Characterization of Factor VII Association with Tissue Factor in Solution

HIGH AND LOW AFFINITY CALCIUM BINDING SITES IN FACTOR VII CONTRIBUTE TO FUNCTIONALLY DISTINCT INTERACTIONS

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Protein-phospholipid as well as protein-protein interactions may be critical for tight binding of the serine protease factor VIIa (VIIa) to its receptor cofactor tissue factor (TF). To elucidate the role of protein-protein interactions, we analyzed the interaction of VII/VIIa with TF in the absence of phospholipid. Binding of VII occurred with similar affinity to solubilized and phospholipid-reconstituted TF. Lack of the γ-carboxyglutamic acid (Gla)-domain (des-(1-38)-VIIa) resulted in a 10- to 30-fold increase of the Kₜ for the interaction, as did blocking the Gla-domain by Fab fragments of a specific monoclonal antibody. These results suggest that the VII Gla-domain can participate in protein-protein interaction with the TF molecule per se rather than only in interactions with the charged phospholipid surface. Gla-domain-independent, low affinity binding of VII to TF required micromolar Ca²⁺, indicating involvement of high affinity calcium ion binding sites suggested to be localized in VII rather than TF. Interference with Gla-domain-dependent interactions with TF did not alter the TF-VIIa-dependent cleavage of a small peptidyl substrate, whereas the proteolytic activation of the protein substrate factor X was markedly decreased, suggesting that the VIIa Gla-domain not only participates in the formation of a more stable TF-VIIa complex but contributes to extended substrate recognition.

Binding of an enzyme to a cell surface receptor is an effective mechanism for localizing enzymatic activity. This activity may be further enhanced if the receptor also serves as a cofactor. The binding of thezymogen coagulation factor VII (VII) to its receptor tissue factor (TF)¹ (1, 2) and the subsequent rapid conversion of bound VII to the active serine protease factor VIIa (VIIa) (3) is the trigger for cell surface activation of the coagulation cascades. VIIa possesses extremely low catalytic activity when free in solution or when associated with a phospholipid surface in the absence of its cofactor TF (4–6). Function of the TF-VIIa complex is optimal when TF is cell surface-expressed or reconstituted into phospholipid vesicles. Binding of VIIa to TF under these conditions results in a profound increase in its catalytic efficiency to activate the substrate factors X and IX (5, 6).

The extracellular domain of TF mediates protein-protein interactions with VIIa which are required for catalytic enhancement of substrate cleavage by the VIIa catalytic domain (6). Binding of VII/VIIa to cell surface TF occurs with high affinity at 2–5 mM CaCl₂ (1, 7, 8), and the VIIa Gla-domain has been implicated in the high affinity binding to cell surface TF (9). From these findings, one can speculate that the Gla-domain of VIIa may interact with charged phospholipid surfaces during assembly with TF. Binding of VII/VIIa to unidentified sites on TF has been suggested to be mediated in part by residues 195–206 located in the catalytic domain of the protease (10). To further evaluate the significance of the Gla-domain and other interactive regions of VIIa for binding to TF, we have estimated binding constants for the interaction of VII with TF in solution. We characterize here the interaction of both full length, detergent-solubilized TF and the isolated TF extracellular domain with VIIa, as well as VIIa lacking the Gla-domain (des-(1–38)-VIIa). We propose participation of both high and low affinity Ca²⁺ binding sites on VIIa which are critical for the formation of the TF-VIIa complex. In addition, we provide evidence that these two types of Ca²⁺-dependent interactions enhance the catalytic activity of VIIa by increasing the function of the catalytic center of VIIa and by improving extended recognition of protein substrates.

EXPERIMENTAL PROCEDURES

Proteins—Human coagulation proteins were purified from plasma as previously described (6). Prothrombin fragment 1 was produced from purified prothrombin as described (6). TF was immunoadfinity-purified from human brain (11) and reconstituted into mixed phosphatidylincholine/phosphatidylserine (70:30, w/w) vesicles (12). The isolated TF extracellular domain (TFα-219) was produced by inserting a stop codon in the TF coding sequence and expressing the recombinant protein in Chinese hamster ovary cells as described (6). The secreted protein was purified from the culture medium using a two-step procedure of monoclonal antibody (mAb) affinity purification followed by gel filtration using Sephadex G-75 Superfine. All proteins were homogeneous by SDS-polyacrylamide gel electrophoresis (13). Recombinant VIIa and the partially proteolyzed des-(1–38)-VIIa which lacks the Gla domain were prepared as described (9, 14). mAbs to VII were produced by immunization of mice with plasma-derived VII/VIIa using standard hybridoma technology. Hybridomas were initially screened by solid phase radioimmunoassay for binding of VII/VII (2 nM). mAb F4-2.1B was selected for binding of fluid phase VII in the presence of CaCl₂ but not in the presence of EDTA.

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Hybridomas were recloned two to three times, and antibody was produced by ascites growth of stable cells. mAbs were purified as previously described (15). Fab fragments of mAbs were produced by cleavage of purified IgG with immobilized papain (16) in 20 mM NaH2PO4, 20 mM cysteine-HCl, 10 mM EDTA, pH 7.0, for 16 h, followed by the use of detergent, phase partition, and uncleaved mAb by binding to immobilized protein A (17).

Functional Assays—Catalytic activity of VIIa bound to TF was analyzed by hydrolysis of a peptide chromogenic substrate, as well as by limited proteolytic activation of factor X. Peptidyl hydrolysis was determined by mixing TF and VIIa in the presence of 1.25 mM Spectrozyme FXa and calcium ions and continuously monitoring the increase in the absorbance at 405 nm in a Molecular Devices (Menlo Park, CA) kinetic microplate reader, essentially as described (16). Cleavage of factor X by the TF-VIIa complex was analyzed by a coupled amidolytic assay as described (18). Briefly, detergent-solubilized TF or TF1.219 and VIIa were preincubated in the presence of 5 mM CaCl2 for 10 min followed by addition of substrate factor X. Samples were removed and quenched in EDTA each minute. Generated factor Xa was determined with a chromogenic substrate, and initial rates of factor Xa formation were calculated.

Analysis of the TF-Factor VII Interaction in Solution—The interaction of VIIa with TF or TF1.219 in solution was analyzed using [125I]-VII, unlabeled TF, and a noninhibitory anti-TF monoclonal antibody (TF9-1OHI0) as a capture antibody (15). Proteins were radiolabeled using the coupled lactoperoxidase/glucose oxidase method as described (18). Falcon MicroTest III plates (Becton Dickinson Labware, Oxnard, CA) were incubated with 10 µg/ml TF9-10H10 (100 µl) overnight followed by blocking with 200 µl of 5% dry milk dissolved in TBS (20 mM Tris, 130 mM NaCl, pH 7.4). After three washes with TBS, a mixture of varying concentrations of [125I]-VII and TF or TF1.219 in the presence of 5 mM CaCl2, 0.5% BSA were added to the wells and incubated at 37 °C for 90 min. Radioactivity bound to the wells was determined after 10 rapid washes of the plates with ice-cold TBS, 5 mM CaCl2, 0.5% BSA. Nonspecific binding was determined for each dilution of radiolabeled ligand from wells which had not been coated with the mAb, but which contained an identical reaction mixture. The specifically bound radioactivity was assumed to be the radioactivity in coated wells which had been coated with the mAb, but which contained an identical reaction mixture. The specifically bound radioactivity was determined by varying the calcium concentration in the incubation mixture from 50 pM to 5 mM. The concentration of calcium in the washing buffer was 50 µM for all the calcium concentrations determined. No difference in the binding data at 5 mM CaCl2 was observed between assays which were washed at 50 µM or 5 mM CaCl2. Displacement of bound ligand was studied by mixing TF or TF1.219 with 100 nM [125I]-VII in the presence of 5 mM CaCl2 followed by an incubation (90 min) in the TF9-10H10-coated wells to allow assembly with the antibody. The mAb TF9-5G4 (1 FM) was added to the reaction mixture from 50 pM to 5 mM. The specifically bound radioactivity was determined after washes as described above. This analysis demonstrated a slow dissociation of the bound TF1.219-VII complex with half-dissociation times of 20 to 40 min at 37 °C, consistent with the slow dissociation of VII from cell surface TF (7). This validates that the rapid washes (<1 min) with cold buffers do not allow substantial dissociation of complexes. Binding analysis of [125I]-VII to cell surface TF was performed as described (18).

Characterization of the Anti-VII mAb F4-2.1B—Binding of solution phase VII to mAb F4-2.1B was analyzed by coating Falcon MicroTest III plates with purified IgG at 10 µg/ml overnight, followed by blocking with 5% nonfat dry milk in TBS. [125I]-VII, [125I]-VIIa, or [125I]-des-(1-38)-VIIa (5 nM) were incubated for 60 min followed by rapid washes with ice-cold buffer. For competition analysis between mAbs, a 100-fold molar excess of competitor over [125I]-VII was added in the incubation mixture. For Western blot analysis of mAb reactivity, 1 µg of reduced VIIa per gel lane was separated by SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose and blocking with 5% nonfat dry milk in TBS. A 1:5 dilution of culture supernatant which was adjusted to 5 mM CaCl2 was incubated with the membrane followed by detection using a secondary, alkaline phosphatase-conjugated antibody as described (6). VII binding to immobilized phospholipid was performed as described (6). Inhibition of VII binding by F4-2.1B was tested by including the mAb (1 µM) in the incubation mixture of VII, 5 mM CaCl2, and the immobilized phospholipid.

NMR Spectroscopy—The effect of Ca2+ on the conformation of TF1.219 was studied by NMR spectroscopy. 1H NMR spectra were acquired on a Bruker AM-500 spectrometer; 1H 2D transients were collected with 8192 data points at 310 K. Solvent suppression was achieved by presaturation of the H2O resonance, and a Juhn-echo pulse scheme was used for detection to eliminate baseline artifacts (20). Ca2+ was removed from the protein sample (TF1.219) by incubation with 10 mM EDTA for 2 h followed by ultrafiltration to adjust to 62 mM NaCl, 50 mM EDTA, and 2.5 mM phosphate, pH 6.5. Analysis was performed with 840 µM TF1.219. Ca2+ was titrated into the sample in 2 aliquots. First, 3.5 mM CaCl2 was added which yielded approximately 1 mM free Ca2+, since 2.5 mM phosphate precipitated upon the addition of CaCl2. The second addition of 9 mM CaCl2 yielded a net Ca2+ concentration of 10 mM.

RESULTS

Analysis of VII Binding to TF in Solution—The noninhibitory anti-TF mAb TF9-10H10 was used to provide an efficient phase separation in studies which characterize the TF-VII interaction in solution. This mAb does not interfere with VII binding to cell surface TF (15). Conversely, binding of detergent-solubilized [125I]-TF or soluble [125I]-TF1.219 to the mAb TF9-10H10 was not inhibited in the presence of 300 nM VII (Fig. 1). If VII binding to TF is analyzed, the TF-VII complexes bound to the mAb should reflect the concentration of complexes formed in solution. However, the fraction of the complexes in the solution which binds to the plate will depend on the concentration of TF in the reaction.

Scatchard analysis can be used to characterize the equilibrium of ligand (L) binding to a receptor (R). The dissociation

**Fig. 1.** Binding analysis of [125I]-TF1.219 and [125I]-TF to immobilized Piab TF9-1OHI0. Plates were incubated for 90 min at 37 °C with the indicated concentrations of TF1.219 (A) or TF (B) and no (O) or 300 nM (D) plasma-derived VII in the presence of 0.5% BSA, 5 mM CaCl2. Mean and standard deviations (n = 3) are given.
constant is given by $[RL] = [R_{total}] - K_d \cdot [RL]/[L_{free}]$. $[RL]$ and $[L_{free}]$ represent the concentrations of receptor-ligand complex and free ligand, $[R_{total}]$ corresponds to the total receptor concentration in the assay. If only a fraction $f$ of the complex is measured, the equation can be transformed to allow determination of the $K_d$ which is given by $f \cdot [RL] = f \cdot [R_{total}] - f \cdot [RL]/[L_{free}]$. Fitting of the data to this equation yields the $K_d$ which is a valid estimate for the interaction, but the calculation only yields the fraction $f$ of the total receptor concentration $[R_{total}]$. At low receptor concentrations, e.g., $[TF] = 2 \text{ nM}$ and $[TF_{1.219}] = 10 \text{ nM}$, the bound fraction $f$ is approximately 50% and 33%, respectively (Fig. 1). We determined the concentration of ligand by counting an aliquot of the supernatant of each well. This concentration corresponds to $[L_{free}] + (1 - f) \cdot [RL]$. At low receptor concentrations, the experimentally observed free receptor-ligand complexes were less than 5% of the free ligand in most of the reactions, and, therefore, in approximation, $[L_{free}] + (1 - f) \cdot [RL] \approx [L_{free}]$, since $[L_{free}] \gg (1 - f) \cdot [RL]$. Parameters for $f$ binding to TF at 2 nM or TF$_{1.219}$ at 10 nM (Table I) were calculated based on this approximation.

For most of the experiments, higher concentrations of TF were used to achieve higher antibody saturation. Increasing the concentration to 100 nM TF$_{1.219}$ and 20 nM TF resulted in a lower fraction of bound complexes which was 7% and 30% of the respective receptor concentration (Fig. 1). The free receptor complexes therefore represented a considerable fraction in the supernatant of the wells, and we obtained $[L_{free}]$ for these experiments by subtracting $(1 - f) \cdot [RL]$ from the total concentration of ligand in the supernatant. Comparison of the binding parameters at high and low TF or TF$_{1.219}$ in Table I demonstrates that the dissociation constants obtained by the two procedures are similar and that the maximal number of sites ($B_{max}$) is in reasonable agreement with the fraction of receptor bound to the mAb $(f \cdot [R_{total}])$ based on Fig. 1.

**TABLE I**

**Binding constants for the TF-VII interaction in solution**

| Ligand | Ca | TF | TF$_{1.219}$ | $K_d$ | $B_{max}$ | n |
|--------|----|----|-------------|-------|-----------|---|
| VII    | 5  | 10 | 116.0 ± 41.4 | 410 ± 140 | 3          |
| VII    | 5  | 100| 120.4 ± 41.8 | 710 ± 240 | 7          |
| VIIa   | 5  | 100| 90.3 ± 26.5  | 850 ± 420 | 3          |
| VII    | 5  | 2  | 10.2 ± 3.5   | 180 ± 30  | 3          |
| VII    | 5  | 20 | 9.2 ± 3.5    | 340 ± 90  | 7          |
| VIIa   | 5  | 20 | 4.2 ± 2.6    | 220 ± 100 | 3          |
| VII (−Triton) | 5 | 20 | 7.4 ± 2.3    | 370 ± 110 | 3          |
| VII + Fragment 1 | 5 | 100| 87.2 ± 38.6  | 850 ± 200 | 3          |
| VII + Fragment 1 | 5 | 100| 84 ± 3.9     | 370 ± 40  | 3          |
| des−(1−38)−VIIa | 5 | 100| 120.0 ± 34.2 | 460 ± 100 | 4          |
| des−(1−38)−VIIa | 5 | 20 | 112.3 ± 17.7 | 500 ± 180 | 4          |
| VII + F4-2.1B | 5 | 100| 225.0 ± 119.0| 420 ± 110 | 4          |
| VII + F4-2.1B | 5 | 20 | 139.8 ± 34.9 | 470 ± 170 | 4          |
| VII    | 1  | 100| 109.7 ± 14.2 | 380 ± 130 | 3          |
| VII    | 1  | 20 | 8.9 ± 3.5    | 340 ± 120 | 3          |
| VII    | 0.2| 100 | 127.3 ± 20.2 | 160 ± 80  | 3          |
| VII    | 0.2| 20 | 51.8 ± 16.5  | 330 ± 30  | 3          |
| VII    | 0.05| 100| 189.0 ± 86.5 | 240 ± 110 | 4          |
| VII    | 0.05| 20 | 142.2 ± 57.5 | 410 ± 60  | 5          |

**VII Binding to Solubilized TF and TF$_{1.219}$**—The binding of plasma-derived human VII to human brain TF which was solubilized with Triton X-100 (<0.02% in the assay) was characterized. We determined a $K_d$ of 9.2 nM for the binding of VII to solubilized TF (Table I, Fig. 2B) which is comparable to the interaction with TF reconstituted into phosphatidylcholine vesicles. This interaction has been shown to occur with a $K_d$ of 13.2 nM at 37 °C and 5 mM CaCl$_2$ (21). Similarly, VIIa bound with a $K_d$ of 4.2 nM (Table I), indicating an apparent affinity slightly greater for the active serine protease. This is comparable to findings for TF associated with phospholipid vesicles (21). In contrast, the TF extracellular domain TF$_{1.219}$ bound VII (Table I, Fig. 2A) and VIIa (Table I) with an approximately 10-fold higher $K_d$. This may indicate that the truncated molecule is slightly less well conformed compared to full length TF resulting in loss of some protein-protein interactions contributing to the tight binding of VII to TF. Alternatively, the tighter binding of VII to detergent-solubilized TF might indicate a favorable interaction of the VII Gla-domain with the detergent associated with the hydrophobic membrane-spanning domain of TF (residues 220−242). To exclude this possibility, we extracted lyophilized TF once with 100% and twice with 80% acetonitrile to remove most of the bound detergent (11). This did not alter the $K_d$ of VII binding to TF (Table I). In addition, we attempted to block potential Gla-domain interactions with detergent by the addition of 100 μM prothrombin fragment 1 which is homologous to the Gla-domain of VII and blocks phospholipid binding of other Gla-domain-containing proteins due to the occupation of the phospholipid binding sites (22, 23). Since we included the prothrombin fragment 1 at a fixed concentration of 100 μM, a 10,000-fold molar excess was present relative to VII at 10 nM. Prothrombin fragment 1 did not alter the $K_d$ of VII binding to TF or TF$_{1.219}$ (Table I). These data suggest contribution of the Gla-domain to specific protein-protein interactions which result in a more stable TF-VII complex, rather than to a less specific type of interaction exemplified by the prothrombin Gla-domain.
**Binding of des-(1-38)-VIIa to TF**—In contrast to cell binding assays (9), the binding assay used here allowed determination of binding parameters at a 10-fold higher Kd, as demonstrated for the TF<sub>1.219</sub>-VII complex. We determined a 30-fold higher Kd for the des-(1-38)-VIIa binding to detergent-solubilized TF as compared to the binding of VIIa (Fig. 3A, Table I). However the dissociation constants of the binding of VIIa and des-(1-38)-VIIa to TF<sub>1.219</sub> were identical (Fig. 3B, Table I). This indicates that at least one interactive site in des-(1-38)-VIIa is properly formed, and it further suggests that the truncated TF molecule has a less conformed structural site participating in binding of the VIIa Gla-domain. These data support our hypothesis that the Gla-domain of VII might directly interact with TF. However, the possibility exists that proteolytic removal of the amino-terminal Gla-domain slightly alters the folding of the protein and/or inter-domainal interactions within VIIa. We explored this further using mAb F4-2.1B which binds to the Gla-domain. This mAb should interfere with the postulated interactions of the Gla-domain with TF.

Hybrdrome F4-2.1B was generated by immunization with human VII/VIIa purified from plasma and identified by screening for binding only in the presence of Ca<sup>2+</sup>. The mAb reacted with the light chain of VIIa. In solution, it bound VII and VIIa at an optimal calcium concentration of 1-5 mM, but not in the presence of 1 mM EDTA. F4-2.1B failed to bind des-(1-38)-VIIa, when analyzed under conditions identical with those where it bound VIIa. Binding of des-(1-38)-VIIa and VIIa was comparable for 19 additional mAbs which were raised against plasma VIIa. In a binding assay with immobilized mixed phosphatidylinerine/phosphatidylcholine vesicles (30/70) (6), addition of 1 μM F4-2.1B decreased the specific binding of 300 nM VII to immobilized phospholipid by 30%. These data are consistent with epitope localization in the Gla-domain. No competition of F4-2.1B with a panel of 19 VII-specific mAbs which were calcium-dependent as well as calcium-independent was observed suggesting that binding of this mAb to VII does not conformationally perturb VII resulting in epitope loss. In addition, the Kd of VII for binding to TF<sub>1.219</sub> was diminished in the presence of this mAb (Table I). However, in the presence of F4-2.1B, VII binding to full length TF occurred with a Kd which was identical with that determined for the interaction with TF<sub>1.219</sub>(Table I). Consistent with these results, binding of VII to cell surface TF was inhibited in the presence of F4-2.1B (data not shown). These data provide an independent line of evidence that selective interference with Gla-domain-mediated interactions prevent high affinity binding of VII to TF.

**Requirement of Calcium for the Low Affinity Interaction of VII with TF**—Previous studies have demonstrated a calcium optimum of 2-5 mM for the interaction of VII with cell surface TF (1, 7, 8). In light of the potential interaction of the Gla-domain of VIIa with TF, these findings indicate that optimal interaction requires a conformation of the Gla-domain imparted by occupancy of the low affinity calcium binding sites. We analyzed the effect of Ca<sup>2+</sup> concentration on the Kd for binding of native VII to both full length TF and the truncated TF<sub>1.219</sub>. The binding to TF<sub>1.219</sub> was not affected by variation of the calcium concentration from 50 μM to 5 mM (Table I). In contrast, the Kd of VII for binding to detergent-solubilized TF approximated the Kd for TF<sub>1.219</sub>, when the calcium concentration was reduced to 50 μM (Table I). The Kd of VII binding to TF at 50 μM calcium was comparable to the Kd of des-(1-38)-VIIa binding to TF. These data demonstrate that the low affinity calcium binding sites of the VII Gla-domain must be saturated for tight binding to TF, consistent with a calcium-dependent structure which participates in this interaction. The low affinity binding of VII to TF as well as TF<sub>1.219</sub> was abolished in the presence of EDTA indicating that the low affinity interaction of VII with TF is dependent on Ca<sup>2+</sup> ions at micromolar concentrations. This indicates involvement of a high affinity calcium ion binding site to properly conform one or more interactive sites on TF or VII or both.

**Conformational Effects of Calcium Ions on TF**—The effect of Ca<sup>2+</sup> on the conformation of TF was analyzed by 1H NMR spectroscopy. Several studies on calcium-binding proteins demonstrated that large spectral changes occurred upon the addition of Ca<sup>2+</sup>. Shifts in aromatic resonances of as much as 0.5 ppm and in methyl resonances in the order of 0.1 to 0.2 ppm are reported (24-26). The NMR spectra obtained for TF<sub>1.219</sub> in the presence of 50 μM EDTA and 10 mM CaCl<sub>2</sub> were similar (Fig. 4A). Both the aromatic (5.0 to 11.0 ppm) (Fig. 4B) and the methyl (−1.0 to 1.0 ppm) (Fig. 4C) regions were monitored during the Ca<sup>2+</sup> additions to TF<sub>1.219</sub> and no detectable differences were observed. These results exclude a major effect of Ca<sup>2+</sup> on the conformation of TF<sub>1.219</sub> and suggest that the Ca<sup>2+</sup> requirement for binding of VII to TF involves one or more calcium binding sites on VII.

**Functional Impact of the High and Low Ca<sup>2+</sup>-dependent Interactions**—We analyzed the characteristics of peptidyl hydrolysis and cleavage of the natural substrate factor X by VIIa when complexed with TF or TF<sub>1.219</sub>. Catalytic activity of TF-VIIa and TF<sub>1.219</sub>-VIIa complexes were not significantly different when peptidyl hydrolysis was examined (Fig. 5A) (6). The apparently lower rate of peptidyl hydrolysis at high TF concentrations is due to inhibition of the assay by the excess of Triton X-100 (>0.25% final concentration) which was used to solubilize the stock solution of TF. These data suggest comparable functional activity of both forms of TF to more effectively enable the VIIa catalytic triad for cleavage of small substrates. The enhancement of peptidyl hydrolysis by VIIa in the presence of TF was abolished in the presence of EDTA (6), consistent with the binding data which demonstrated dissociation of the complex upon removal of Ca<sup>2+</sup>. Peptidyl hydrolysis mediated by 200 nM des-(1-38)-VIIa was approximately 60% of that mediated by an identical concen-
Fig. 4. 1H NMR analysis of TF1-219 in the presence and absence of Ca2+. A, the acquired 1H NMR spectra for TF1-219 (840 μM) in the presence of 50 μM EDTA or 1 and 10 mM Ca2+ are given. An enlarged view of the aromatic (B) and the methyl (C) regions shows no resonance shifting upon the addition of Ca2+ to TF1-219.

Fig. 5. Peptide hydrolysis by VIIa and des-(1-38)-VIIa. A fixed concentration (200 nM) of VIIa (A) or des-(1-38)-VIIa (B) was assembled with the indicated concentrations of TF (●) or TF1-219 (○) in the presence of 0.5% BSA, 5 mM CaCl2, and 1.25 mM Spectrozyme FXa at ambient temperature. Mean and standard deviation (n = 3) for the rate of absorbance increase at 405 nm (mOD/min) is given as a measure for peptidyl hydrolysis by the catalytic complex.

Limit proteolytic cleavage of the protein substrate factor X by VIIa was approximately 5- to 10-fold slower when TF was replaced by TF1-219 (Fig. 6A), consistent with previous analysis (6). The rate of factor X cleavage by 200 nM des-(1-38)-VIIa was approximately 100-fold less when in complex with TF as compared to the TF-VIIa complex (Fig. 6B). Similar rates were obtained with TF1-219 when very high concentrations of the cofactor were added to ensure saturation of des-(1-38)-VIIa. These data suggest that function of the catalytic site of des-(1-38) VIIa for peptidyl substrates is readily imparted by complex formation with TF and equally effective with TF1-219, whereas extended substrate recognition, as for protein substrates, is severely diminished. This also applied to phospholipid-bound factor X; in the presence of 1 mM phospholipid-reconstituted TF, the rate of activation of 1 μM factor X by 500 nM des-(1-38)-VIIa was 80% reduced compared to an identical concentration of VIIa. The binding analysis indicated that TF1-219 was deficient in providing a complementary interactive site for at least a Gla-domain-dependent interactive site on VII. Similarly, interaction of VII with TF1-219 appeared to result in a less efficient interaction with the protein substrate factor X. However, rates in the presence of TF1-219 were different for VIIa and des-(1-38)-VIIa suggesting that TF1-219 may provide transient interactions for the VII-Gla domain resulting in more efficient substrate interaction with VIIa. This could be consistent with the concept that conformational entropy is modified in
TF-VIIa providing a less optimal complementary structure for VIIa Glα-domain-dependent interactions. Further evidence for this idea is provided by the finding that cleavage of phospholipid-associated factor X by TF-VIIa and TFa(VII) complexes occurred with similar catalytic efficiency (6), further emphasizing that, at least transiently, a good fit does occur between VIIa and TFa(VII) to form a functional TFa(VII)-VIIa complex.

**DISCUSSION**

The assembly of VIIa with its cellular receptor TF is the critical initial reaction leading to efficient limited proteolytic activation of the substrate factors X and IX on cell surfaces. Insertion of TF into a phospholipid bilayer is critical for full functional activity (6, 6, 27). The preferential cleavage of factor X that is associated with the phospholipid surface may contribute largely to the increase in catalytic activity of the TF-VIIa complex in a phospholipid environment (6). By analogy to homologous proteins, interactions of the VIIa Glα-domain with charged phospholipid surfaces could additionally facilitate the assembly of the protease with its receptor cofactor. The Glα-domain of VIIa has been suspected to mediate interactions with the phospholipid surface resulting in conformational changes which facilitate binding of additional sites on VII to the TF extracellular domain (9, 10). However, inhibitors of protein interactions with phospholipid have not been shown to inhibit VII binding to TF, whether cell-surface-expressed or reconstituted into phospholipid (28); thus, the importance of VII Glα-domain interactions with phospholipid for high affinity binding to TF and for functional activity has not been established. We address here the functional significance of the Glα-domain of VII and its interaction with TF by analyzing the formation of the TF-VII complex in solution.

Binding constants for the TF-VII interaction were estimated from an assay which uses a noninhibitory anti-TF mAb to capture TF-VII complexes which formed in solution. Both plasma VII and recombinant VIIa bound to detergent-solubilized full length TF with affinities similar to those observed for TF reconstituted into uncharged phospholipid (21). The affinity was not significantly influenced by the absence of detergent or by competition for potential Glα-domain interactions by a high molar excess of prothrombin fragment 1. These data provide evidence that insertion of TF into a phospholipid bilayer is not critical for the TF structure to provide binding function and that a lipid surface is not required for high affinity binding of VII to TF. This suggests that the functional importance of the VIIa Glα-domain (9) may be explained by the contribution of the Glα-domain to specific protein-protein interactions with TF. This analysis does not exclude additional complexity as indicated by the observation of positive cooperativity in the presence of negatively charged phospholipid (21) which suggests a functional noncovalent dimerization of TF (7, 21). Our data suggest that the Glα-domain of VII may participate in interactions with TF which contribute to the high affinity binding. Similar structural requirements have also been described for binding of factor IX to its endothelial cell receptor, a ligand-receptor interaction of comparable affinity to the TF-VII assembly (29, 30). The factor IX Glα-domain in conjunction with the first epidermal growth factor domain has been proposed as the site for these protein-protein interactions (30), suggesting that Glα-domain-dependent interactions are not restricted to interactions with phospholipid.

Consistent with the proposed participation of the Glα-domain in the high affinity binding of VII to TF, tight binding of VII to TF was observed at 1–5 mM CaCl2. Further, VIIa lacking the amino-terminal Glα-domain (des-(1–38)-VIIa) did not bind with high affinity to full length TF in solution, consistent with the lack of high affinity binding to cell surface TF (9). Similarly, interfering with Glα-domain interactions by binding of Fab fragments of a Glα-domain-specific mAb abolished high affinity binding. Low affinity binding of VII to full length TF was observed using plasma VII in the presence of Glα-domain-specific mAb (Kd = 140 nM) or des-(1–38)-VIIa (Kd = 112 nM). A previous study using a cell binding assay failed to demonstrate binding of des-(1–38)-VIIa to TF (9). From the data presented in that study, as well as from our own experience, it is evident that nonspecific binding of VII/VIIa in the cell binding assay presents use of sufficiently high concentrations of VII to observe des-(1–38)-VIIa binding to TF at the Kd determined in our study. The present study demonstrates TF-VII interaction which is independent of the Glα-domain. Binding with similar affinity was also observed at low (50 μM) CaCl2 or when binding of plasma VII or VIIa to the isolated extracellular domain of TF (TFa(VII)) was analyzed. Binding affinity of VII and des-(1–38)-VIIa to TFa(VII) was identical, and the affinity of VII for TFa(VII) did not change at 50 μM CaCl2 or in the presence of the Glα-domain-specific mAb.

These data support the hypothesis of multiple interactions between VII and TF and suggest that TF and TFa(VII) express a binding site which allows low affinity association of VII independent of a properly conformed Glα-domain. Low affinity interaction of VIIa with TF or TFa(VII) was sufficient to enhance peptidase substrate hydrolysis. In addition, des-(1–38)-VIIa hydrolyzed the same peptidyl substrate at an identical rate with either TF or TFa(VII) as cofactor. This suggests that the low affinity interaction with TF must influence the catalytic domain of VIIa to lead to increased accessibility or alignment of the catalytic triad. Whether this low affinity interaction involves interactions of TF with the catalytic domain of VIIa or whether the effects are allosteric due to interactions in other domains of VIIa has not been established. However, an interactive region in the VIIa catalytic domain has been suggested to involve residues 195–206 (10). The catalytic triad His195 is adjacent to residues of this peptide. Association of TF with VIIa through certain residues in the sequence 195–206 could therefore be conceived to enhance peptidyl hydrolysis by a rather local conformational alteration of the catalytic triad or, alternatively, by more global conformational rearrangements of the catalytic domain, as demonstrated to occur spontaneously during the activation of tryspinogen or upon binding of an active site inhibitor or tryspinogen (31). However, experimental proof in support of these speculations remains to be established. The cleavage of the substrate factor X in solution by TFa(VII)-VIIa or TFa(VII)-des-(1–38)-VIIa was markedly reduced compared to complexes formed with VIIa. Under identical conditions, a less than 2-fold difference in peptidyl hydrolysis was observed, whereas the cleavage of factor X was reduced approximately 100-fold. This is consistent with the observed 97% loss of function, when des-(1–38)-VIIa was analyzed at 10 nM with phospholipid-reconstituted TF (9). Since the use of a 20-fold higher des-(1–38)-VIIa concentration in our study did not result in a substantial increase of factor Xa generation, a decreased number of catalytic TFa(VII)-des-(1–38)-VIIa complexes due to the lower affinity of the interaction is unlikely to account for the decrease in functional activity. Rather, these findings suggest that the interaction of the Glα-domain of VIIa with TF contributes to more optimal extended recognition of the protease substrate by allosteric effects or direct interaction with factor X. This conclusion is further substantiated by the slightly reduced rate of solution-phase factor X...
activation by TF\textsubscript{1.219} compared to TF-VIIa. Since TF\textsubscript{1.219} presumably does not provide an entirely equivalent fit for the VIIa Gla-domain, the decreased rate of factor X activation would be consistent with a reduction in contacts of the Gla-domain with TF\textsubscript{1.219}.

The low affinity interaction of VII with TF or TF\textsubscript{1.219} is dependent on calcium ions and is fully supported by 50 \(\mu\text{M} \text{Ca}^{2+}\). This suggests one or more high affinity calcium binding sites which participate in the interaction with TF directly or by conferring stability to the interactive domain. No apparent homology with calcium-binding proteins is found in the TF extracellular domain. Using \(^1\text{H} \text{NMR spectroscopy, we provide here evidence that the conformation of the TF binding structures is not demonstrably influenced by the presence of Ca}^{2+}\). This indicates that a high affinity binding site in VII could be critical for interaction with TF. Such high affinity sites could be located in the first EGF domain (32) or in residues 210–220 of VII which are homologous to the calcium binding site in trypsin (33, 34). This homologous sequence is adjacent to the proposed interactive region in the catalytic domain (10) and peptides encompassing the sequence have been reported to inhibit the function of the TF-VIIa complex (35), although not confirmed by others (10).

Our results are consistent with the participation of at least two sites on VII in the interaction with TF (35), one which is dependent on a proper conformation of the Gla-domain or which may involve the Gla-domain itself and another which is functionally independent of the Gla-domain. Distant homology search revealed structural similarity of TF with the interferon receptor and cytokine receptor superfamily (36). A tandem repeat of two immunoglobulin-like \(\beta\) barrel domains has been proposed as the global structural folding characteristic of this receptor family and interactive regions for the respective ligands have been hypothesized for both domains. Interactive sites in the amino- and carboxyl-terminal region of the TF extracellular domain may therefore provide the structural counterpart for the two sites proposed here for VII.

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