Tissue Factor Residues 157–167 Are Required for Efficient Proteolytic Activation of Factor X and Factor VII*

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(Received for publication, April 28, 1992)

The cell surface receptor tissue factor (TF) initiates coagulation by supporting the proteolytic activation of factors X and IX as well as VII to active serine proteases. Architectural similarity of TF to the cytokine receptor family suggests a strand-loop-strand architecture for TF residues 151–174. Site-directed Ala exchanges in the predicted surface loop demonstrated that residues Tyr157, Lys159, Ser163, Gly164, Lys165, and Lys166 are important for function. Addition of side chain atoms at the Ser162 position decreased function, whereas the Ala exchange was tolerated. The dysfunctional mutants bound VII with high affinity and fully supported the catalytic activation of small peptide substrates by the mutant TF-VIIa complex. Lys159→Ala substitution was compatible with efficient activation of factor X, whereas the Tyr157→Ala exchange and mutations in the carboxyl aspect of the predicted loop resulted in diminished activation of factor X. The specific plasma procoagulant activity of all functionally deficient mutants increased 7- to 200-fold upon the supplementation of VII suggesting that TF residues 157–167 also provide important interactions that accelerate the activation of VII to VIIa. These data are consistent with assignment of the TF 157–167 region as contributing to protein substrate recognition and cleavage by the TF-VIIa complex.

Initiation of the coagulation cascades in vivo is mediated by the cell surface expression of tissue factor (TF) (1) which serves as the receptor and catalytic cofactor for the serine protease factor VIIa (VIIa) as well as a mediator for the autoactivation of VII to VIIa (2). Upon assembly with TF, VIIa exhibits enhanced catalytic activity evidenced by hydrolysis of small peptideyl (3, 4) as well as protein substrates (3, 5–8). Cleavage of small peptideyl substrates is efficient at ≈50 μM Ca2+, whereas the activation of macromolecular substrates such as the zymogen factor X (X) require the presence of Ca2+ at concentrations consistent with saturation of the γ-carboxylated amino-terminal domain of VIIa (9). These observations indicate that the structural requirements for catalytic enhancement in the VIIa protease domain are distinct from other interactions which are required for efficient assembly, hydrolysis, and release of protein substrates. In addition, TF residues Lys159 and Lys166 have been identified as important in this cofactor for specific proteolytic activation of X, but they are required neither for cleavage of peptideyl substrates nor for high affinity binding of VIIa (10). Structural predictive algorithms have suggested a similarity of the TF extracellular domain with the two module domains of the cytokine receptors (11), and the evaluation of secondary structure by circular dichroism analysis of the isolated TF surface domain (12) as well as the recently elucidated three-dimensional structure of the hypothetically homologous growth hormone receptor (13) are consistent with a β-strand organization. Based on this structural model, Lys159 and Lys166 are localized to a predicted surface loop between strands C and D in the immunoglobulin nomenclature. This surface loop has also been shown to be in proximity to VIIa, based on chemical cross-linking from VIIa to TF (12). Further, one of the tripeptide motifs Trp-Lys-Ser occurring three times in TF is found in the amino aspect of the same loop, and we have identified Lys159 in the motif as a functionally important residue (14). We here present a mutational search and analysis of functional residues in this predicted surface loop and provide evidence that the entire sequence span from Tyr157 to Thr167 is not required for high affinity binding of VIIa. In addition, we demonstrate that residues 157–167 contribute to efficient hydrolysis of the protein substrate X by the TF-VIIa complex as well as to the conversion of VII to VIIa during the initiation of coagulation.

MATERIALS AND METHODS

Reagents—Coagulation proteins were purified as described (3). VIIa was purchased from Novo Nordisk (Gentofte, Denmark), and the functional activity and binding characteristics of the recombinant protein have previously been described (9). Coagulation factor-deficient plasmas were purchased from George King Bio-medical. Spectrozyme FXa was from American Diagnostica Inc. (Greenwich, CT).

Mutagenesis and Expression of TF—Oligonucleotide-directed mutagenesis was performed according to Kunkel (15) with previously described modifications (16). Mutant TFADGnas was generated with the oligonucleotide TATACACTTTTACCGGCATCTTCAACT (triplet for amino acid change italicized), TFADGnas departure

Gene ID 22206

http://www.jbc.org/content/267/5/22206.full.pdf
resistance gene (pMAMneo) with the TF-encoding plasmid followed by selection in G418 as previously described (16).

Functional Analysis—Specific functional activity was determined for transiently expressed mutants. First, TF antigen in a detergent (CHAPS) cell lysate was determined by immunosassay using two non-overlapping monoclonal antibodies or, alternatively, polyvalent antibody purified by affinity for immobilized TF as capture antibody followed by detection with monoclonal antibody (3). The assay was calibrated with recombinant human TF. Initiation of coagulation by wild-type and mutant TF in recalcified plasma was determined after lysis of cell pellets with 15 mM octyl glucopyranoside (15 min at 37 °C) followed by 3-fold dilution (14). Activity was determined from a calibration curve established with purified TF reconstituted in phospholipid vesicles (70% phosphatidylycholine, 30% phosphatidylserine) using detergent solubilization and dialysis, as described in detail (11). Both the enzyme-linked immunosorbent assay and clotting assay were quality controlled using cell pellets of a stable cell line expressing wild-type TF. Samples of this cell line were subjected to the same lysis and analysis procedure as the test samples. The coefficient of variation calculated for a 1-month sampling period was 10.1% (n = 6) for the enzyme-linked immunosorbent assay and 10.8% (n = 16) for the clotting assay. Radioligand binding analysis with plasma-derived 125I-VIIa was carried out on cell monolayers, and Scatchard analysis was performed using the LIGAND program, as described (16). Factor X activation was analyzed by incubating a freshly prepared octyl glucopyranoside cell lysate (0.03-0.12 mM TF) with excess VIIa (5 nM) at 5 mM CaCl2 for 5 min at 37 °C followed by addition of Xa (100) Samples were removed from the reaction and quenched in 100 mM EDTA in TBS (20 mM Tris, 140 mM NaCl, pH 7.4). Xa in the quenched reaction was determined with Spectrozyme FXa, and the rate of Xa generation was calculated for several points in the initial linear portion of the progress curve (3). For all the stable cell lines described in this report, the activation of X on viable cell surfaces was comparable to the experiments with octyl glucopyranoside-lysed cells. This indicated that no loss of functional activity was introduced by the detergent lysis procedure. Cleavage of small peptide substrates by the TF-VIIa complex was analyzed using lysates of cell lines stably expressing wild-type or mutant TFs which were lysed with 4 mM CHAPS dissolved in TBS, and this lysate was diluted 2-fold in the final reaction (200 μl) which contained VIIa (10 nM), CaCl2 (5 mM), and Spectrozyme FXa (1.25 mM). The rate of Spectrozyme hydrolysis was determined at ambient temperature in a kinetic plate reader (Molecular Devices, Mountain View, CA). Clotting activity of TF mutants with or without added VIIa was evaluated with freshly prepared octyl glucopyranoside lysates. Cell lysate (100 μl), normal or coagulation factor-deficient plasma (50 μl), and 500 nM VIIa or buffer (50 μl) were equilibrated at 37 °C for 1 min followed by initiation of the reaction by adding 20 mM CaCl2 (100 μl). The VIIa concentration was chosen to provide a 50- to 100-fold excess over VII in the plasma. Control experiments with a 10-fold lower concentration of VIIa gave similar results. Further, preincubation of wild-type and mutant TF with VIIa in the presence of CaCl2 followed by the addition of plasma did not reveal differences compared to the assay where the reaction was started by the addition of Ca2+. This suggests that a slower assembly of VIIa with the TF mutants does not contribute to the functional defect. Functional activity was derived from double logarithmic calibration curves of serial dilutions of purified and phospholipid-reconstituted TF versus the clotting times in normal or factor IX (IX)-deficient plasma. The concentration of TF which produced a 50-s clotting time in normal or IX-deficient plasma was set to 1 unit/ml of TF activity. Specific functional activity was based on the TF antigen concentration of the cells determined by enzyme-linked immunosorbent assay. Several dilutions of mutant TF (30 to 200 pm) were used to establish the functional activities for each experimental condition, and mean and standard deviation were calculated for three independent experiments.

RESULTS

Mutations in the Predicted C and D β-Strand in the Carboxyl Module of TF—Structural prediction suggested that residues 157 through 167 are localized in a surface loop of the carboxyl module between the equivalents of the C and D β-strands in the immunoglobulin nomenclature (11). Based on this structural model, residues 151–156 should form a β-strand, and residues Leu153, Tyr155, and Leu156 in TF are indeed alternating hydrophobic residues consistent with the model. Residues Ile152, Thr154, and Tyr156 may form the hydrophilic side of the β-strand, and hydrophilic substitutions for Ile152 and Tyr156 are found in the TF sequence of other species (18). The hydrophobic residues Leu153, Tyr155, and Leu156 were each replaced by Ala, and the triple mutant TF was transiently expressed. In several experiments, TF153,155,156 was expressed at levels one-tenth or less of a wild-type TF control transfected in parallel. This suggested diminished efficiency of cellular processing which may indicate alteration of the protein fold (19, 20). Further, Western blot analysis of the mutant protein exhibited increased electrophoretic mobility characteristic of incomplete glycosylation of TF. These data are consistent with a structurally altered mutant protein which undergoes less than normal cellular processing. The specific functional activity of TF153,155,156 was greatly reduced (Table I). Since 3 alternating hydrophobic residues in a predicted β-strand had been replaced, one or more side chains which are critical for the hydrophobic core of the carboxyl module may have been removed. This mutational analysis is consistent with the predicted β-strand architecture of the TF extracellular domain. We have previously shown by mutational Cys→Ser exchange that lack of covalent stabilization of the disulfide-bonded loop (residues 186–209) resulted in a protein with quantitatively reduced cell surface expression and diminished specific functional activity (16). These two examples of an apparent structural defect coupled with loss of functional activity demonstrate an important functional role of the carboxyl module of TF.

The sequence 168–174 in TF which corresponds to the predicted D β-strand (11) is hydrophilic. The D-strand in immunoglobulins (21) and structurally related proteins (22) is located at the edge of the opposed two β-sheets and often lacks residues which interact with the hydrophobic core. In the growth hormone structure, the corresponding residues were found to form a short strand which aligned with the opposite β-sheet (13). We generated two mutants which replace alternating residues. In the first mutant, Thr170, Thr172, and Glu74 were replaced by Ala, the second mutant had replacements of Lys89, Asn171, and Asn173 oriented to the opposite site of the predicted β-strand. Both triple mutants exhibited quantitatively normal cellular expression, indicating little if any structural alterations. In addition, residues

### Table I

**Functional activity of TF mutants**

Mutants were expressed in transient transfection experiments using CHO-K1 cells. Cell lysates were analyzed for TF antigen by enzyme-linked immunosorbent assay and for functional activity in a one-stage clotting assay. The specific functional activity was calculated based on these determinations, and mean and standard deviation calculated for the indicated number (n) of duplicate determinations is given. The specific functional activity relative to wild-type TF is given as % wild-type.

| TF mutant | % Wild-type | Specific activity | n |
|-----------|-------------|------------------|---|
| Wild-type | 100         | 298 ± 87         | 6 |
| TF153A155A156 | 3  | 10 ± 8          | 3 |
| TF153A155A156 | 66 | 197 ± 99        | 5 |
| TF153A155A156 | 130 | 322 ± 130     | 7 |
| TF153A155A156 | 2 | 5 ± 8          | 4 |
| TF153A155A156 | 128 | 383 ± 168      | 4 |
| TF153A155A156 | 82 | 244 ± 107      | 4 |
| TF153A155A156 | 11 | 33 ± 9        | 4 |
| TF153A155A156 | 11 | 8 ± 7        | 4 |
| TF153A155A156 | 96 | 282 ± 89      | 3 |
| TF153A155A156 | 19 | 58 ± 15      | 4 |
| TF153A155A156 | 21 | 64 ± 51      | 4 |
| TF153A155A156 | 3 | 8 ± 5      | 3 |
| TF153A155A156 | 2 | 7 ± 2      | 4 |
168–174 were not required for function of TF, as indicated by normal specific functional activity (Table I). Although the effect of mutational exchange of residues 169–174 can be considered as consistent with the proposed structural model, elucidation of the three-dimensional structure of the TF extracellular domain will be required to establish the structural alignment of residues in this region of TF.

**Functionally Important Residues in the 157–167 Sequence**—Tyr<sup>163</sup> and Lys<sup>168</sup> have previously been identified as functionally important and are flanked in linear sequence by the noncritical residues Tyr<sup>160</sup> and Ser<sup>169</sup> (14). Ala replacements for Tyr<sup>167</sup> or Lys<sup>168</sup> resulted in a 87% or 92% respective loss of specific functional activity by plasma coagulation assay. Replacement of both residues in one mutant reduced the functional activity by 98% (Table I) which may indicate an additive effect of the two mutations. Ala replacements for Ser<sup>161</sup> and Ser<sup>162</sup> did not result in a significant loss of function. In contrast, Ala substitution for Ser<sup>163</sup> reduced specific functional activity by 89%, indicating importance of the Ser<sup>163</sup> side chain (Table I).

A recent study indicated that the positively charged residues Lys<sup>168</sup> and Lys<sup>169</sup> are important for recognition and activation of the protein substrate X (10). We further explored the charge requirements in the vicinity of these 2 Lys residues by introducing Asp, thus a negative charge in place of Ser<sup>162</sup>. Although Ser<sup>162</sup> could be replaced by Ala without loss of function, the TF<sub>D162</sub> mutant demonstrated a significant loss of function (Table I). A similar loss of function was also observed with the triple mutant TF<sub>AD161AD162</sub>. Within the experimental error of this analysis, the additional Ser<sup>163</sup> → Ala substitution thus appeared to be silent in this latter mutant. Other replacements for Ser<sup>162</sup> further demonstrate that substitution by bulkier side chains, as in Thr or Asn, resulted in a nearly 80% loss of function. This suggests that the packing of the Ser<sup>162</sup> side chain may be critical for local conformation. Alternatively, Ser<sup>162</sup> could be localized within an interactive surface without direct functional contribution, and the additional side chain atoms may not be accommodated upon assembly of the interacting protein surfaces (23). Since there was an additional effect when a negative charge was introduced at the Ser<sup>162</sup> position, this may indicate that the local charge field contribution of the adjacent Lys<sup>168</sup> and Lys<sup>169</sup> was perturbed or the side chain orientation of these residues was distorted. These 2 Lys residues are separated in linear sequence from the stretch of Ser residues by Gly<sup>164</sup>.

Gly<sup>164</sup> appears to be necessary for function of the 157–167 region, since the Gly<sup>164</sup> → Ala substitution resulted in very low specific functional activity (Table I). Gly residues are often found in reverse turns (24), because of the lack of a C<sup>α</sup>-atom, the increased flexibility of their backbone and their more favorable φ and ψ angles. The functional defect resulting from the Gly<sup>164</sup> → Ala exchange is likely to reflect local perturbation of the orientation of adjacent functionally important residues. Thr<sup>167</sup> could be replaced by Ala without alterations in the functional properties of TF. This analysis therefore provides evidence for several functionally important residues in the 157–167 region of TF (Fig. 1). Analysis of selected mutants stably expressed on CHO cells was performed to further define functional defects.

**VII Binding by Mutants in the 157–167 Sequence**—Disruption of a TF mutant could follow from reduced affinity for its ligand VII/VIIa. To examine this possibility, we analyzed VII binding to the various mutant proteins in radioligand binding analyses on cell monolayers. Previously, characterization of the TF<sub>AD161</sub> mutant had demonstrated normal binding of VII, based on radioligand binding to cell surface TF and to detergent-solubilized TF in the absence of phospholipid, as well as that based on dissociation analysis of the TF·VIIa complex (10). These observations indicated that the basic residues Lys<sup>168</sup> and Lys<sup>169</sup> do not contribute significantly to the binding energy required for assembly of the TF·VIIa complex. Since removal of charged side chains, as in the TF<sub>D162</sub> mutant, may be tolerated more readily than the addition of an oppositely charged or bulkier side chain in the same region, we analyzed the VII binding characteristics of TF<sub>D161D162</sub>. This mutant was chosen because it exhibited the greatest loss of function, when mutants at the Ser<sup>162</sup> and

![Fig. 1. Schematic representation of residues 151–172 in TF. Alignment to strand C according to Bazan (11) is indicated, and the functionally important residues are highlighted. The single-letter code for amino acids is used.](http://www.jbc.org/content/157/167/Fig1.tei.html)

![Fig. 2. VII binding to TF<sub>AD161AD162</sub>. Specific binding of VII to cell surface TF<sub>AD161AD162</sub> (A) and wild-type TF (B) is shown. The insets give the Scatchard analysis for the same data obtained in a representative experiment.](http://www.jbc.org/content/157/167/Fig2.tei.html)

| Mutant | K<sub>D</sub> (nM) | Binding sites (pmol/well) | n |
|--------|-----------------|--------------------------|---|
| Wild-type | 10.2 ± 1.4 | 800 ± 200 | 4 |
| TF<sub>D161</sub> | 5.8 ± 2.0 | 500 ± 200 | 5 |
| TF<sub>D162</sub> | 8.9 ± 1.7 | 1700 ± 200 | 3 |
| TF<sub>D161D162</sub> | 6.8 ± 0.7 | 900 ± 100 | 8 |
We further analyzed VII binding of the TF_{A150} and TF_{A160} mutants. These two mutants lack the side chains of the functionally important residues Tyr^{157} and Lys^{160}. Both mutants bound VII with undiminished affinity compared to wild-type TF which was analyzed in a 200-nM reaction with a kinetic plate reader and is given as the increase in absorbance (mOD/min). TF_{A150} and TF_{A160} demonstrated rates of X activation which were indistinguishable from that of the wild-type TF. This suggests that the mutants have a selective defect either in extended recognition and hydrolysis of protein substrates or in the release of Xa, the cleaved product.

To evaluate the role of the alternative substrate for TF-VIIa, namely IX, during the activation of the extrinsic coagulation pathway by the TF mutants, we compared their specific functional procoagulant activity in normal and IX-deficient plasma. Both wild-type TF and the TF_{A150} mutant demonstrated a similar and less than 2-fold decrease of specific procoagulant activity in IX-deficient plasma compared to normal plasma (Fig. 4). The diminished function in IX-deficient plasma was more pronounced with the TF_{A157}, TF_{A159}, TF_{A162}, and TF_{A166} mutants resulting in a 5-, 2.5-, and 3.1-fold reduction of specific functional activity (Fig. 4). Thus, it appears that mutants with a defect in X activation are even less active in the absence of IX suggesting modest compensation of the functional defect by IX. This is consistent with the proposal that the generation of IXa by Xa and the accelerated activation of IXa to IXa$\beta$ by TF-VIIa plays a role in the initiation of the coagulation cascade by TF (8).

Importance of Cofactor Residues 157–167 for the Conversion of VII to VIIa—The potential contribution of TF residues 157–167 to the conversion of VII to VIIa (2, 25) was analyzed in a plasma coagulation assay. The specific functional activity of the TF mutants relative to wild-type TF was determined in normal and IX-deficient plasma with or without a 50- to 100-fold molar excess of recombinant VIIa relative to VII present in the plasma. With wild-type TF, addition of VIIa accelerated the clotting times in both normal and IX-deficient plasma. Based on calibration curves with purified TF in normal or IX-deficient plasma, specific functional activity increased 3-fold in normal human plasma and 5.4-fold in IX-deficient plasma (Fig. 4). The specific activity of all TF mutants increased to a much greater extent in the presence of VIIa, suggesting a contribution of residues in the 157–167 surface loop to the activation of VII during the TF-dependent initiation of coagulation. In the presence of VIIa, the functional activity of TF_{A157} or TF_{A160} increased 200-, 198-, or 7-fold in normal plasma and 566-, 653-, or 37-fold in IX-deficient plasma. The specific functional activity catalytic function of VIIa when assembled with TF was analyzed with detergent lysates of cells expressing the mutant TFs. When VIIa in access was incubated with identical concentrations of wild-type or mutant TF, comparable hydrolysis of the chromogenic substrate Spectrozyme FXa was observed (Fig. 3A). This further supports the binding analysis that all TF mutants in the 157–167 region form equivalent complexes with VIIa. This analysis also excludes that a significant fraction of the mutant TF is misfolded and noninteracting with VIIa. In addition, these data demonstrate that the catalytic function of VIIa toward small peptidyl substrates is normal indicating a fully functional catalytic triad in VIIa when complexed with the mutant TFs.

Proteolytic Activity of Mutant TF-VIIa Complexes—The proteolytic activity of wild-type and mutant TF-VIIa complexes was analyzed using the protein substrate X (Fig. 3B). At identical TF concentrations, TF_{A150}–VIIa and wild-type TF-VIIa complexes activated X at rates that were indistinguishable. In contrast, complexes formed with TF_{A151} or TF_{A159} demonstrated rates of X activation which were reduced by 85%, 55%, and 59%, respectively (Fig. 3). Thus, all dysfunctional mutants with the exception of TF_{A150} formed catalytic complexes with VIIa that exhibited some loss of proteolytic activation of the natural protein substrate. Amidoless activity of the mutant TF-VIIa complexes was indistinguishable from that of the wild-type TF-VIIa complex suggesting that the mutants have a selective defect either in extended recognition and hydrolysis of protein substrates or in the release of Xa, the cleaved product.

Support of Catalytic Function by Mutants in the 157–167 Region—The ability of the dysfunctional mutants to induce the ability of the dysfunctional mutants to induce serine-specific proteolytic activity when assembled with TF was analyzed with detergent lysates of cells expressing the mutant TFs. When VIIa in access was incubated with identical concentrations of wild-type or mutant TF, comparable hydrolysis of the chromogenic substrate Spectrozyme FXa was observed (Fig. 3A). This further supports the binding analysis that all TF mutants in the 157–167 region form equivalent complexes with VIIa. This analysis also excludes that a significant fraction of the mutant TF is misfolded and noninteracting with VIIa. In addition, these data demonstrate that the catalytic function of VIIa toward small peptidyl substrates is normal indicating a fully functional catalytic triad in VIIa when complexed with the mutant TFs.

Proteolytic Activity of Mutant TF-VIIa Complexes—The proteolytic activity of wild-type and mutant TF-VIIa complexes was analyzed using the protein substrate X (Fig. 3B). At identical TF concentrations, TF_{A150}–VIIa and wild-type TF-VIIa complexes activated X at rates that were indistinguishable. In contrast, complexes formed with TF_{A151} or TF_{A159} demonstrated rates of X activation which were reduced by 85%, 55%, and 59%, respectively (Fig. 3). Thus, all dysfunctional mutants with the exception of TF_{A150} formed catalytic complexes with VIIa that exhibited some loss of proteolytic activation of the natural protein substrate. Amidoless activity of the mutant TF-VIIa complexes was indistinguishable from that of the wild-type TF-VIIa complex suggesting that the mutants have a selective defect either in extended recognition and hydrolysis of protein substrates or in the release of Xa, the cleaved product.
of these mutants was not completely normalized by the addition of VIIa, but remained reduced 40- to 45-fold for TF<sub>α161Δ162Δ166</sub> and 14-fold for TF<sub>α150</sub> and 5-fold for TF<sub>α157</sub> in comparison to wild-type TF (Fig. 4). The decreased activity in the presence of VIIa is consistent with the diminished rate of proteolytic activation of X demonstrated for all three mutants. Since addition of VIIa increased the specific functional activity of all mutants defective in X activation, it is suggested that the feedback loop of Xa cleaving VII bound to TF (26) may be of importance.

The same residues of TF which are important for TF cofactor function in the activation of X may also contribute to recognition of VII as a substrate by the TF-VIIa complex. Evidence for a specific contribution of cofactor residues to this latter activation is provided by the mutant TF<sub>α150</sub> which fully supported activation of X. In both normal and IX-deficient plasma, VIIa increased the specific functional activity of TF<sub>α150</sub> 11- to 20-fold which is a 4- to 5-fold greater increase than observed with wild-type TF. The activity of TF<sub>α150</sub> in the presence of high concentrations of VIIa was similar to wild-type TF without added VIIa, and a 2.8-fold difference remained when mutant and wild-type TF activity were compared in the presence of excess VIIa. Despite this unexplained difference, the normalization of the functional defect of TF<sub>α150</sub> by the addition of VIIa suggests that Lys<sup>150</sup> is important for the conversion of VII to VIIa. Conceivably, this residue may be critical for assembly of Xa with the TF-VII complex during activation of the bound VII, or Lys<sup>150</sup> may be important for recognition and hydrolysis of the substrate VII by the TF-VIIa complex. The TF mutants described here may aid in elucidating the specific contribution of cofactor residues to the autoactivation of VII by VIIa.

**DISCUSSION**

The region of TF which includes residues 151–174 has been predicted to adopt a strand-loop-strand structure, from sequence-based secondary structure predictive algorithms (11) and apparent homology to the growth hormone receptor structure (13). The previously demonstrated predominant β-strand secondary structure of TF (12) in conjunction with the mutations of residues 151–174 analyzed in this study are consistent with this hypothesized immunoglobulin-like fold of TF. Residues Tyr<sup>157</sup>, Lys<sup>166</sup>, Ser<sup>167</sup>, Gly<sup>164</sup>, Lys<sup>160</sup>, and Lys<sup>166</sup> were identified as important for function, either directly or indirectly through maintenance of a functional structure in the predicted 157–167 loop. Replacement of Gly<sup>164</sup> with the larger and more rigid Ala resulted in severe loss of function consistent with location of Gly<sup>164</sup> in a turn which may be required for the proper conformation of the putative 157–167 loop. All dysfunctional mutants in the 157–167 region were characterized by high affinity binding of VII/VIIa and the ability to form mutant TF-VIIa complexes which efficiently hydrolyzed small peptidyl substrates. These data are consistent with expression of mutant proteins with proper overall fold. The functionally defective mutants displayed two phenotypes. Whereas removal of the Lys<sup>160</sup> side chain only affected the conversion of VII to VIIa, the other mutants formed catalytic binary complexes with selectively reduced proteolytic activity for X and a suggested consecutive defect in VII activation.

The Tyr<sup>157</sup> → Ala mutation in the amino aspect of the predicted loop resulted in a functional phenotype similar to the charge-modifying mutations in the carboxyl aspect. It must be considered whether Tyr<sup>157</sup> may be important for stabilization of the loop by providing a hydrophobic center with its aromatic side chain. Consistent with this possibility, we have previously demonstrated that the Tyr<sup>157</sup> → Phe substitution is compatible with full functional activity of TF (14). A supporting role for the structure of the 157–167 loop can also be considered for residues Ser<sup>162</sup> and Ser<sup>165</sup>. Although functionally important interactions for the Ser<sup>162</sup> side chain cannot be excluded, loss of function due to side chain capping (Ser<sup>165</sup>) or introduction of additional side chain atoms (Ser<sup>162</sup>) would also be consistent with a perturbation of the structural integrity of the 157–167 loop resulting in loss of proper alignment of adjacent functionally important residues.

This study provides evidence for a discrete structure in TF that is required for efficient hydrolysis of protein substrates. This function may be mediated through specific contacts with VIIa which contribute minimally to the binding energy. Alternatively, this region in TF may form direct contacts with the protein substrates contributing significantly to extended proteolytic substrate recognition. The present evidence is consistent with a model of an interactive surface on the TF-VIIa complex for protein substrates which is composed entirely or in part by TF residues. It appears that these residues contribute differently to the activation of X and VII. The mutants characterized here thus help to define the molecular structures which mediate the well documented roles of TF as an enhancer of VII activation (2, 25) and as a catalytic cofactor for specific proteolysis of protein substrates X and IX (3, 5–8) which trigger the subsequent procoagulant events in the coagulation cascade.

**Acknowledgments**—We thank Pablo Tejada and David Revak for technical assistance and Barbara Parker for preparation of the manuscript.

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*J. Biol. Chem.* 1992, 267:22206-22210.

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