l of cephalin were incubated for 2 min at 37 °C, followed by the addition of 75 μ l of 25 mM CaCl₂ (prewarmed to 37 °C). The clotting times measured were converted into factor IXa concentrations using a calibration curve constructed using purified factor IXa.

Activation of factor IX was further analysed by resolving the reaction product by SDS/PAGE. Fixed concentrations of wild-type and mutant TF (5 nM) were incubated with VIIa (5 nM) or, in control experiments, Xa (5 nM), in TBS/0.01 % BSA/5 mM CaCl₂ for 10 min at 37 °C. The reaction was started by the addition of factor IX (15 μ M) which was purified from plasma. At designated time points (0–120 min), aliquots were removed, quenched in reducing sample buffer and 5 μ g of factor IX/Xa per lane was separated by SDS/PAGE. Protein bands were visualized by staining with Coomassie Brilliant Blue, and the extent of factor IX activation was quantified by densitometric scanning of dried gels and analysed by the ImageQuant program. To exclude the possibility that contaminating factor Xa contributed to the activation of factor IX, TAP, a highly selective Xa inhibitor [24], was included in some experiments at a concentration of 1 mg/ml. This concentration is 100-fold higher than that required to inhibit the amidolytic activity of 10 nM Xa by more than 99 %.

RESULTS

Effects of VIIa on the coagulant function of TF_{R158G160}

In previous studies, we demonstrated a partial correction of the defect in coagulant function of several of the TF mutants after addition of a high concentration of VIIa (500 nM) to the plasma, suggesting that these mutants are defective in supporting the activation of the bound zymogen VII [17]. High concentrations of VIIa can overcome dependence on TF cofactor function [36]. We therefore evaluated the effect of lower VIIa concentrations on the coagulant defect of selected TF mutants. Addition of 10 nM VIIa to normal plasma resulted in a modest increase in the specific activity of wild-type TF from 1100 to 2100 mU/ng TF. The procoagulant function of the double mutant TF_{A165A166} was severely reduced and exhibited only a 3-fold increase (from 9 to 27 mU/ng TF) on addition of 10 nM VIIa, consistent with the notion that the predominant functional defect of this mutant is diminished activation of the substrate zymogen factor X [16]. In contrast, the functional activity of the mutant TF_{R158G160} increased 10-fold (from 60 to 610 mU/ng TF) on addition of 10 nM VIIa, providing evidence for a selective role of these residues in the activation of the zymogen VII.

The improvement of coagulant function of TF_{R158G160} by VIIa was dose-dependent (Figure 1), and at 10 nM VIIa the functional defect of the mutant was only 3-fold as compared with wild-type TF. When the coagulant function of TF_{R158G160} and wild-type TF were compared in VII-depleted plasma supplemented with various concentrations of enzyme VIIa, a constant 2–3-fold difference in coagulant function was found (results not shown). This indicates that TF_{R158G160} is slightly defective in activating the downstream protease zymogens and that the affinity of VIIa binding is largely unaffected. These data further substantiate the hypothesis that the mutant is defective in supporting the activation of the zymogen VII in normal plasma. Since a dose-dependence similar to that depicted in Figure 1 has previously been demonstrated for soluble TF [37], which is used as a reagent

Figure 1 Effect of VIIa added to normal plasma on the procoagulant activity of wild-type TF and TF_{R158G160}

Chinese hamster ovary cells which stably express wild-type TF or TF_{R158G160} were lysed with octyl β -D-glycopyranoside to homogeneously disperse TF. Wild-type TF (□) or TF_{R158G160} (◇) was added at 20 ng/ml to pooled normal human plasma which was supplemented with the indicated concentrations of VIIa (10^3 –10 nM), followed by recalcification. Mean $\pm$ S.D. of the clotting time determined in triplicates in three independent experiments is shown in dependence of the exogenously added VIIa. The lines are least-square fits to the depicted data points.

Table 1 Characterization of VII and VIIa binding to wild-type or mutant sTF

The results for the amidolytic assay are means $\pm$ S.D. ($n = 3$); the maximum rate of Spectrozyme FXa hydrolysis at saturation of VIIa is given. The results for surface plasmon resonance are means $\pm$ S.D. for two determinations.

	Amidolytic assay		Surface plasmon resonance					
	Factor VIIa		Factor VII _{A344}			Factor VIIa		
	Maximum rate (mOD/min per sTF–VIIa)	$K_D(\text{app})$ (nM)	$10^5 \times k_a$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^{-3} \times k_d$ (s^{-1})	K_D (nM)	$10^5 \times k_a$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^{-3} \times k_d$ (s^{-1})	K_D (nM)
Wild-type sTF	0.88 ± 0.11	2.4 ± 0.3	1.7 ± 0.4	2.2 ± 0.6	12.3	1.5 ± 0.5	1.4 ± 0.2	9.3
sTF _{R158G160}	0.67 ± 0.07	2.2 ± 0.2	1.6 ± 0.4	1.8 ± 0.5	11.3	1.6 ± 0.3	2.5 ± 0.3	15.5

for quantification of VIIa levels in plasma [38], the TF_{R158G160} mutant provides an example of impairment of VII activation without elimination of the phospholipid anchoring of TF.

Characterization of VII and VIIa binding to soluble and full-length TF_{R158G160}

Previous studies demonstrated that TF_{A165A166} binds VIIa with an affinity indistinguishable from that of wild-type TF. Since Trp¹⁵⁸ is located in the interface with the Gla domain of VIIa, we determined the affinity of TF_{R158G160} for VII and VIIa, in order to exclude the possibility that the local conformational effect of the replacement of the Trp¹⁵⁸ side chain affects VIIa binding. The $K_D(\text{app})$ determined by amidolytic assay demonstrated similar affinity of sTF_{R158G160} and wild-type sTF for VIIa (Table 1). By surface plasmon resonance, the association rate constants (Table 1) for VIIa binding to wild-type sTF or sTF_{R158G160} were indistinguishable and in good agreement with previously reported values [32]. The dissociation rates determined for wild-type sTF were within the range of previously reported values [32,33]. However, the dissociation kinetics do not show a perfect fit to a single kinetic phase, as previously reported [33]. Because of these limitations in data analysis, it is uncertain whether the 2-fold increase in rate of dissociation of VIIa from sTF_{R158G160} can be considered a significant difference (Table 1). Indeed, no difference between this mutant and wild-type sTF was observed in the binding analysis with the zymogen mutant VII_{A344}, and the calculated equilibrium dissociation constants (K_D) for VII binding by mutant and wild-type sTF were similar (Table 1). These data provide evidence that the mutant sTF is not selectively defective in binding of VIIa or zymogen VII.

Binding of VIIa to phospholipid-reconstituted full-length TF was further analysed using assays in the absence or presence of macromolecular substrate. In the first assay, complex-formation was determined with the sensitive fluorogenic substrate Mes-L-G-R-ANSN(C₂H₅)₂, which is hydrolysed at a substantially accelerated rate when VIIa is bound to TF [31]. We determined a similar $K_D(\text{app})$ for wild-type TF and TF_{R158G160} with this assay (Table 2). It is noteworthy that the $K_D(\text{app})$ is below the TF concentration, the lower limit of which was dictated by the sensitivity of the detection method. The $K_D(\text{app})$ from this analysis must therefore be considered as an upper-limit estimate. The second functional assay used factor X to detect TF–VIIa complex-formation; this $K_D(\text{app})$ may be influenced by macromolecular substrate interaction. There was no difference in the affinity of VIIa binding between wild-type TF and TF_{R158G160}, when analysed at receptor concentrations well below the $K_D(\text{app})$ (Table 2). Taken together, these data demonstrate that binding

Table 2 Binding of factor VIIa to full-length wild-type TF or TF_{R158G160}

Results are means $\pm$ S.D. for at least three determinations.

	Fluorogenic assay		Enzyme-linked assay	
	Maximum rate (nmol of ANSN(C ₂ H ₅) ₂ /min per nmol of TF–VIIa)	$K_D(\text{app})$ (pM)	Maximum rate (nmol of Xa/s per nmol of TF–VIIa)	$K_D(\text{app})$ (pM)
Wild-type TF	0.13 ± 0.02	27.8 ± 2.3	2.10 ± 0.31	2.1 ± 0.5
TF _{R158G160}	0.12 ± 0.02	35.0 ± 4.7	0.64 ± 0.14	4.4 ± 3.1

Table 3 Kinetic parameters for the activation of factor X by phospholipid-reconstituted TF–VIIa

Results are means $\pm$ S.D. ($n = 4$).

	K_m (nM)	k_{cat} (nmol of Xa/s per nmol of TF–VIIa)	k_{cat}/K_m ($\text{nM}^{-1} \cdot \text{s}^{-1}$)
Wild-type TF	73 ± 1	4.1 ± 0.8	$(5.6 \pm 0.90) \times 10^{-2}$
TF _{R158G160}	118 ± 12	1.9 ± 0.7	$(1.6 \pm 0.05) \times 10^{-2}$
TF _{A165A166}	643 ± 196	0.7 ± 0.1	$(0.1 \pm 0.01) \times 10^{-2}$

of VIIa or zymogen VII is not affected by the mutation of Trp¹⁵⁸ and Ser¹⁶⁰ in TF.

Support of VIIa catalytic function by TF_{R158G160}

The binding analyses employing chromogenic *p*-nitroanilide substrates (Table 1) and fluorogenic substrates (Table 2) demonstrated that VIIa in complex with TF_{R158G160} retained normal hydrolytic function towards small peptide pseudo-substrates. The enzyme-linked assay, however, revealed that the proteolytic function of the TF_{R158G160}–VIIa complex was reduced 3-fold compared with wild-type (Table 2). We determined the Michaelis–Menten parameters, which demonstrated a 2-fold increase in K_m and a 2-fold decrease in k_{cat} for the activation of factor X by TF_{R158G160}–VIIa compared with wild-type TF–VIIa in phospholipid (Table 3). The diminished efficiency of activation of factor X probably accounts for the difference in coagulant function which persisted when this mutant was compared with wild-type TF at high concentrations of VIIa (Figure 1). The 3.5-

Figure 2 Analysis of factor IX activation by mutant or wild-type TF–VIIa complexes

The activation of 15 μ M factor IX by 5 nM VIIa in the presence of phospholipid-reconstituted wild-type TF, TF_{R158G160} or TF_{A165A166} (5 nM) was analysed in TBS/5 mM CaCl₂ at 37 °C. Aliquots of 5 μ g of factor IX were quenched at the indicated time points in reducing sample buffer and separated on 8–16% gradient acrylamide gels, followed by Coomassie Blue staining. The bands correspond to (from the top): uncleaved factor IX, the heavy chain of factor IX α and the heavy chain of factor IX $\alpha\beta$. The light chain of factor IX stains poorly with the dye and is not well reproduced.

fold reduction in enzymic efficiency (k_{cat}/K_m) with TF_{R158G160} is modest in comparison with the 56-fold reduction for the double-replacement mutant TF_{A165A166}, resulting from the 9-fold increase in K_m and the 6-fold decrease in k_{cat} . These data indicate that the decreased activation of factor X is largely responsible for the coagulant defect of TF_{A165A166}.

Activation of factor IX by mutant or wild-type TF–VIIa

We further characterized the activation of factor IX (20 nM) by VIIa (50 pM) bound to stoichiometric amounts of TF, TF_{R158G160} or TF_{A165A166}. In the first experimental approach, factor IXa generation was quantified by a clotting assay. The rates of activation were indistinguishable for wild-type TF (3.2 ± 0.5 mol of factor IXa/min per mol of TF–VIIa) and TF_{R158G160} (2.8 ± 0.7 mol of factor IXa/min per mol of TF–VIIa), but a 2-fold decrease was observed for TF_{A165A166} (1.4 ± 0.2 mol of factor IXa/min per mol of TF–VIIa). Factor IX activation proceeds through two consecutive proteolytic steps where the intermediate factor IX α is non-enzymic and thus undetectable in clotting assays. We studied the cleavage of the two peptide bonds in factor IX by resolving the reaction products by SDS/PAGE under reducing conditions, as exemplified in Figure 2. Cleavage of the Arg¹⁴⁵–Ala¹⁴⁶ peptide bond yields the intermediate heavy-chain fragment, indicative of factor IX α . Subsequent cleavage of the Arg¹⁸⁰–Val¹⁸¹ bond releases the 35-amino acid activation peptide and generates the 30 kDa heavy-chain fragment of active factor IXa β . The generation of factor IX α and IXa β was quantified by laser densitometry of the gels (Figure 3). Factor IXa β formation by complexes of VIIa with wild-type TF or TF_{R158G160} were indistinguishable (Figure 3B), with activation

rates of 13 ± 6 mol of factor IXa β /min formed per mol of TF_{R158G160}–VIIa and 12 ± 5 mol of factor IXa β /min formed per mol of wild-type TF–VIIa. In contrast, the rate of factor IXa β formation (5.7 ± 2.3 mol/min per mol of complex) was reduced 2-fold for TF_{A165A166}–VIIa (Figure 3B).

The reduced rate of factor IXa β formation together with normal factor IX α generation in the presence of TF_{A165A166} resulted in the accumulation of intermediate product (Figure 3A). Intermediate product also accumulated with TF_{R158G160} (Figure 3A), despite normal generation of factor IXa β . These data indicate that the latter mutations in TF selectively enhanced the Arg¹⁴⁵–Ala¹⁴⁶ bond cleavage in factor IX. To exclude the possibility that factor IX α accumulation in these experiments occurred as the result of variations other than the mutations in TF, the following control experiments were performed. First, to exclude factor IX α generation by factor Xa, factor IX activation was studied in the presence of the potent factor Xa inhibitor, rTAP. Factor IX α formation was unchanged in the presence of rTAP (results not shown), excluding cooperation by factor Xa [23]. Second, to exclude the possibility that variations in the phospholipid content in the TF preparations influenced the factor IX activation kinetics, the relipidated TF was used as a phospholipid source in an assay that measured factor Xa-dependent activation of factor IX. Phospholipid in all TF preparations supported factor IX activation to the same extent (results not shown), excluding phospholipid composition as a factor that influences factor IX activation. The observed differences in the sequential cleavage of the activation bonds in factor IX are thus attributable to modifications resulting from the mutations in TF. The accumulation of intermediate product in the case of TF_{R158G160}, however, is not responsible for the VIIa dependence of the mutant's procoagulant function, because VIIa produced similar dose-responses in normal and factor IX-deficient plasma (results not shown).

Support of activation of zymogen VII by TF mutants

TF supports the activation of the bound zymogen VII by two proteases, i.e. factor Xa and VIIa in complex with TF. To eliminate a contribution from newly generated VIIa in the experiments, all activation reactions were performed with an active-site-mutated zymogen, VII_{A344}, which is devoid of catalytic activity because of mutation of the catalytic triad Ser. Activation of ¹²⁵I-labelled VII_{A344} was monitored by autoradiography. When a complex of VII_{A344} with TF was formed in the absence of added proteases, VII_{A344} was not converted into the two-chain enzyme VIIa, demonstrating that VII_{A344} indeed lacks proteolytic functions necessary for autoactivation. Under the reaction conditions employed, free ¹²⁵I-VII_{A344} also was not demonstrably converted by VIIa or factor Xa (results not shown), demonstrating a necessary role for TF. The activation of VII_{A344} was studied with a fixed concentration of wild-type or mutant TF reconstituted in mixed phosphatidylserine/phosphatidylcholine vesicles.

Figure 4 illustrates the progress curves of activation of the single-chain zymogen VII_{A344} to two-chain VIIa_{A344} by TF–VIIa (A) or factor Xa (B). We calculated half-maximal activation times of 53.3 ± 17.6 min for wild-type TF, 115.5 ± 38.5 min for TF_{R158G160}, and 346.5 ± 173.3 min for TF_{A165A166} when activation was studied with 1 nM VIIa. There was no apparent diffusion limitation in these experiments, as the rates of activation at 5-fold higher concentrations of VIIa were concordantly higher for all TFs tested. Half-maximal activation times were 10.7 ± 1.0 min with wild-type TF, 26.6 ± 2.2 min with TF_{R158G160} and 86.6 ± 10.8 min with TF_{A165A166}. These data thus establish that

Figure 3 Quantitative analysis of activation of factor IX by mutant and wild-type TF-VIIa complexes

Data from three experiments as exemplified in Figure 2 were quantified by laser densitometry of the stained gels. Serial dilutions of factor IX stained with Coomassie Blue produced linear densitometric values. The production of factor IXα (A) and IXαβ (B) over time is shown as means $\pm$ S.D. for wild-type TF (□), TF_{R158G160} (◇) and TF_{A165A166} (○).

the two mutants are deficient in supporting the activation of the TF-bound zymogen VII by proteolytically active TF-VIIa complexes.

Activation of VII bound to TF by factor Xa appeared to be greatly enhanced compared with the VIIa-mediated activation pathway. At 100 pM factor Xa, half-maximal activation times were 6.9 ± 0.2 min with wild-type TF, 16.1 ± 1.0 min with TF_{R158G160} and 53.3 ± 4.1 min with TF_{A165A166}. The rate of activation in the presence of TF_{A165A166} was still faster than the reaction in the presence of 100 μM mixed phospholipid, with half-maximal activation after 173.3 ± 43.3 min. TF thus enhances the activation of bound VII by factor Xa approx. 25-fold, and the support by the TF_{R158G160} and TF_{A165A166} mutants was reduced 2- and 8-fold respectively. When the factor Xa concentration was increased 10-fold, the rate of VII activation increased approx. 2-fold in each case, and half-maximal activation was observed after 2.8 ± 0.4 min with wild-type TF, 5.8 ± 1.0 min with TF_{R158G160} and 30.1 ± 6.6 min with TF_{A165A166}. The only slightly accelerated rate in the presence of higher concentrations of factor Xa suggests that enzyme binding in the reaction approaches saturation at subnanomolar concentrations. However, the relative loss of support of VII-activating function introduced by the mutations in TF was similar at each concentration of enzyme tested, demonstrating a role for the C-terminal module of TF in enzyme assembly during VII activation.

Figure 4 Activation of TF-VII_{A344} by VIIa (A) and factor Xa (B)

Activation of 10 nM ¹²⁵I-VII_{A344} by 1 nM VIIa (A) or 100 pM factor Xa (B) in the presence of 10 nM wild-type TF (□), TF_{R158G160} (◇) or TF_{A165A166} (○) is shown. The activation of free ¹²⁵I-VII_{A344} by 100 pM factor Xa in the absence of TF but presence of 100 μM phosphatidylserine/phosphatidylcholine vesicles is also shown (Δ) (B). Results are means $\pm$ S.D. ($n = 4$).

DISCUSSION

In this study, we characterized the activation of macromolecular substrates by VIIa in complex with two TF mutants, TF_{A165A166} and TF_{R158G160}. Both TF mutants had no apparent defect in binding of VII or VIIa, but they exhibited striking differences when analysed in a plasma-coagulation assay. Whereas the coagulant defect of TF_{A165A166} was only minimally affected by exogenously added VIIa, the TF_{R158G160} mutant had significantly improved coagulant function when analysed in the presence of 10 nM VIIa. This suggests that the coagulant defect of the latter mutant is largely due to a deficiency in the conversion of zymogen VII to the active enzyme VIIa.

Kinetic analysis of factor X activation demonstrated that the functional defect of TF_{A165A166} results from an increase in K_m combined with a decrease in k_{cat} of similar magnitude, leading to a greater than 50-fold reduction in enzymic efficiency. Concordant, but much more subtle, changes in K_m and k_{cat} were found with TF_{R158G160}, resulting in a 3.5-fold reduced enzymic efficiency of factor X activation. These data thus demonstrate a prominent role for Lys¹⁶⁵ and Lys¹⁶⁶ in the activation of factor X. Notably, factor IX activation was reduced only 2-fold in the presence of TF_{A165A166} and was normal with TF_{R158G160}, providing evidence for fine specificity of macromolecular substrate recognition by the TF-VIIa complex.

Both mutants inefficiently supported the activation of bound zymogen VII. Rates of activation to VIIa were reduced 2-fold when bound to TF_{R158G160} and 6-fold with TF_{A165A166}. The magnitude of the defect thus parallels the reduction in catalytic efficiency for the activation of factor X. Taken together with the virtually normal activation of factor IX, these data point to similarities in molecular recognition which are selectively shared by the macromolecular substrates factor X and VII. The Gla domain of VIIa binds to the C-terminal module of TF in close proximity to Lys¹⁶⁵ and Lys¹⁶⁶, the latter residue being largely exposed to solvent in the three-dimensional structure of the TF–VIIa complex [14]. The functional defect of the TF_{A165A166} mutant thus probably results from direct interactions with substrate. In contrast, Arg¹⁵⁸ is buried in the interface with the Gla domain and the mutational defect of TF_{R158G160} probably results from subtle reorientations of the VIIa Gla domain. We have previously shown that the Gla domain of VIIa is required for efficient activation of macromolecular substrate, but not for the activation of small peptide *p*-nitroanilide substrates [19], consistent with inhibition studies using VIIa Gla peptides [20]. These data support a hypothesis that residues of TF and the VIIa Gla domain co-operate to form a recognition structure for macromolecular substrate proteins.

It appears that the first EGF domain in factor X has little impact on the activation of factor X by TF–VIIa [39], whereas the first EGF domain of factor IX is important for the interaction with TF–VIIa [40]. This is consistent with the observed differences of the mutations in TF on the activation of these two different substrates. Binding of factor X to phospholipid through its Gla domain facilitates the activation by TF–VIIa [2,41], and deletion of the Gla domain of factor X markedly decreases proteolytic cleavage of factor X by sTF–VIIa [39], suggesting that the Gla domain of substrate factor X provides a complementary site for interaction with the Gla domain of enzyme VIIa aligned with the C-terminal module of TF. It may be possible that the high-affinity Ca²⁺-binding site of the first EGF domain is important for proper alignment of the Gla domain and the EGF domain, and thus helps to orient the substrate for assembly with TF–VIIa. From the similar effect of the TF mutations on VII and factor X activation by TF–VIIa, we infer that the Gla domain of substrate VII is possibly involved in the interaction with TF–VIIa because of the localization in a membrane proximal position after binding to TF.

It was surprising that the activation of VII complexed with each of the TF mutants was similarly impaired when factor Xa or TF–VIIa were the activating proteases. This implies that TF plays a role in presentation of the substrate VII scissile bond to the activating protease. We have previously shown that TF_{A165A166} is also deficient in allowing the rapid assembly of the TF pathway inhibitor in the presence of factor Xa [26]. These data indicate that, under certain conditions, factor Xa assembles in a substrate-like manner with TF. In the case of TF pathway inhibitor, factor Xa provides facilitated assembly of the first Kunitz-type domain with TF–VIIa, whereas the assembly of factor Xa with TF–VII is energetically favoured by the availability of the scissile bond in the zymogen VII. The data presented are consistent with a predominant role for the C-terminal module of TF in providing support for macromolecular substrate and inhibitor assembly.

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