The Tissue Factor Region That Interacts with Factor Xa in the Activation of Factor VII

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ABSTRACT: Tissue factor is the cell membrane-anchored cofactor for factor VIIa and triggers the coagulation reactions. The initial step is the conversion of factor VII to factor VIIa which, in vitro, is efficiently catalyzed by low concentrations of factor Xa. To identify the tissue factor region that interacts with the activator factor Xa during this process, we evaluated a panel of soluble tissue factor (1−219) mutants for their ability to support factor Xa-mediated activation of factor VII. The tissue factor residues identified as most important for this interaction (Tyr157, Lys159, Ser163, Gly164, Lys165, Lys166, and Tyr185) were identical to those found to be important for the interaction of substrate factor X with the tissue factor·factor VIIa complex. The residues form a continuous surface-exposed patch with an area of about 500 Å^2, which appears to be located outside the tissue factor−factor VII contact zone. In agreement, the two monoclonal antibodies 5G6 and D3H44-F(ab′)$_2$, whose epitopes overlap with this identified region, inhibited the rates of factor VII activation by 86% and 95%, respectively. These antibodies also strongly inhibited the conversion of ^{125}I-labeled factor VII when cell membrane-expressed, full-length tissue factor (1−263) was employed. Together the results suggest the usage of a common surface region of tissue factor in its dual role—as a cofactor for factor Xa-mediated factor VII activation and as a cofactor for factor VIIa-mediated factor X activation. The finding that factor Xa and factor X may engage in similar, if not identical, molecular interactions with tissue factor further indicates that factor Xa and factor X are similarly oriented toward their respective interaction partners in the ternary catalytic complexes.

Tissue factor (TF)[1] is the cell membrane-anchored cofactor of the serine protease factor VIIa (VIIa) and is localized in the vascular wall (*1*). Injury exposes TF to circulating blood, resulting in the formation of TF·VIIa complex, which initiates the coagulation cascade by activating substrates factor IX (IX) (*2*), factor X (X) (*3*), and factor VII (VII) (*4, 5*). The coagulation reactions culminate in the generation of thrombin, which processes fibrinogen to fibrin and also activates platelets. This leads to the formation of a hemostatic plug consisting of an insoluble fibrin meshwork and platelet aggregates. TF-initiated coagulation may also be involved in various cardiovascular disease processes including atherosclerosis (*6, 7*) and ensuing acute thrombotic events (*8, 9*), restenosis (*10*), reperfusion injury (*11*), disseminated intravascular coagulation (*12, 13*), and venous thrombosis (*14, 15*).

The initial step in the TF pathway of coagulation is the conversion of VII (zymogen) to VIIa (activated VII). A number of proteases are capable of converting VII in vitro, including factor Xa (Xa) (*16*−*19*), factor IXa (IXa) (*20*−*22*), factor XIIa and fragments thereof (*20, 23, 24*), thrombin (*18*), hepsin (*25*), and a hepatocyte growth factor activator-like serine protease from human plasma (*26*). In addition, VII is activated in an autocatalytic manner by VIIa or TF·VIIa (*4, 5, 27, 28*). It has been suggested that autoactivation of VII could be important under certain physiological conditions (*5*), but it remains unclear to what degree this pathway contributes to VII conversion in hemostasis and thrombosis. On the other hand, Xa, at picomolar concentrations, very efficiently catalyzes the conversion of VII to VIIa and, in all likelihood, is the physiologically relevant VII activator (*29*−*31*).

VII activation by Xa was shown to be dependent on the presence of TF (*29, 30, 32*). VII binds to TF with about the same affinity as VIIa (*33*−*36*), which may indicate that the molecular interactions of VII with TF are similar to those of VIIa. Biochemical studies (*36*−*43*) and the crystal structures of TF (*44, 45*), VIIa (*46*−*48*), and TF·VIIa (*49, 50*) provide a structural context for considering the molecular interactions of TF with VIIa and of TF·VIIa complex with substrates X and IX. An important function of TF is to properly orient the VIIa heavy and light chains with respect to substrate. The active site of VIIa is thereby positioned at a distance of about 70−80 Å from the membrane surface (*49, 51, 52*).

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[1] Abbreviations: TF, tissue factor; sTF, soluble tissue factor (1−219); sTF$_{wt}$, wild-type sTF; VII/VIIa, factor VII/VIIa; IX/IXa, factor IX/IXa; X/Xa, factor X/Xa; PCPS phosphatidylcholine/phosphatidylserine; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; HBSA, 20 mM Hepes, pH 7.5, 0.5 mg/mL BSA, 150 mM NaCl, and 5 mM $CaCl_2$; SPR, surface plasmon resonance; 293-wt, wild-type 293 cells; 293-TF, TF-expressing 293 cells.

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TF not only binds VIIa but also interacts with substrates X and IX. This involves a defined surface-exposed region with the size of about half of the TF−VIIa contact area (*53*). This region is located in the C-terminal fibronectin type III domain of TF and includes the lysine residues 165 and 166 (*54*−*56*). It borders the γ-carboxyglutamic acid (Gla) domain of VIIa which, together with TF, may form an extended substrate recognition region (*56*−*59*). This TF region interacts with the Gla domain of the substrate X (*56*, *57*) and might also interact with other proteins and protein complexes, such as with VII/VIIa in autoactivation (*60*, *61*) and with TFPI−Xa in the inhibition of TF·VIIa (*62*).

We reasoned that since this TF region appears optimally suited to interact with Gla-containing proteins, Xa may also interact with this TF region in the activation of TF-bound VII. To test this hypothesis, we investigated a panel of soluble TF (sTF) mutants for their ability to support VII activation by Xa. The results suggested that Xa (as activator) and X (as substrate) utilize similar, if not identical, molecular interactions with TF in two different enzymatic processes. We discuss the implications on our understanding of the structural relationship between the ternary complexes TF·VII·Xa and TF·VIIa·X.

MATERIALS AND METHODS

Reagents. Fatty acid-free BSA was from Calbiochem (La Jolla, CA). Purified human recombinant VII, expressed in human 293 cells (*48*), was a gift from Mark O'Connell (Genentech, Inc.). Xa was from Haematologic Technologies Inc. (Essex Junction, VT). Chromozym t-PA was from Boehringer Mannheim (Indianapolis, IN). The anti-TF antibody 5G6 was described recently (*63*). The IgG-matched anti-TF control antibody 1H7 came from the same fusion as described for 5G6 (*63*). The anti-TF antibody D3H44-F(ab′)$_2$ (*64*) is a humanized version of the originally described murine antibody D3 (*63*, *65*), and the corresponding control F(ab′)$_2$, humanized anti-neurturin (NTN), was described previously (*66*).

Site-Directed Mutagenesis, Expression, and Purification of Soluble TF (sTF) Mutants. Expression of sTF (1−219) mutants in *E. coli* and subsequent purification on a D3 antibody affinity column were carried out as described earlier (*41*, *53*). For the two double mutants N199A:R200A and K201A:D204A, a 7G11 antibody affinity column was used for purification (*53*). Protein concentrations were determined by absorbance measurements using an ϵ_{280} of 29.4 mM^{-1} cm^{-1} calculated from quantitative amino acid analysis data. An ϵ_{280} of 24 mM^{-1} cm^{-1} was used for the W158F mutant.

Preparation of Membrane Fractions of Wild-Type 293 Cells and of TF (1−*263)-Expressing 293 Cells.* Human wild-type 293 cells (293-wt) (*65*, *67*) were cultured in serum-supplemented medium. Production of human 293 cells expressing recombinant full-length TF (1−263) (293-TF) was as described (*68*). Cells were expanded in 850 cm^2 roller bottles (Corning Inc., Corning, NY) until they reached confluence. The cell layers were washed in PBS, detached with 10 mM EDTA, and centrifuged twice (2500 rpm on a Beckman GSA) for 5 min, with a PBS wash after the first centrifugation run. The cell pellet [(4−5) × 10^7 cells/mL] was suspended in 50 mM Tris, pH 7.5, and homogenized using a Dounce homogenizer, followed by centrifugation (2500 rpm) for 5 min at 4 °C. The membrane-containing supernatant fraction was collected and centrifuged at 45000*g* (Sorvall RC 5B, DuPont Co., Newtown, CT). The protein concentration of the cell membrane fraction was determined using the BCA assay (Pierce, Rockford, IL), and the membranes were stored in aliquots at −80 °C until use.

VII Activation Assay with Wild-Type 293 Cell Membranes. Membrane fractions of wild-type 293 cells (293-wt) were incubated for 20 min in 20 mM Hepes, pH 7.5, containing 150 mM NaCl, 0.5 mg/mL BSA, and 5 mM $CaCl_2$ (HBSA buffer) together with VII and sTF mutants. The reaction was started by adding Xa. The concentrations of reactants in this mixture were as follows: 50 nM VII, 150 μg/mL 293-wt cell membranes (total protein concentration), 100 nM sTF, and 0.2 nM Xa. For the two double mutants N199A:R200A and K201A:D204A, the concentration was 200 nM instead of 100 nM; 100 μL aliquots of the reaction mixture were taken at different time points up to 2 min and quenched in 125 μL of 3 μM Xa active site inhibitor G8053 (gift from Tom Rawson, Department of Bioorganic Chemistry, Genentech, Inc.) in HBSA buffer. In the second stage of the assay, 25 μL of 5 mM Chromozym t-PA was added and the absorption at 405 nm measured on a kinetic microplate reader (Molecular Devices). The linear rates of VIIa generation were expressed as relative activities (v_{mut}/v_{wt}) with respect to the activity of wild-type sTF (sTF_{wt}). The contaminating VIIa in the VII preparations was estimated to be about 7% of VII, according to amidolytic assays.

All tested sTF mutants maintained sTF_{wt} activity in separately performed amidolytic assays with VIIa using identical conditions as in the VII activation assays. These results suggested that the sTF mutants maintained sTF_{wt} binding to VIIa as reported recently (*53*) and that reduced VII activation observed for several tested sTF mutants was not due to impaired amidolytic activity. Furthermore, additional experiments demonstrated that the employed Xa active site inhibitor G8053 had no effect on sTF_{wt}·VIIa amidolytic activity at the concentrations used in VII activation assays, yet it completely inhibited Xa enzymatic activity (data not shown).

For testing murine anti-human TF antibodies, the conditions were as follows: 1 μM antibody, 200 nM sTF_{wt}, 50 nM VII, 150 μg/mL 293-wt cell membranes, and 0.2 nM Xa. For testing the humanized D3H44-F(ab′)$_2$, we used a higher concentration of sTF_{wt} (1 μM) and of antibody (3 μM). Under these conditions, neither the D3H44-F(ab′)$_2$ nor the murine anti-TF antibodies affected the amidolytic activity of TF·VIIa toward Chromozym t-PA measured in the second stage of the assay. The humanized control antibody F(ab′)$_2$ (anti-neurturin, NTN) and the murine control antibody 1H7 had no effects on sTF_{wt}·VIIa amidolytic activity.

^{125}I-Labeling of VII. Two hundred fifty microliters of a solution of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) (Pierce Chemical Co., Rockford, IL) in chloroform (0.5 mg/mL) was placed into 5 mL borosilicate glass tubes. Solvent was evaporated at room temperature under a steady stream of nitrogen gas and the dried material stored in a desiccator until further use.

VII (170 μg) in 50 mM Tris, 150 mM NaCl, pH 7.5, buffer (TBS) was added to the dried Iodogen material. ^{125}I-Labeled sodium solution (NEN Life Sciences Inc., Boston, MA) was added (5 μCi/μg of protein), and the reaction mixture was

incubated on ice for 5 min with gentle swirling. The material was then applied onto a PD-10 column (Pharmacia, Uppsala, Sweden) which had been equilibrated with 20 column volumes of TBS buffer containing 5 mg/mL BSA. Fractions containing the ^{125}I-labeled VII were collected and pooled. The specific activity was 0.6 μCi/μg of VII.

Iodinated VII was compared with unlabeled VII in a X activation assay using relipidated TF(1−243) (*69*). Using a wide range of VII concentrations, the initial rates of X activation for unlabeled and labeled VII were identical, suggesting that the iodination of VII did not impair the functional properties.

Activation of ^{125}I-Labeled VII Using TF(1−263)-Expressing 293 Cell Membranes. The anti-TF antibodies were incubated in HBSA buffer together with cell membranes of TF(1−263)-expressing 293 cells (293-TF) and ^{125}I-VII for 20 min before Xa was added to start VII conversion. To prevent autoactivation of TF·VII complexes by the newly generated TF·VIIa, we added the specific, reversible VIIa active site inhibitor G8119 (gift from Alan Olivero, Department of Bioorganic Chemistry, Genentech Inc.). The concentration of the active site inhibitor used (5 μM) completely inhibited autoactivation. This concentration of G8119 did not interfere with Xa enzymatic activity, since a 4-fold higher concentration (20 μM) did not result in any significant changes of VII activation. Furthermore, the employed concentration of 5 μM had no effect on ^{125}I-VII activation in assays that used sTF and 293-wt cell membranes, further indicating that G8119 did not affect Xa enzymatic activity. The concentrations of the reactants in the reaction mixture were as follows: 1 μM antibody, 100 μg/mL membrane TF_{1-263} (total protein concentration), 10 nM ^{125}I-VII, 0.2 nM Xa, and 5 μM G8119. At different time points, aliquots were removed and added to sample buffer (Bio-Rad Laboratories, Hercules, CA) containing the reducing agent dithiothreitol (Bio-Rad). After a brief heating, samples (approximately 10^5-cpm/lane) were loaded onto a 4−20% gradient polyacrylamide gel (Invitrogen Corp., Carlsbad, CA). After electrophoresis, the dried gels were exposed on X-ray films (X-OMAT AR, Eastman Kodak Co., Rochester, NY) for 12−24 h. Films were developed (Kodak M35A X-OMAT Processor), scanned (Umax S-12, Umax Data Systems, Inc., Fremont, CA), and further processed with Adobe Photoshop V.5.5 software (Adobe Systems Inc., San Jose, CA).

Activation of ^{125}I-Labeled VII Using sTF Mutants and Wild-Type 293 Cell Membranes. ^{125}I-Labeled VII was incubated in HBSA buffer with sTF_{wt} or sTF mutants in the presence of 293-wt cell membranes for 20 min, after which Xa was added. The concentrations in this reaction mixture were the following: 100 μg/mL (total protein concentration) 293-wt cell membranes, 50 nM ^{125}I-VII, 100 nM sTF_{wt} or sTF mutant. At different time points, aliquots were removed and added to sample buffer and further analyzed by SDS−PAGE as described above.

Determination of Binding Constants for VII/VIIa Binding to sTF. The binding affinity of VII or VIIa to immobilized sTF was determined by surface plasmon resonance (SPR) measurements on a Pharmacia BIAcore 2000 instrument (Pharmacia Biosensor). Both the K165A·K166A mutant of sTF and the E219C-sTF mutant (*70*) were immobilized as previously described (*70*). Briefly, the K165A·K166A mutant was immobilized at a level of 1400 resonance units (RU) via random coupling through amino groups. E219C-sTF was covalently linked at a level of 700 RU by thiol-directed coupling. Sensorgrams were recorded for VII or VIIa binding at concentrations ranging from 15.6 to 500 nM in 2-fold increments. Software supplied by the manufacturer was used to perform a nonlinear regression analysis of the data according to a 1:1 binding model. Dissociation constants were calculated from the kinetic constants determined in this fashion.

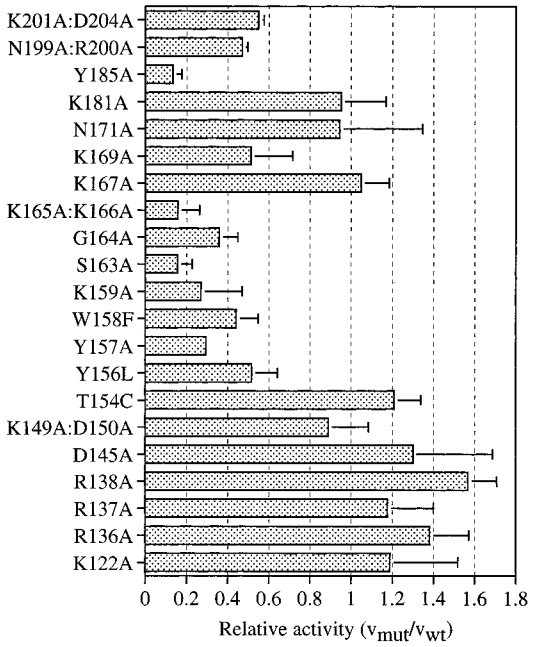

FIGURE 1: Effects of sTF mutants on VII activation by Xa. Activation rates were determined in a two-stage assay using purified membranes of wild-type 293 cells (293-wt) as a phospholipid source. The reaction was started by adding Xa (0.2 nM) to a mixture of VII (50 nM) and molar excess of sTF and 293-wt cell membranes in HBSA buffer. Timed aliquots were quenched in a Xa inhibitor solution and concentrations of generated VIIa measured with chromogenic substrate. The effects of sTF mutants were expressed as relative activities (v_{mut}/v_{wt}). The values are the mean ± SD of three experiments.

RESULTS

To measure the sTF-dependent activation of zymogen VII by Xa, we employed cell membranes as a phospholipid source. The wild-type 293 (293-wt) cell membranes used neither express TF (*67*) nor support autoactivation of sTF·VII complexes by sTF·VIIa, in accord with results from using sTF and PCPS vesicles (*28*, *71*). To study the effect of substitutions in the C-terminal TF domain on VII activation catalyzed by Xa, molar excess of sTF was used, so that all VII molecules were bound to sTF. Under these conditions, the rate of VII conversion with 293-wt cell membranes was 19.6 ± 0.75 nM VIIa (nM Xa)$^{-1}$ min^{-1}. As shown in Figure 1, the set of substitutions in sTF affecting Xa-dependent VII activation are similar to those previously identified to affect utilization of substrate X (*53*). The five sTF mutants Y157A, K159A, S163A, K165A:K166A, and Y185A had less than 30% of sTF_{wt} activity. Moreover, six additional sTF mutants (Y156L, W158F, G164A, K169A, N199A:R200A, K201A:D204A) showed activities of 30−75% relative to sTF_{wt}. The remaining sTF mutants displayed activities of more than 75% and were considered to possess normal activities. Earlier

Table 1: Binding Constants for FVII/FVIIa Binding to sTF

immobilized sTF variant	ligand	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})	K_D (nM)
E219C (sTF_{wt})	FVIIa	9.0	1.3	1.4
E219C (sTF_{wt})	FVII	8.3	2.1	2.6
K165A:K166A	FVIIa	2.9	0.84	2.9
K165A:K166A	FVII	2.2	1.7	7.9

experiments with most of these sTF mutants (except for K122A, D145A, and K181A) demonstrated that they maintained wild-type levels of VIIa binding affinity and support of amidolytic activity (*53*). Thus, the reduced activities of sTF mutants were not due to impaired amidolytic activity of sTF mutant·VIIa complexes in the second stage of the assay.

It remained possible that zymogen VII bound to sTF in a different manner than VIIa. If, for example, VII bound with its light chain to TF residues examined in our mutagenesis study, then the effects of sTF mutants may result from interference with VII binding rather than with Xa docking. To address this issue, we performed SPR experiments comparing the binding of VII or VIIa to mutant (K165A:K166A) or wild-type sTF. For preparation of the wild-type sTF surface, we used site-specific coupling via an engineered Cys residue (E219C). Thiol-directed coupling is more efficient than amine coupling, and the E219C-sTF has affinity for VIIa identical to wild-type sTF (*70*). The results showed that zymogens VII and VIIa bound to wild-type sTF about equally well (Table 1), which agrees with published results (*33*−*36*). More importantly, the affinity difference between wild-type sTF−VII binding and K165A:K166A−VII binding was only 3-fold (Table 1). Also, the binding affinity of zymogen VII to the sTF mutant K165A:K166A was less than 3-fold weaker than that of VIIa (Table 1). These results suggested that the effects of the investigated sTF mutants are not due to impaired VII−sTF mutant binding.

The activities of sTF mutants were also determined by analyzing the rates of ^{125}I-VII conversion using SDS−PAGE. The results obtained were in agreement with the relative activities determined in the two-stage amidolytic assay. The sTF mutants Y185A and K165A:K166A are shown as representative examples in Figure 2. The conversion of the zymogen VII into the two-chain VIIa form was significantly delayed with both sTF mutants. In the presence of sTF_{wt}, substantial amounts of VII were already converted after 15 min, whereas none or very little VIIa was generated with K165A:K166A and the Y185 sTF mutants (Figure 2). There was some residual VIIa present in the labeled VII preparation (zero time point in Figure 2). This was consistent with the small amounts of VIIa present in the VII material that was labeled. Using amidolytic assays with Chromozym t-PA substrate, it was estimated that about 7% of the VII was present as VIIa. Because the experimental conditions with 293-wt cell membranes did not allow for any VII autoactivation to occur, even after addition of unlabeled VIIa (data not shown), this residual VIIa activity was of no consequence.

The TF residues identified as important for Xa-mediated activation of VII were mapped onto the crystal structure of TF (Figure 3a). The residues identified as important for the Xa-catalyzed activation of VII form a nearly continuous surface on the C-terminal fibronectin type III domain of TF

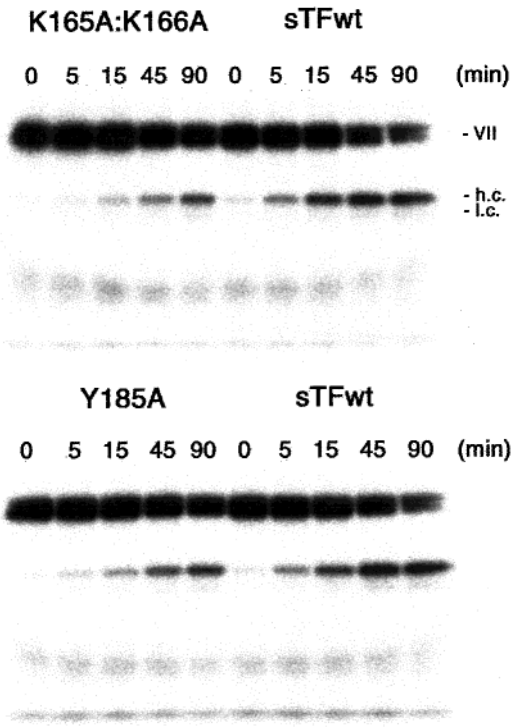

FIGURE 2: Effects of sTF mutants on the activation of ^{125}I-labeled VII by Xa. The sTF mutants or sTF_{wt} (100 nM) was incubated together with ^{125}I-VII (50 nM; specific activity 0.6 μCi/μg of VII) and 293-wt cell membranes. The reaction was started by adding Xa (0.2 nM), and aliquots were removed at 0, 5, 15, 45, and 90 min and directly added to sample buffer. Polyacrylamide gel electrophoresis was carried out under reducing conditions on 4−20% gradient gels, and the dried gels were exposed on X-ray films. Indicated in the top panel are the zymogen VII (VII) and the heavy chain (h.c.) and light chain (l.c.) of activated VII.

(Figure 3a). If this TF region was indeed important for interaction with Xa during VII activation, then antibodies which bind to this region should interfere with VII conversion. To test this hypothesis, we used two recently described anti-TF antibodies, 5G6 and D3, which bind to this TF region (*63*). The antibody epitopes include residues Lys165 and Lys 166; antibody D3 additionally interacts with Tyr156 (*63*). For these experiments, we employed the murine 5G6 antibody and a humanized $F(ab')_2$ variant of the D3 antibody, D3H44-$F(ab')_2$, which binds with higher affinity than the original murine D3 (*64*). In VII activation experiments, 5G6 and D3H44-$F(ab')_2$ inhibited the rates of VII activation by 86% and 95%, respectively (Figure 4). In contrast, a nonrelevant humanized control $F(ab')_2$ (NTN) and a TF-binding, IgG-matched murine control antibody (1H7) had no effect (Figure 4). Unlike 5G6, the D3 antibody (*63*) and its humanized counterpart slightly interfered with sTF−VIIa interaction at sTF concentrations (100 nM) normally used in the assay. Therefore, a relatively high concentration of sTF (1 μM) was used to minimize this effect. Additional experiments were carried out with ^{125}I-labeled VII using identical conditions. We found that, consistent with the amidolytic assay data, ^{125}I-VII conversion was strongly inhibited by both antibodies (data not shown).

We performed additional experiments with 293 cells expressing full-length TF (1−263) (293-TF), because it was possible that sTF might not entirely recapitulate all aspects of membrane-anchored TF. As observed by others using membrane-expressed TF (*4*, *28*), there was substantial

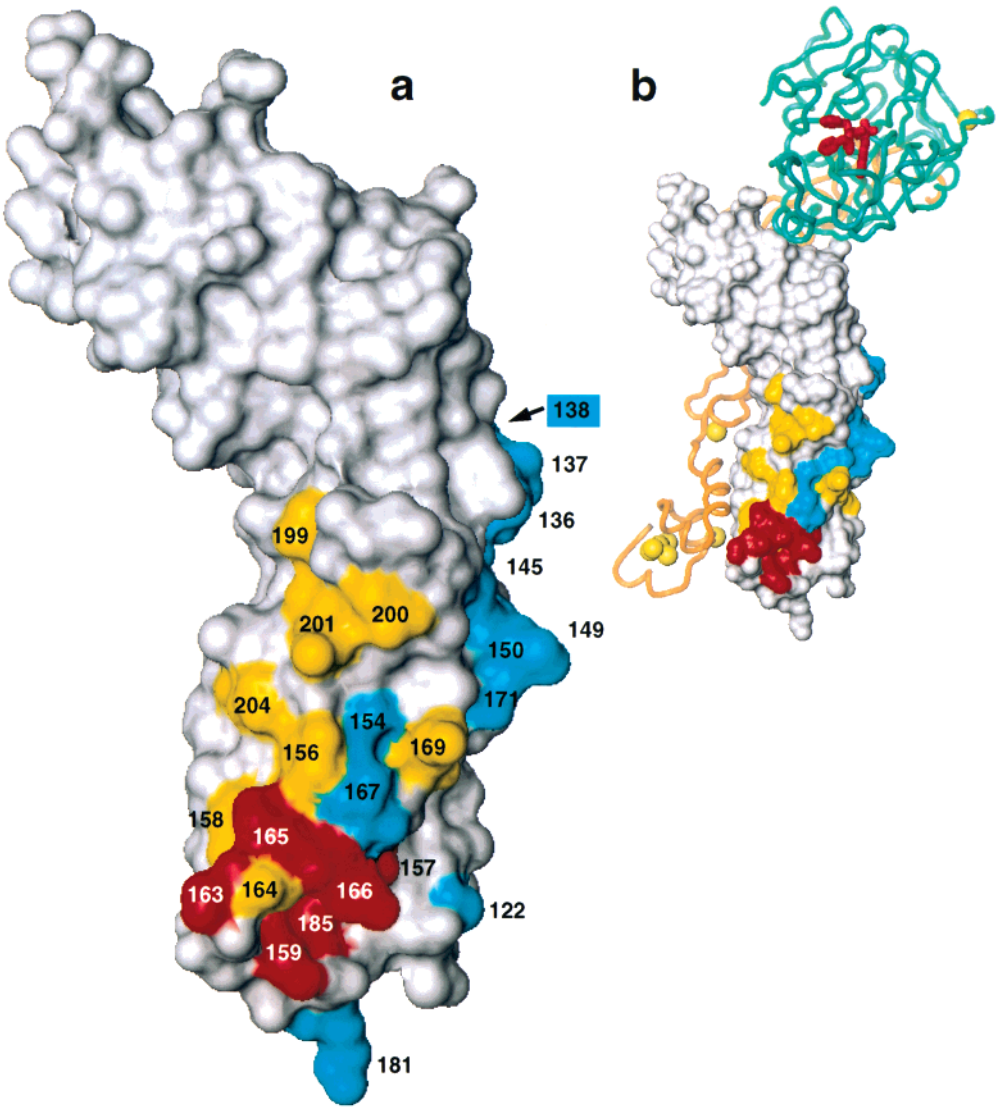

FIGURE 3: Localization of the Xa interaction region on tissue factor in comparison to the X interaction region. The orientation of TF is identical for both figures. (a) Crystal structure of TF (*72*). TF residues, which upon mutation resulted in relative activities (v_{mut}/v_{wt}) in VII activation assays (see Figure 1) of less than 0.3, are in red, those with relative activities of 0.3−0.75 are in yellow, and those >0.75 (considered as not different from TF_{wt} activity) are in blue. For the double mutants K165A:K166A, N199A:R200A, and K201A:D204A, both residues were assigned as equally important for VII activation, even though a more specific single-residue analysis was not performed. (b) Crystal structure of the TF·VIIa complex (*49*) depicting the X interaction region of TF (*53*). The indicated TF residues were analyzed in X activation assays (*53*) and are color-coded according to panel a.

autoactivation with 293-TF membranes. By adding a specific VIIa active site inhibitor (G8119), autoactivation was completely inhibited, yet allowed TF·VII activation by Xa to proceed unabated (Figure 5a). As for experiments with sTF, the anti-TF antibodies 5G6 and D3H44-F(ab′)$_2$ were used to examine the role of the identified TF region in the Xa-mediated VII activation process. As displayed in Figure 5, both antibodies strongly inhibited conversion of ^{125}I-labeled VII to VIIa. For example, in the presence of D3H44-F(ab′)$_2$, the amount of generated VIIa (heavy and light chain staining) after a 90 min reaction time appeared less than that in uninhibited reactions at the earliest time point of 5 min. Similar degrees of inhibition were seen with 5G6 (Figure 5c). As control antibodies, we used NTN, a humanized F(ab′)$_2$ directed against neurturin, and the 1H7 antibody that binds to a different TF region than 5G6 and D3 (data not shown). In separate experiments, the control antibodies did not show any inhibition of ^{125}I-labeled VII conversion in comparison to buffer controls. Experiments were repeated at least 3 times, and results were identical to those shown in Figure 5.

DISCUSSION

A distinct surface-exposed region located in the C-terminal TF domain was previously shown to be important for the recognition of substrate X (*53*−*55*, *60*). Here, we provide evidence for an additional role of this TF region in the interaction of the TF·VII complex with its activator Xa. The surprising identity of the TF residues involved in these two enzymatic reactions suggested that nearly identical molecular contacts occur in the dual role of TF in the TF·VII and the TF·VIIa complex.

The evidence for the presence of a distinct TF region that interacts with Xa in the VII activation process was derived from three different experimental approaches. First, the TF residues which played an important role in interacting with Xa were identified by using sTF mutants in cell membrane-

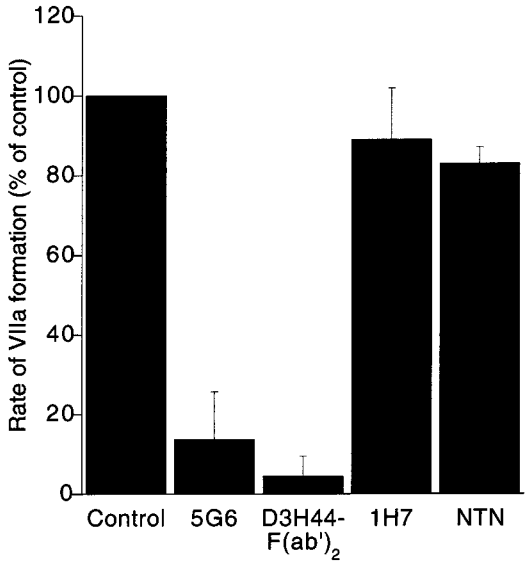

FIGURE 4: Inhibition of Xa-mediated VII activation by monoclonal anti-TF antibodies. VII (50 nM), sTF_{wt} (0.2 μM for murine and 1 μM for humanized antibodies), and molar excess of antibodies were incubated for 20 min in the presence of 293-wt cell membranes. The reaction was started by adding Xa (0.2 nM), and aliquots were removed and quenched at different time points. Using a VIIa amidolytic substrate in the second stage of the assay, the rates of VIIa generation were determined and expressed as percent of uninhibited (control with no antibody present) rates. 5G6, murine anti-TF antibody; 1H7, murine anti-TF control; D3H44-F(ab′)$_2$, humanized anti-TF F(ab′)$_2$; NTN, humanized F(ab′)$_2$ control (anti-neurturin).

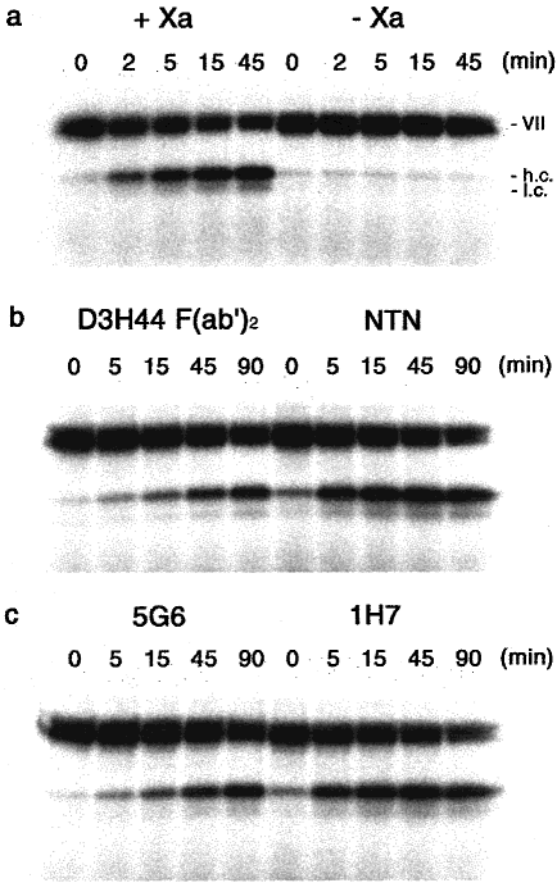

FIGURE 5: Xa-mediated conversion of ^{125}I-labeled VII in complex with 293 membrane-expressed full-length TF (1−263). The antibodies (1 μM) were incubated together with 293-TF cell membranes and ^{125}I-VII (10 nM). The reaction was started by adding Xa (0.2 nM). To prevent VII autoactivation, a VIIa active site inhibitor (5 μM) was added. Aliquots were removed at different time points, added to sample buffer, and analyzed by SDS−PAGE (reducing conditions) using a 4−20% gradient gel, followed by exposure on X-ray films. (a) Dependence of ^{125}I-VII activation on Xa (+Xa). Note that the time points are different from those in panels b and c. (b) Effects of humanized D3H44-F(ab′)$_2$ and the control F(ab′)$_2$ NTN. (c) Effects of murine antibody 5G6 and the isotype-matched control murine 1H7.

based assays. The effects were specific and were interpreted as stemming from a perturbed interaction of sTF with Xa. The use of a soluble form of TF precluded autoactivation (*28*, *71*) by preexisting and newly formed TF·VIIa complexes, which might have obscured such an interpretation. Second, two different antibodies, 5G6 and D3H44-F(ab′)$_2$, whose epitopes overlap with the identified TF region, strongly inhibited VII conversion. Third, experiments with cell membrane-expressed TF (1−263) were carried out to confirm that the obtained results were not due to possible shortcomings of sTF in recapitulating the functional properties of full-length TF. The observed strong inhibition of VII conversion by the antibodies 5G6 and D3H44-F(ab′)$_2$ was entirely consistent with the mutagenesis data.

In the crystal structure of TF (*72*), the 14 identified residues are located in the earlier identified substrate contact region of TF (Figure 3) (*53*). Our findings agree with studies by Ruf et al. (*60*) and Dittmar et al. (*61*), suggesting a role for some of these residues in Xa-mediated activation of VII. The seven most important TF residues (Tyr157, Lys159, Ser163, Gly164, Lys165, Lys166, Tyr185) for this reaction form a continuous surface-exposed patch and were also found as the most important ones in TF·VIIa-mediated activation of X (Figure 3b) (*53*). This region covers an area of about 500 Å^2 and, in the TF·VIIa complex, is located outside the TF−VIIa contact area. We assume that this is also true for the TF·VII complex, although it has not yet been demonstrated that the interactions of TF with VII are exactly the same as with VIIa. The comparable binding affinities of VII and VIIa to TF and those of VII and VIIa protease domain mutants (*36*) would suggest that the interactions may indeed be very similar (*33*−*36*). In addition, the sTF mutant K165A:K166A bound about equally well to VII and VIIa, further supporting the view that, at least with respect to the light-chain interactions with the C-terminal TF domain, VII binds largely in the same way as VIIa.

The X-Gla domain was described to contact TF during TF·VIIa-mediated activation of X (*56*, *57*). The Gla domain conformations of Xa and X are likely to be the same, and, therefore, the residues of the Xa- and X-Gla domains that interact with this common TF region could be similar or even identical. This has further implications on how to view the global docking events of Xa and of X with their respective substrate and enzyme complexes. If the Gla domains of Xa and X are oriented the same way when contacting the TF interaction region, then the entire Xa and X molecules may be oriented in a similar fashion. A (hypothetical) schematic of the relative protein orientations in the ternary catalytic complexes is depicted in Figure 6. The active sites of both enzymes involved in these two enzymatic reactions, Xa and TF·VIIa, are located about 60−80 Å above the phospholipid surface (*49*, *51*, *52*, *73*). This implies that the P1 residues of the scissile peptide bonds of the zymogens TF·VII and X are at about the same distance from the phospholipid surface.

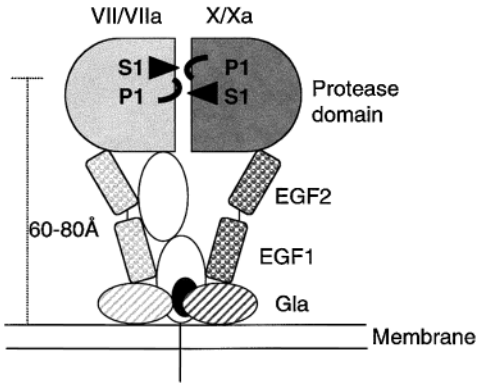

FIGURE 6: Schematic of the possible zymogen vs active enzyme orientations in the ternary catalytic complexes TF·VII·Xa and TF·VIIa·X. TF is depicted as two open ovals (N- and C-terminal domains). The identified TF region (~500 Å^2) is shown as a filled black oval in the C-terminal (membrane proximal) TF domain. The serine proteases (zymogens and active enzyme) are composed of the light chains (Gla, EGF1, EGF2) and heavy chains (protease domains). The substrate specificity pocket (S1) for the active enzymes and the P1 residues (Arg152 for VII and Arg52 for X) of the scissile bonds of the zymogen are indicated. Although the scheme depicts a monomeric TF·VII complex interacting with Xa, it is possible that, similar to predictions made for TF·VII autoactivation (*83*), the substrates for Xa might be TF·VII dimers $(TF·VII)_2$.

Therefore, the P1 residue of VII (Arg152) has to be located somewhere on the surface of the VII heavy chain at a distance of 60−80 Å from the membrane, as has the P1 residue of X in the TF·VIIa·X complex (Figure 6). Given the premise that the global heavy chain orientations (VII vs Xa and VIIa vs X) are similar, then one could conclude that the P1 residue (Arg152) in VII zymogen is topographically proximal to the location of the active site, which is formed upon conversion to active enzyme. The same could hold for the relationship of the X P1 residue vs Xa active site. Zymogen VII (or X) and the active enzyme VIIa (or Xa) likely differ in the position of several surface-exposed loops, i.e., the canonical 'activation domain' (*74*, *75*). Therefore, even with the assumption of similar heavy chain orientations (as depicted in Figure 6), one would predict that on a molecular level the interaction points between the heavy chains of Xa and VII and those between VIIa and X are not the same.

Alternatively, the heavy chain orientations (VII vs Xa and VIIa vs X) may differ significantly, and this could be accomplished by rotational movements of X and Xa along the 'hinges', two short amino acid stretches between Gla−EGF1 and EGF1−EGF2. In such a case, P1 residues and active sites must not necessarily be in proximity. Unfortunately, the structures of the zymogens VII and X are unknown, and the published structures of VIIa (*46*−*50*) do not provide information on the P1 location, since the C-terminal portions of the light chains (8−10 residues) were not resolved. In the case of Xa, the P1 residue (Arg52) is part of the activation peptide that is released upon X activation and, therefore, not present in Xa crystal structures (*76*, *77*). This precluded any useful modeling attempts to further elucidate these topographical relationships. Additional mutagenesis data and structural information on the zymogens may provide more insight into the precise molecular interactions governing these enzymatic reactions.

A number of serine proteases, including the Gla domain containing enzymes IXa (*20*−*22*) and VIIa/TF·VIIa (*4*, *5*, *28*), were shown to activate VII in vitro. The importance of the TF region described for interacting with Gla domains of substrates suggests that IXa and TF·VIIa also utilize the same TF region when processing TF-bound VII. Preliminary experiments in our laboratory support this assumption, consistent with studies that used an anti-TF antibody (*78*) and two TF mutants (*61*).

The results presented in this study provide evidence for the usage of a common TF surface region for divergent TF functions, i.e., as cofactor for VII activation by Xa and as cofactor for X activation by VIIa. Since the dual role of this TF region serves to greatly amplify the coagulation reactions, antibodies such as D3 (*63*) and TF8-5G9 (*79*) which target this TF site are excellent candidates for the development of new antithrombotic agents. Moreover, it will be of interest to examine whether this TF region has a role in other biological processes, such as in cell adhesion (*80*, *81*) and in activation of signaling receptors (*82*).

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