Cofactor Residues Lysine 165 and 166 Are Critical for Protein Substrate Recognition by the Tissue Factor-Factor VIIa Protease Complex*

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High affinity binding of factor VIIa (VIIa) to its cellular receptor tissue factor (TF), as well as association of factor X with phospholipid are required for optimal assembly of the extrinsic activation complex. In addition to the interactions of substrate with phospholipid and enzyme, we here provide evidence that cofactor residues Lys-165 and Lys-166 specifically contribute to the recognition of macromolecular substrate. Ala for Lys replacement in TF\textsubscript{A165A166} was compatible with high affinity binding of VIIa when analyzed on cell surfaces as well as in the absence of phospholipid. Dissociation of TF\textsubscript{A165A166} VIIa did not occur with a faster rate compared to TF-VIIa, further supporting unaltered VIIa binding function of TF\textsubscript{A165A166}. Cleavage of chromogenic peptidyl substrate by TF\textsubscript{A165A166} VIIa complexes was not diminished, demonstrating that TF\textsubscript{A165A166} supported enhancement of catalytic function of the VIIa protease domain. In contrast, factor X activation was reduced in the presence and absence of phospholipid. Further, TF\textsubscript{A165A166} effectively competed with wild-type TF in the cleavage of factor X at limited VIIa concentrations. Selective reduction in macromolecular substrate hydrolysis combined with normal VIIa binding by TF\textsubscript{A165A166} indicates that the cofactor TF does contribute, either directly or indirectly via specific interactions with VIIa, to factor X recognition.

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Tissue factor (TF), a transmembrane cell surface receptor, provides cofactor function in the initiation of the coagulation pathways by the formation of a binary complex with the coagulation serine protease factor VIIa (VIIa) (1, 2), or the zymogen VII which is rapidly converted to VIIa when bound to TF (3). Like other cofactor-protease complexes, assembly of VIIa with TF markedly enhances specific functional proteolysis of substrates. VIIa binds with high affinity to TF whether TF is inserted into a phospholipid bilayer or is in solution suggesting that protein-protein interactions are sufficient for tight binding of the protease to its receptor (4). High affinity binding of VIIa to TF is dependent on a properly conformed and Ca\textsuperscript{2+}-saturated Gla domain of VIIa (4, 5), as well as, other sites of VIIa (4, 6, 7) and is a requisite for optimal recognition and proteolytic activation of protein substrate by the TF-VIIa complex (4). Substrate assembly with cofactor-enzyme complexes in the coagulation pathways may require either substrate-phospholipid or substrate-cofactor interactions. The former is exemplified by the prothrombinase complex assembly (8) which is enhanced by local concentration of the reactants on the phospholipid surface. In contrast, protein-protein interactions of the substrate protein C with the cofactor thrombomodulin are critical for substrate recognition by the thrombomodulin-thrombin complex, as demonstrated by the dissection of enzyme-binding sites and critical regions for cofactor function using proteolytic fragmentation (9-11) or recombinant deletion proteins based on the domain structure (12, 13). Phospholipid is required for full functional activity of the TF-VIIa complex. This reflects, at least in part, the contribution of substrate-phospholipid interactions, as demonstrated by the preferential activation of phospholipid-associated factor X by soluble TF-VIIa (14). In addition to these structural requirements for assembly and substrate recognition by TF-VIIa, we now identify 2 amino acid residues that are required for optimal recognition and cleavage of protein substrate by TF-VIIa, but are not required for binding of VIIa or the induced enhancement of function of the catalytic site of VIIa.

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EXPERIMENTAL PROCEDURES

Reagents—Coagulation proteins and anti-TF mAbs were purified as described (14). Cross-linking reagents were obtained from Pierce Chemical Co. and Sequenase from U.S. Biochemical. VIIa was from Novo Nordisk (Gentofte, Denmark) and the purification and characterization of this recombinant protein has been previously described (15, 16). The binding constants of this recombinant VIIa for TF in the absence of phospholipid have been shown to be similar to those of plasma-derived VIIa (4).

Site-directed Mutagenesis and Generation of Stable Cell Lines—Site-directed mutagenesis was performed with the TF coding sequence inserted in CDM8 using the uracil substitution method described by Kunz (17) with modifications as previously reported (18). To generate TF\textsubscript{A165A166}, the oligonucleotide AAGTTCAGGC\textsubscript{m}AAAACAGCC and TGGAAATCTTCCTCG\textsubscript{(underlined)} for Ala in place of Lys-165 and Lys-166. Similarly, Ala substitutions for Lys-165 and Lys-166, respectively. Purified plasmid DNA was sequenced using Sequenase and transfected into Chinese hamster ovary cells using the calcium phosphate method. In transient transfection experiments, cells were harvested after 2 days for analysis. To establish stable cell lines, pMAMneo was cotrans-
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acted with the TF coding sequence, and clones were selected for resistance to the neomycin analogue G418 (GIBCO). Stable clones were tested for epitope expression of a panel of anti-TF mAbs using flow cytometry as described (18).

Chemical Cross-linking of TF—Chemical cross-linking of TF on viable cells was performed as previously described (18) with the following modifications. The water-soluble, non-reducible, homobifunctional, and amino-reactive cross-linking reagent Bixa(sulfosuccinimidyl)suberate (BS') and the reducible reagent di-thio-bis(sulfosuccinimidylpropionate) (DTSSP) were used at 5 mM in HEPES-buffered saline (HBS, 10 mM HEPES, 140 mM NaCl, pH 7.4). Cells (5-10 x 10^6 cells/ml) were incubated with cross-linker or buffer control for 1 h on ice followed by washing with 100 mM Tris, pH 7.4, to quench the reaction. Samples which were not cross-linked and those which were cross-linked with DTSSP were applied to sodium dodecyl sulfate-polyacrylamide gels under non-reducing conditions; samples cross-linked with BS' were reduced prior to gel electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels (20). TF was detected using a polyclonal primary antibody which had been affinity purified on immobilized TF, followed by alkaline phosphatase-conjugated secondary antibody and color development as described previously (21).

Extraction of TF from Cells—TF or TF_{Ala165,166} were extracted from cell lysate. Frozen cells were subjected to the depilation and solubilization protocol which had previously been used for the purification of TF from brain (22). This included the preparation of an acetone powder by extracting the cells five times with acetone followed by heptane/ butanol (2:1, v/v) extraction of the acetone powder to remove phospholipid. The protein pellet was then extracted with 0.1% Triton X-100 to reduce contaminating proteins, and TF was solubilized in a final extraction with 10 mM CHAPS in TBS (20 mM Tris, 140 mM NaCl, pH 7.4). The concentration of TF and TF_{Ala165,166} in this extraction was determined by ELISA (16) using 2 mAbs which have been shown to be reactive with both molecules by flow cytometry.

Functional Characterization of TF Mutants—Initial characterization of the TF mutants was performed in a one-stage clotting assay. Briefly, cells were lysed at 2 x 10^6 cells/ml with 15 mM octyl-β-D-glucopyranoside in HBS at 37 °C for 20 min followed by a 5-fold dilution to HBS. Clotting times were determined for these cell lysates in a one-stage clotting assay (equal volumes of sample, plasma, and 20 mM CaCl_2) and converted to units based on a calibration curve using purified and phospholipid reconstituted TF. The clotting activity of viable cells which stably expressed TF_{Ala165,166} was also compared with cells expressing wild-type TF to exclude artifacts introduced by the lysis method. The clotting times obtained in the one-stage clotting assay were converted into units of TF activity using a calibration curve based on serial dilutions of the cell line which expressed wild-type TF, essentially as previously described (23).

Binding of VIIa to Cell Surface TF—Binding of VIIa to cell surface TF was analyzed on a cell monolayer, and Scatchard analysis was performed as previously described (24). Binding of VIIa to TF or TF_{Ala165,166} in the absence of phospholipid was determined using the previously described binding assay based on a noninhibitory anti-TF capture mAb (4). Briefly, 125I-VIIa and TF (2 nM) solubilized by 0.4 or 4 mM CHAPS as a detergent were assembled in the presence of 5 mM Ca^2+ in a microtiter well which had been coated with the anti-TF mAb TF9-10H10. After equilibrium, free and bound radioactivity was separated by rapid washes, and bound VIIa was determined from the captured radioactivity in the well, whereas the free ligand was determined from the radioactivity in the supernatant. Scatchard analysis was performed on these experimental data. Dissociation of VIIa from TF and TF_{Ala165,166} was studied on cell surfaces, with variations from previously described procedures (24). VIIa (20 nM) was assembled with TF or TF_{Ala165,166} at 5 mM CaCl_2, 0.5% bovine serum albumin for 2 h at 37 °C. Displacement of VIIa bound to TF was analyzed by two different procedures. First, displacement was initiated by dilution jump which included removal of the reaction mixture (400 μl) followed by addition of 4 ml of buffer containing 5 mM CaCl_2, 0.5% bovine serum albumin at 37 °C to each well. Second, displacement was studied by addition of the inhibitory anti-TF mAb TF9-6B4 in a small volume (10-fold molar excess of VIIa in the reaction) in order to prevent the dissociation of VIIa after dissociation from TF. The residual 125I-VIIa bound after various times was determined based on control reactions which were incubated in parallel.

Substrate Hydrolysis—Chromogenic substrate (Spectrozyme FXa, American Diagnostica) hydrolysis was measured with VIIa (50 nM) bound to TF or TF_{Ala165,166} (1.5-25 nM) at 5 mM Ca^2+ in a kinetic plate reader as described (14). TF or TF_{Ala165,166} were from delipidated cell extracts solubilized by 10 mM CHAPS. The detergent concentration in the assay was adjusted to 4 and 0.4 mM. Factor Xa generation by TF·VIIa was determined with TF expressed on viable cells in a coupled amidolytic assay, and data were fitted to the Michaelis-Menten equation as previously described (14, 18). For fluid-phase analysis, TF was transiently transfected with plasmid DNA for the mutants TF_{Ala165,166}, TF_{Ala165}, and TF_{Ala166}. The cells were harvested after 2 days, and cell lysate was analyzed immunologically for TF expression by ELISA and for function in a one-stage clotting assay. From these data, specific activities were calculated. Single alanine substitutions for Lys-165 resulted in a 70 ± 27% (n = 4) and for Lys-166 in a 88 ± 4% (n = 3) loss of specific functional activity in comparison to wild-type TF. The specific activity of the double mutant TF_{Ala165,166} was reduced by 98 ± 2% (n = 4) indicating that the phenotype of this mutant reflects the additive effect of mutations in both Lys residues. This double mutant was further characterized after generation of a stable cell line which expressed this mutant.

Generation of a Stable Cell Line Expressing TF_{Ala165,166}—The TF protein coding sequence in the vector CDMS was mutated to replace both Lys-165 and Lys-166 by Ala. The mutated sequence was confirmed by DNA sequencing of the plasmid which was then used for transfection of Chinese hamster ovary cells. Stable clones were obtained by G418 selection for the cotransfected neomycin resistance plasmid pMAMneo. Based on flow cytometry, mutant TF_{Ala165,166} was expressed at 2-fold higher levels on the cell surface compared to the wild-type TF cell line used in this study (Fig. 1A). This is consistent with antigen determination in cell lysate from the two lines which demonstrated 1.50 ± 0.31 pmol/10^6 cells for TF_{Ala165,166} and 0.77 ± 0.07 pmol/10^6 cells for TF (mean ± standard deviation, n = 4). The viable cells expressing TF_{Ala165,166} demonstrated a 99 ± 1% (n = 3) decrease of functional activity compared to the wild-type cell line when analyzed in a plasma clotting assay at concentrations from 3 x 10^6 to 1 x 10^6 cells/ml. The functional defect observed in the initial screening with cell lysate was therefore reproduced with TF_{Ala165,166} expressed on a viable cell suggesting that the reduced function is not due to inactivation of TF_{Ala165,166} during the detergent cell-lysing procedure.

No Evidence for Global Conformational Alterations of TF_{Ala165,166}—The epitopes for three non-overlapping mAbs (21) on the TF extracellular domain were equivalently expressed on both TF and TF_{Ala165,166} as demonstrated by flow cytometry (Fig. 1A). The TF9-6B4 epitope was mapped to residues 40-83, and the TF8-5G9 epitope is in the carboxyl-terminal aspect of TF (residues 106-219). The latter epitope is discontinuous and conformation-dependent (21). Similarly, TF9-9C3 could not be assigned to a linear epitope suggesting a discontinuous epitope for this mAb. Reactivity with these mAbs therefore indicates a proper overall conformation of the
wild-type molecule (19). TFA165A166 cross-linked with the reductant DTSSP demonstrated dimer formation to an extent similar to wild-type TF, whereas the non-cross-linked sample lacked a band migrating at a position corresponding to the dimer of TF (Fig. 1B). This cross-linking to analyze whether TFA165A166 is expressed in a dimeric organization on the cell surface as described for the wild-type molecule (19). TFA165A166 cross-linked with the reducible reagent DTSSP demonstrated dimer formation to an extent similar to wild-type TF, whereas the non-cross-linked samples lacked a band migrating at a position corresponding to a dimer of TF (Fig. 1B). We also used the non-reducible cross-linker BS3 to demonstrate that the dimerization observed in the presence of DTSSP is not due to disulfide bond formation via the Cys residues in the cytoplasmic domain of TF after exposure of the cells to the cross-linking reagent. When samples cross-linked with BS3 were reduced to disrupt cystines, the analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting demonstrated consistent with our previous analysis (4). This analysis demonstrates that CHAPS at the indicated concentrations did not demonstrably influence the TF-VIIa interaction.

**VII Binding to Cell Surface TF**—Binding of $^{125}$I-VII to TF and TFA165A166 was similar (Fig. 2) and saturable with twice the number of binding sites on cells expressing TFA165A166 compared to cells expressing TF, consistent with the higher surface expression on the TFA165A166 cell line demonstrated by flow cytometry. The dissociation constants ($K_d$) were comparable with 13.8 nM for TF and 14.3 nM for TFA165A166 (Fig. 2). The number of sites was 0.81 pmol/10^6 cells for TF and 1.62 pmol/10^6 cells for TFA165A166 consistent with the antigen determinations in cell lysates by ELISA (see above). This is consistent with a 1:1 stoichiometry of VII binding to TF and TFA165A166, as previously demonstrated for wild-type TF (23, 27).

**VII Binding in the Absence of Phospholipid**—Similar affinity of VIIa binding to TF and TFA165A166 was further demonstrated by binding analysis in the absence of phospholipid. Crude TF and TFA165A166 were obtained from the stable cell lines by organic extractions to remove lipids followed by solubilization of TF with 10 mM CHAPS as a detergent. Binding of VIIa to 2 nM detergent-solubilized TF or TFA165A166 was analyzed in a mAb-based binding assay at high (4 mM CHAPS) and low (0.4 mM CHAPS) detergent concentrations. The affinities of VIIa binding to TF and TFA165A166 were similar and were not influenced by the detergent concentrations (Table I). The binding constants for binding to cell surface TF and detergent-solubilized TF were similar, consistent with our previous analysis (4). This analysis demonstrates that CHAPS at the indicated concentrations did not demonstrably influence the TF-VIIa interaction.

**Equilibrium binding of VII to TF and TFA165A166**—Binding of $^{125}$I-VII to a cell monolayer expressing TF was analyzed. The graphs show binding to TF (A) or TFA165A166 (B) in dependence of the added radiolabeled ligand VII. The insets depict Scatchard analysis and the curve fit obtained from the Ligand program for five independent experiments. Binding constants: $TF, K_c = 13.8 \pm 4.8$ nM, 0.81 ± 0.31 pmol/10^6 cells bound at saturation; $TFA165A166, K_c = 14.3 \pm 4.1$ nM, 1.62 ± 0.38 pmol/10^6 cells bound at saturation (estimates and standard error determined by the Ligand program are given).
The dissociation constants ($K_d$) and maximal number of sites ($B_{max}$) were determined for the indicated number of experiments ($n$) at 0.4 and 4 mM CHAPS which was used to solubilize TF or TF$_{A165A166}$ (2 nM). Since the reaction was 100 µl, 200 fmol of TF and TF$_{A165A166}$ were added and quantitatively bound to the wells, consistent with previous experiments (4).

Dissociation of VIIa from Cell Surface TF—To further substantiate the unaltered binding of VIIa to TF$_{A165A166}$, the dissociation of the TF-VIIa and TF$_{A165A166}$-VIIa complex was studied. First, dissociation was induced by dilution jump after VIIa was assembled with TF or TF$_{A165A166}$. The rate of dissociation from TF$_{A165A166}$ was similar to the reaction with the wild-type TF (Fig. 3). A 100-fold molar excess of the inhibitory anti-TF mAb TF-96B4 was used in a second set of experiments to prevent the reassociation with TF$_{A165A166}$. The dissociation of VIIa from TF$_{A165A166}$ occurred with a slightly slower rate compared to TF, thus demonstrating no evidence for a lower affinity of the VIIa-TF$_{A165A166}$ interaction. The analysis of dissociation therefore provides additional support for high affinity VIIa binding by TF$_{A165A166}$.

Substrate Cleavage by the TF$_{A165A166}$-VIIa Complex—We analyzed the hydrolysis of a small peptidyl chromogenic substrate (Spectrozyme FXa) as well as the proteolytic activation of the protein substrate factor X by complexes of VIIa formed with either TF or TF$_{A165A166}$. Peptide hydrolysis by VIIa (50 nM) was analyzed in the presence of various concentrations of TF or TF$_{A165A166}$ Solubilized with detergent. High concentrations (4 mM) of CHAPS did not influence the TF- or TF$_{A165A166}$-enhanced catalysis of chromogenic peptidyl substrates compared to 0.4 mM CHAPS (Fig. 4A). There was no difference in the rate of peptide hydrolysis between the TF-VIIa and the TF$_{A165A166}$-VIIa complex over the range of TF concentrations tested. This demonstrates that both cofactor proteins support the catalytic enhancement of the serine protease domain of VIIa to a similar extent. However, the analysis of factor X cleavage by cell surface complexes of TF or TF$_{A165A166}$ with VIIa revealed that the TF$_{A165A166}$-VIIa complex had a considerably slower rate of factor X activation compared to TF-VIIa (Fig. 4B). Kinetic analysis of the presented data demonstrated an increase in the $K_{m}$ of 205 ± 102 nM (TF-VIIa) to 522 ± 209 nM (TF$_{A165A166}$-VIIa) and a decrease in the $V_{max}$ of 181 ± 45 nM/min (TF-VIIa) to 110 ± 58 nM/min (TF$_{A165A166}$-VIIa) (mean ± standard deviation, n = 6). When corrected for the 2-fold difference in cell surface expression of the molecules on the cell populations used, these data reflect 3-fold reduction in the maximal rate of substrate cleavage and an 8-fold reduction in the catalytic efficiency for factor X cleavage by TF$_{A165A166}$-VIIa compared to TF-VIIa.

Factor X activation was also analyzed in the absence of phospholipid. Dose titration of VIIa at a fixed concentration of factor X (5 µM) was performed with TF (4 nM) in the presence of either 4 or 0.4 mM CHAPS. The concentrations of VIIa, CHAPS, and factor X were determined for the indicated number of experiments (4). Since the reaction was 100 µl, 200 fmol of TF and VIIa were added and quantitatively bound to the wells, consistent with previous experiments (4).

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**TABLE I**

| TF               | CHAPS | $n$  | $K_d$ (nM) | $B_{max}$ (fmol/well) |
|------------------|-------|------|------------|----------------------|
| Wild-type        | 0.4   | 10   | 9.1 ± 4.7  | 140 ± 50             |
| Wild-type        | 4     | 6    | 7.9 ± 2.9  | 230 ± 70             |
| TF$_{A165A166}$  | 0.4   | 9    | 10.6 ± 5.9 | 210 ± 60             |
| TF$_{A165A166}$  | 4     | 6    | 8.0 ± 5.1  | 260 ± 100            |

FIG. 3. Dissociation of VIIa from TF and TF$_{A165A166}$. VIIa (20 nM) was assembled with cell surface TF or TF$_{A165A166}$ for 2 h followed by displacement analysis at 37 °C. Dilution jump was used in one set of experiments to prevent the reassociation of VIIa dissociated from TF (A) or TF$_{A165A166}$ (G). A 100-fold molar excess of the inhibitory anti-TF mAb TF-96B4 was used in a second set of experiments to prevent the reassociation with TF (C) or TF$_{A165A166}$ (I).

FIG. 4. Substrate cleavage by TF-VIIa and TF$_{A165A166}$-VIIa. A, cleavage of the peptide chromogenic substrate Spectrozyme FXa (1 nM) by a complex formed with VIIa (50 nM) and various concentrations of TF (O) or TF$_{A165A166}$ (I) in the presence of 0.4 mM CHAPS. The inset shows the same experiment performed in the presence of 4 mM CHAPS. The concentrations of VIIa, CHAPS, and factor X were determined for the indicated number of experiments (4).
of VIIa which were required to achieve half-maximal rates were similar for TF and TF\textsubscript{A165A166} at 4 and 0.4 mM CHAPS (Fig. 5). However, the maximal rate of factor X activation in the presence of TF\textsubscript{A165A166} was reduced 3-fold compared to the reaction with an identical concentration of wild-type TF, when analyzed at 0.4 mM CHAPS. This is consistent with the difference in reaction rates observed on cell surfaces and further supports the proposed selective reduction in protein substrate cleavage by TF\textsubscript{A165A166}-VIIa. In addition, the maximal rates were influenced by the detergent concentration. The rate of factor X activation was slightly reduced by 30% when wild-type TF at 4 mM CHAPS was compared with reactions at 0.4 mM detergent. This indicates that detergent interferes with the transient protein substrate assembly and cleavage by TF-VIIa, and the reduction appears to be selective, since cleavage of chromogenic peptidyl substrates was not altered at high detergent concentrations (Fig. 4A). The reaction rate for factor X cleavage by TF\textsubscript{A165A166}-VIIa decreased even more, and a 70% reduction was observed when reactions at 4 mM CHAPS were compared to the low detergent conditions at saturating concentrations of VIIa (Fig. 5B). The slight alteration of the cleavage of protein substrate by TF-VIIa due to detergent thus appeared to be exaggerated with the mutant TF consistent with the idea that protein substrate recognition was further diminished in the presence of detergent. The differences between TF and TF\textsubscript{A165A166} were also apparent when the concentration of factor X was varied at a fixed concentration of VIIa (Fig. 6A). Consistently, high concentrations of detergent inhibited TF-VIIa complex to a lesser extent than the TF\textsubscript{A165A166}-VIIa complex (Fig. 6B), and increasing concentrations of factor X did not compensate for the reduced rate of protein substrate hydrolysis.

**Fig. 6. Effect of factor X concentration on the activation by TF-VIIa and TF\textsubscript{A165A166}-VIIa.** The initial rate for cleavage of factor X in the presence of 15 nM VIIa and 4 nM TF (C) or TF\textsubscript{A165A166} (○) was determined at 37 °C and 5 mM Ca\textsuperscript{2+}. Means and standard deviations from three experiment are given. The analysis was performed in the presence of 0.4 mM (A) or 4 mM (B) CHAPS.

**Fig. 7. Competition of TF\textsubscript{A165A166} with TF.** The hypothesis of selective reduction in protein substrate cleavage without loss of VIIa binding function by TF\textsubscript{A165A166} was further tested by competition analysis. At both, 4 and 0.4 mM CHAPS, TF\textsubscript{A165A166} effectively competed with TF for a limited amount of VIIa (2 nM) in the assay (Fig. 7). A 10-fold excess of the mutant over wild-type TF reduced the rate of factor X activation to levels which were observed at 2 nM TF\textsubscript{A165A166}, and a 50% reduction of the rate of factor X activation by TF-VIIa was observed at approximately equal concentrations of TF (2 nM) and the mutant (Fig. 7). These data are consistent with unaltered VIIa binding by TF\textsubscript{A165A166} even in the presence of substrate and at different detergent concentrations and provide further support that the TF\textsubscript{A165A166}-VIIa complex exhibits a selective defect in protein substrate recognition.

**DISCUSSION**

Assembly of a protease with a cofactor receptor and subsequent oriented association of substrate is an efficient mechanism to generate proteolytic activity on cell surfaces. Fora-
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tion of the TF·VIIa complex by high affinity binding of VIIa to TF is a requisite for full catalytic and proteolytic activity of VIIa. A functional GlA domain in VIIa appears to be required for high affinity binding of VIIa to TF and for the resulting efficient recognition of protein substrates (4). We here characterize a TF mutant which demonstrates normal VIIa binding, but a selective defect in the support of macro-molecular substrate recognition in the TFΔ165A166·VIIa complex suggesting a contribution of the cofactor to substrate recognition which is independent of the high affinity binding of VIIa.

TFΔ165A166 appeared to adopt a proper global structure based on the lack of defects in the expression of three non-overlapping mAb epitopes, in apparent glycosylation and in proper cellular processing including high levels of cell surface expression. Proper global folding of TFΔ165A166 is also suggested by unaltered high affinity binding of VIIa and by efficient chromogenic peptidyl substrate cleavage by the TFΔ165A166·VIIa complex. Normal VII binding function of TFΔ165A166 was substantiated by radioligand binding analysis using TF expressed on viable cells as well as TF extracted from the cells and solubilized with detergent. Dissociation of VIIa bound to TFΔ165A166 was similar to wild-type TF, and TFΔ165A166 effectively competed with TF in functional assays. The discrete functional defect resulting from the deletion of the side chains of Lys-165 and Lys-166 was a diminished rate of proteolytic activation of the protein substrate factor X by the TFΔ165A166·VIIa complex. This reduced activation of factor X was observed both in the presence and in the absence of a phospholipid surface. High concentrations of detergent interfered with factor X activation by TFΔ165A166·VIIa to a larger extent than with the activation by TF·VIIa. These observations converge upon the conclusion that macromolecular recognition of factor X by the binary complex is selectively altered by the mutations in Lys-165 and Lys-166 consistent with the possibility of a direct contribution of cofactor residues to interactions with substrate.

There appears to be a quantitative difference when the reduction in coagulation of plasma is compared with analysis in the purified protein system. Clotting activity of TFΔ165A166 on viable cells was reduced almost 100-fold, whereas the catalytic efficiency of TFΔ165A166 on viable cells was reduced only 8-fold compared to wild-type TF. This difference arises in part from the much lower factor X concentration (∼50 nM) in the clotting assay compared to the high concentrations (up to 5 μM) in the purified system. Since there appears to be a slight increase in the $K_{M app}$ for factor X activation by TFΔ165A166·VIIa in comparison to TF·VIIa, the loss of function would be more pronounced at lower substrate concentrations. In addition, the activation of factor IX by TF·VIIa is enhanced in the presence of factor X suggesting interactions of the two substrates during the initiation of the extrinsic pathway of coagulation (30). The cooperative activation of factor IX may contribute to the overall procoagulant activity determined in a clotting assay, and the demonstrated defect in factor X recognition may lead to a greater functional loss due to an indirect effect on the factor IX activation. However, direct loss of factor IX substrate recognition cannot be excluded based on our analysis.

The present study demonstrates critical contributions of 2 lysine side chains in the carboxyl half of the extracellular domain to extended recognition of factor X by the TF·VIIa complex. Epitope mapping of an inhibitory anti-TF mAb also implicated structures in exons 4 and 5 in binding of factor VII (21), and residues 129–169 have been shown to be in proximity to VIIa by photoaffinity cross-linking (31). Lysine 165 and 166 may be in some spatial proximity to VIIa interactive sites on TF. However, no alterations in the VII binding to TFΔ165A166 were observed suggesting that the side chains of lysine residues 165 and 166 do not significantly contribute to the proposed VII interactive site in the carboxyl half of the TF extracellular domain (18). Kinetic analysis has previously provided evidence that association of substrate in the ternary complex diminishes dissociation of the TF·VIIa complex (32) suggesting that substrate interaction with the binary complex either tightens the binding of VIIa to TF by allosteric alterations of VIIa or prevents dissociation of VIIa by binding to a site on TF which is spatially close to VIIa interactive sites and thereby sterically hinders VIIa mobility and dissociation of the complex. Lys-165 and Lys-166 could form specific contacts with VIIa which are critical for extended recognition of substrates and which do not significantly contribute to the binding energy in the absence of substrate. In the presence of substrate, however, these contacts may prevent dissociation of VIIa from the TF·VIIa complex. Alternatively, these 2 lysine residues in TF could provide critical contacts in an interactive site for substrate on TF. Macromolecular assembly of substrate with the TF·VIIa complex may involve residues in VIIa as well as in TF which, once associated, provide a contiguous region for extended recognition of substrate. This analysis provides evidence for multiple alignments during the assembly of substrate with an enzyme-cofactor complex. A model for efficient substrate assembly is suggested which involves both enhancement of function of the catalytic site and association of specific cofactor and enzyme residues in a site for extended recognition of substrate.

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Note Added in Proof—After analysis of the data presented in the recently published analysis of the TFΔ165A166 mutant (Roy, S., Hass, P. E., Bourell, J. H., Henzel, W. J., Vehar, G. A. (1991) J. Biol. Chem. 266, 22063-22066), we cannot concur with their conclusion that these two Lys residues contribute to interaction with phospholipid, but rather consider our conclusion that macromolecular substrate recognition in the TFΔ165A166·VIIa complex is selective and is valid.

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