Membrane anchoring of tissue factor (TF), the cell receptor for coagulation factor VIIa (VIIa), exemplifies an effective mechanism to localize proteolysis at the cell surface. A recombinant TF mutant (TF₁₋₂₁⁹), deleted of membrane spanning and intracellular domains, was used to evaluate the role of phospholipid interactions for assembly of substrate with the catalytic TF⋅VIIa complex. TF₁₋₂₁⁹ was secreted by cells rather than expressed as a cell membrane protein. Unlike free VIIa, TF₁₋₂₁⁹ as well as the TF₁₋₂₁⁹⋅VIIa complex demonstrated no stable association with phospholipid. In the absence of lipid, kinetic evaluation of substrate factor X cleavage by free VIIa, TF⋅VIIa, and TF₁₋₂₁⁹⋅VIIa suggests that the catalytic function of VIIa rather than substrate recognition is enhanced by complex formation. Furthermore, compared with free factor X, factor X on phospholipid was preferentially cleaved as a substrate by TF₁₋₂₁⁹⋅VIIa. TF-dependent initiation of the coagulation protease cascade thus involves an enhancement of the activation of factor X on the cell surface by a crucial role of the TF transmembrane domain to membrane anchor the reaction, by the TF extracellular domain to provide protein-protein interactions with VIIa to enhance the activity of the catalytic domain of VIIa, and the preferential presentation of factor X as a substrate when associated with phospholipid surfaces.

Cellular initiation of the coagulation serine protease cascades is mediated by tissue factor (TF),¹ the cell surface receptor and cofactor for coagulation factor VII (VII) and its derivative VIIa (VIIa). The amino acid sequence of TF is encoded by an mRNA transcribed from a 6-exon gene (1). Exons 2 to 5 encode the extracellular domain implicated in receptor and cofactor functions. In the presence of calcium ions, VII and VIIa bind with high affinity to cell surface TF (2–4) as well as purified TF reconstituted into phospholipid vesicles (5). It has been postulated that upon association with TF, allosteric changes in VIIa enable or markedly enhance catalytic function of the bound serine protease domain of VIIa. The functional assembly of TF, VIIa, and factor X as a ternary complex of activator, enzyme, and substrate, respectively, has been characterized as an ordered addition model (6) in which substrate associates with a relatively stable activator-enzyme complex to form a transient ternary complex. The catalytic function of the binary TF⋅VIIa complex may be markedly reduced if the surface organization of the catalytic complex is disturbed by solubilizing TF, as deduced from reactions in the presence of nonionic detergent (7). This enhancing effect of a charged phospholipid surface could be ascribed (i) to secondary VIIa association with the phospholipid surface to enhance formation of the proteolytically active TF⋅VIIa complex, or (ii) to effects of association of factor X with phospholipid on its presentation as a substrate for the TF⋅VIIa complex.

Analysis of a soluble form of cell surface receptors provides novel insight into structure-function relationships. Proteolytic digestion and isolation of soluble domains of thrombomodulin, the anticoagulant thrombin receptor and cofactor, have been successfully applied to provide insight into the functional domains and their phospholipid interactions (8, 9). A truncated form of the major histocompatibility complex has also been adopted for solution of the three-dimensional structure and function (10). Functional analysis of the isolated extracellular domain of TF should further an understanding of the cell surface interactions of TF that may be required for full receptor and cofactor function. A soluble form of the cell surface domain of TF would provide a model for analysis of the interaction of TF with VIIa independent of surface assembly and potential secondary phospholipid-induced effects. To this end, a TF mutant deleted of membrane spanning and intracellular domains was produced and used to characterize primary assembly of the TF⋅VIIa complex and its catalytic function towards the substrate factor X in solution in comparison with phospholipid-bound factor X. These data provide evidence for catalytic function of TF⋅VIIa independent of assembly on phospholipid and further demonstrate that the primary protein:protein interactions of VIIa with the surface domains of TF alone are sufficient for marked enhancement of the catalytic function of VIIa. They also indicate that association of factor X with phospholipid renders it a more effective substrate than when free in solution. The results have been incorporated into a model for assembly-dependent initiation of coagulation on cell surfaces.

**EXPERIMENTAL PROCEDURES**

*Proteins—Factor VII (VII) (11), factor X (12), factor IX (13), and factor Xa (13) activated by Russell's viper venom (14) were prepared as described and were homogeneous as judged by SDS-polyacrylamide gel electrophoresis (15). Fibrinogen was prepared from fractions obtained during the factor X purification using adsorption to Hepa-

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¹ The abbreviations used are: TF, tissue factor; VIIa, factor VIIa; MES, 2-(N-morpholino)ethanesulfonic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; MAb, monoclonal antibody; βp, base pair(s); CHO, Chinese hamster ovary; Gla, γ-carboxyglutamic acid.
Rin-Sepharose (18) and elution with a NaCl gradient from 0 to 0.2 M in 0.02 M MES, Tris, pH 5.9, in the presence of 2.5 mM CaCl2 and 1 mM benzamidine-HCl. Prothrombin fragment 1 was prepared by the procedure of Malhotra (17) with the following modifications. Prothrombin was digested with thrombin at a 100:1 (w/w) ratio for 15 h to yield uncleaved prothrombin and prothrombin fragment 1 by ion-exchange chromatography on Mono Q (Pharmacia LKB Biotechnology, Inc.) using adsorption at 300 mM NaCl and elution at 400 mM NaCl in 20 mM Bio-Tris, pH 6.1. Prothrombin was removed from the preparation by gel filtration using Sephadex G-75 Superfine. Homogeneous preparation of prothrombin fragment 1 was obtained by this procedure as judged by SDS-gel electrophoresis. VII was converted to VIIa by incubation of the purified protein with factor Xa immobilized on Affi-Gel 15 beads (Bio-Rad). Conversion was monitored by SDS-polyacrylamide gel electrophoresis of reduced 5 mM benzamidine HCl. Prothrombin fragment 1 was prepared by the purification of TFXa, American Diagnostics, New York) at 0.2 mM final concentration with anti-TF beads for 3 h or more. After sequential washes with 5G9, coupled to Affigel beads was used for the purification of TFXa immobilized on Affi-Gel15 beads (Bio-Rad). Conversion was monitored by SDS-polyacrylamide gel electrophoresis of reduced 5 mM benzamidine HCl. The monoclonal antibody TF9-6B4 which blocks VII binding to TF (19) was coupled to Affigel-10 (Bio-Rad) at pH 6.5 in 0.1 M NaCl, 0.5% NaCl, 0.5% MgCl2, pH 5.5. Following washes, bound alkaline phosphatase was visualized by a color reaction from a substrate mixture of 330 μM nitro blue tetrazolium (Sigma) and 165 μM MgCl2, 4-chloro-3-indolylphosphate (Sigma), 0.1 M Tris, 0.1 M NaCl, 5 mM CaCl2, pH 5.5.

Construction of the TF1-219 Deletion Mutant and Stable Expression in Eukaryotic Cells—A 775-bp EcoRI fragment of the TF cDNA from nucleotides 1 to 775 (numbering based on Ref. 1) and encoding amino acids 32 through 216 as described (19), was ligated with the synthetic oligonucleotide fragments ATTTTAGAATAAC and ATCCCTTAACG. The fragment was cloned in the expression of the triplet for Phe217 from TTC to TTT, destroyed the original EcoRI site at the 3' end of the cDNA fragment, converted the triplet for Leu to Leu to a termination codon, and provided a new EcoRI cloning site at the 3' end. This fragment was ligated to EcoRI-digested pBR322. This EcoRI insert was subcloned into CDM8 (20) modified to contain an EcoRI cloning site (a gift from Dr. D. Diaylanas, Research Institute of Scripps Clinic). The nucleotide changes introduced into the cDNA were confirmed by sequencing using single-stranded DNA rescued using the helper phage R408 (21) and deoxynucleotide sequencing with Sequenase (U. S. Biotechnical Corp.). Chinese hamster ovary cells (CHO-K1 cells, CCL 61, ATCC, Rockville, MD) were used for transient expression and, as a selection marker, pMAM-neo (Clonetech, San Francisco, CA) at 20μl ratio using the calcium phosphate method (22). The neomycin analogue G418 (GIBCO) was used at 600 μg/ml to select for cells which stably incorporated foreign DNA. Stable single cell-derived colonies were further selected by analyzing the medium for non-N-linked glycosylation was obtained from cells which were grown in the presence of a predetermined optimal concentration (1-2 μg/ml) of tunicamycin (Sigma).

Purification of TF and TF219—TF219 was purified by immunoaffinity chromatography as previously described (23). The same MAB, TF8-5G9, coupled to Affigel beads was used for the purification of TF219 from the culture supernatant. Detergent was not used during the purification of TF219. After removal of cellular debris by centrifugation and filtration (0.45 μm), the culture medium was incubated with anti-TF beads for 3 h or more. After sequential washes with PBS the beads were resolubilized with 0.1 M NaCl, 0.1% SDS, 0.5 M glycine, pH 10 followed by addition of the substrate factor Xa generation assay with phospholipid vesicles (see below). Enzyme reactions were monitored for 2 h at 37°C. Following washes, bound alkaline phosphatase was visualized by a color reaction from a substrate mixture of 330 μM nitro blue tetrazolium (Sigma) and 165 μM MgCl2, 4-chloro-3-indolyl phosphate (Sigma) in 0.1 M Tris, 0.1 M NaCl, 5 mM MgCl2, pH 5.5.

Assay of Factor Xa Activity—Factor Xa activity was monitored by the Spectrozyme FXa. Various concentrations of VIIa and nonphospholipid-associated TF (sobulbilized in Triton X-100) were incubated in 0.2% BSA, 5 mM CaCl2, and 1.25 mM Spectrozyme FXa. The rate of product formation was monitored at 405 nm and corrected for minor (<10%) background hydrolysis which occurred in the absence of TF at high VIIa concentrations. The velocity of absorbance increase was determined in a kinetic plate reader and three or more periods of 1 min followed by addition of the substrate factor X. Aliquots were removed every minute and the reaction stopped in 100 mM EDTA. Initial rates of factor X formation were linear in the first 3 min and an average rate was typically calculated from individual samples obtained during that period. From these initial rates apparent Michaelis-Menten parameters were estimated using the Wilman4 com-
computer program (27). To analyze the cleavage of solution-phase factor X by VIIa in the absence of TF, but presence of 5 mM CaCl$_2$, aliquots were removed after 20, 40, and 60 min from the reaction mixture at 37 °C and stopped in 100 mM EDTA. Rates of factor Xa formation were calculated from these samples and catalytic parameters determined as mentioned above. (ii) To analyze the effect of phospholipid on the cleavage of factor X, purified human TF reconstituted into a 7800-fold molar excess of phosphatidylcholine and phosphatidylserine (70/30, w/w) (Sigma) vesicles using deoxycholate solubilization and dialysis (28) was compared to TF$_{1-219}$ in the presence of vesicles of identical phospholipid composition prepared by the same method. The assay procedure was as above. (iii) To study the effect of prothrombin fragment 1 on the reaction rate of TF$_{1-219}$, VIIa cleavage of phospholipid-bound factor X, factor X, prothrombin fragment 1, and phospholipid were incubated in the presence of 5 mM CaCl$_2$ for 3 min at 37 °C and the reaction initiated by the addition of 1 μM TF$_{1-219}$ and 10 nM VIIa. Rates were determined as above. (iv) Cleavage of factor X by the factor X activator from Russell’s viper venom was studied by prewarming the enzyme (36.8 ng/ml) with 100 μM mixed phospholipid vesicles or buffer in the presence of 5 mM CaCl$_2$ for 3 min followed by addition of the substrate factor X. Rates of factor Xa formation were determined and kinetic parameters calculated as described above.

RESULTS

Isolation of TF$_{1-219}$—A truncated TF DNA coding sequence was generated by replacing the Ile$^{200}$ codon by a stop codon. The entire coding sequence of the mutant was confirmed by DNA sequencing of the construct which was subsequently cloned into the vector CDM8 for expression. A stable cell line was derived from a single cell which contained the integrated DNA for TF$_{1-219}$. The synthesized TF$_{1-219}$ protein was secreted into the culture medium at 0.5–1 mg/liter per 24 h and less than 10% of the immunoreactive protein remained cell-associated. In contrast, no immunoreactive material was found in the media from cells which expressed the natural full-length TF. This demonstrated that TF$_{1-219}$ lacked the ability to stably localize as a transmembrane cell surface protein, which is consistent with the removal of the predicted transmembrane domain in TF$_{1-219}$. TF$_{1-219}$ was purified from the medium by affinity chromatography on an immobilized monoclonal antibody specific for human TF. This single-step purification yielded one major band of 40 kDa and three minor bands, the most rapidly migrating having an apparent molecular mass of 34.5 kDa under nonreducing conditions (Fig. 1, lane A). To investigate whether the minor bands were incompletely glycosylated forms of TF$_{1-219}$, cells were incubated with tunicamycin to arrest N-linked glycosylation and the secreted TF$_{1-219}$ was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. A single protein band with an apparent molecular mass of 34.5 kDa was observed under these conditions, consistent with a single protein species among which the predominant 40-kDa band was the most highly glycosylated form. All molecular forms reacted (Fig. 1, lane B) on Western blots with polyclonal rabbit anti-TF antibodies affinity-purified on immobilized human brain TF (19). Unlike natural intact TF isolated from human brain (23, 28), no dimeric forms of TF$_{1-219}$ were observed, presumably since the cysteine residue of the intracellular domain was deleted. TF$_{1-219}$ reacted with an entire panel of 24 MAbs (18) against human TF (data not shown), suggesting proper folding of TF$_{1-219}$. Amino-terminal protein sequencing of TF$_{1-219}$ established Ser$^1$-Gly$^2$-Thr$^3$-Thr$^4$-Asn$^5$-Thr$^6$ and Thr$^3$-Thr$^4$-Asn$^5$-Thr$^6$-Val$^7$-Ala$^8$ as the amino-terminal residues with two alternative processing sites as described for natural full-length TF purified from human brain (19). The amino acid composition was concordant with the composition of the expected TF$_{1-219}$ protein, thus excluding deletions at the carboxyl terminus due to intracellular processing or extracellular carboxypeptidases.

VII Binding to TF$_{1-219}$—Ligand blotting of TF and TF$_{1-219}$ with $^{125}$I-VII was used to analyze direct protein:protein association. TF or TF$_{1-219}$ were separated by SDS-polyacrylamide gel electrophoresis using nonreducing conditions, then transferred to nitrocellulose membranes. Incubation of the filters with $^{125}$I-VII in the presence of 5 mM CaCl$_2$ demonstrated binding of VII to TF and TF-dimer. VII bound to TF$_{1-219}$ independent of the degree of glycosylation (Fig. 1, lane C) indicating that neither full glycosylation nor phospholipid association is a requisite for the VII association with TF or TF$_{1-219}$ under these conditions. The binding of VII to both proteins was dependent on calcium ions, since addition of 5 mM EDTA prevented the interaction (Fig. 1, lane E). Specificity was further demonstrated by the following. (i) Monoclonal antibody TF9-6B4, specific for human brain TF and demonstrated to block binding of VII to TF (18, 25), inhibited (>80%) association of VII with both proteins when included during the incubation in a 50-fold molar excess over VII (Fig. 1, lane F). (ii) A 50-fold molar excess of factor X, factor IX (Fig. 1, lane D) or prothrombin did not inhibit (<20%) the association of VII with TF and TF$_{1-219}$. (iii) TF$_{1-219}$ included in the incubation at 2.5 μM inhibited binding of VII to TF and TF$_{1-219}$ to more than 80% (Fig. 1, lane G). These results are consistent with specific interactions of VII with TF$_{1-219}$ in the absence of phospholipid.

TF$_{1-219}$ Interaction with Phospholipid—To determine whether the predicted TF$_{1-219}$ protein interacts with relative stability with phospholipid surfaces, a direct binding assay was adopted with the following considerations. (i) The TF extracellular domain might interact with a phospholipid surface such that the surface domains of the molecule fold back onto the surface in addition to the transmembrane anchoring domain. This interactive site might be expressed on TF alone or after complex formation with VIIa. (ii) TF$_{1-219}$ binding to VIIa might result in a stable binary complex which could associate with the phospholipid surface via the γ-carboxyglutamic acid (Gla) domain of VIIa or other induced conformational site.

For analysis, we used a binding assay which employs surface-immobilized, mixed phosphatidylcholine and phosphatidylserine. In contrast to the reported low affinity ($K_d = 9$ μM) of VII binding to phospholipid (5, 29), we observed binding of
VIIa to phospholipid at 200 nM (Fig. 2). In addition, prothrombin fragment 1 at 200 μM effectively inhibited binding of VIIa (Fig. 2), suggesting that the observed binding of VIIa might be Gla domain mediated. Although off-rates of proteins which bind via the Gla domain to phospholipid surfaces are relatively rapid if studied at 37°C, the use of calcium containing cold buffers for washing in the assay apparently decreased the off-rate to allow demonstration of interaction of VIIa with the phospholipid surface. Under these conditions, we were not able to demonstrate binding to phospholipid for: (i) TF1-219 alone; (ii) TF1-219 in the presence of VIIa (Fig. 2); or (iii) when the catalytic complex of TF1-219-VIIa together with the substrate factor X was analyzed. At VII concentrations as high as 1 μM and TF1-219 as high as 2.9 μM demonstrable specific binding of TF1-219 to phospholipid was not observed. These data indicate lack of relatively stable interaction of TF1-219-VIIa with phospholipid under conditions where binding of free VIIa was demonstrated.

**Amidolytic Activity of VIIa Bound to TF1-219**—Hydrolysis of the peptidyl substrate methoxy-carbonyl-D-cyclohexylglycyl-glycyl-arginine-p-nitroanilide (Spectrozyme FXa) was used to monitor accessibility and function of the VIIa catalytic triad in the absence of the effects of extended substrate recognition of factor X. This assay is specific for VIIa in complex with TF by the following criteria. (i) Hydrolysis of Spectrozyme FXa by factor Xa was not enhanced by TF; whereas measured hydrolysis by VIIa required the presence of TF. TF alone did not hydrolyze the substrate. (ii) Addition of EDTA (50 mM) to the VIIa amidolytic assay decreased cleavage of Spectrozyme FXa to background levels; whereas cleavage by purified factor Xa continued at approximately half the velocity, in accordance with the limited cation-dependence of peptidyl hydrolysis by factor Xa (30, 31). (iii) Results were identical with recombinant VIIa. This substantially excludes that the observed amidolytic activity in the VIIa assay is due to factor Xa generated from traces of factor X in VIIa preparations purified from plasma. (iv) The factor Xa-specific substrate S2222 (Helena Laboratories, Beaumont, TX) was not cleaved by TF-VIIa (<1.5 μM 35 mOD/min (rate of absorbance increase at 405 nm) with Spectrozyme FXa under identical conditions). (v) Inactivation of VIIa by 1 mM D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (Calbiochem) (32) followed by extensive dialysis to remove residual free D-phenylalanyl-L-prolyl-L-arginine chloromethylketone abolished all activity in the assay, demonstrating that the activity originates from the VIIa and not the TF or TF1-219 preparations. Taken together, these observations support the specificity of this assay for catalytic activity of VIIa in complex with TF.

We used hydrolysis of Spectrozyme FXa to compare expression of cofactor function by TF and TF1-219. Increasing concentrations of detergent-solubilized TF as well as TF1-219 progressively increased the catalytic efficiency of VIIa. The rate of peptidyl hydrolysis was comparable for equivalent concentrations of TF or TF1-219 (Fig. 3A). Rates were also identical when at a given TF and TF1-219 concentration the VIIa concentration was varied from 50 to 1000 nM. We conclude that the interaction of VIIa with the TF extracellular domain appears to be sufficient to induce accessibility and function of the catalytic site of VIIa for recognition and hydrolysis of the peptidyl substrate.

**Cleavage of Factor X by the TF1-219-VIIa Complex in Solution**—We investigated limited proteolytic activation of the natural substrate factor X in experiments similar to those described above. With VIIa held constant at 100 nM, TF was more efficient as the cofactor for VIIa-mediated proteolytic activation of factor X than was TF1-219 (Fig. 3B). This difference could not be attributed to residual detergent in the TF preparation, since addition of equal amounts of detergent (up to 0.35% Triton X-100) to TF1-219 did not influence the catalytic rate (data not shown). Catalytic parameters for the reactions yielded a comparable $K_{cat,app}$ for TF VIIa and TF1-219 VIIa with only small differences in the $V_{max,app}$ (Table 1). The identity of the $K_{cat,app}$ is consistent with indistinguishable substrate association with the two different binary complexes in solution. Comparison with the $K_{cat,app}$ for factor X cleavage by free VIIa showed that the association of VIIa with TF in solution induced only minor changes in the $K_{cat,app}$, while considerably increasing the velocity of the reaction (Table 1). This indicates that binding of VIIa to TF does not facilitate substrate recognition per se by the TF-VIIa or TF1-219 VIIa complexes; rather it enhances catalysis. Assuming that the $V_{max,app}$ at 50 nM TF or TF1-219 and saturating concentrations of VIIa (1000 nM) corresponds to the rate of

![Fig. 2. Binding of TF1-219 and VIIa to mixed phospholipid immobilized on microtiter wells. Specific binding of VIIa with or without prothrombin fragment 1 (PT Frag. 1) and TF1-219 alone or with 100 nM VIIa is shown. Values are given for a representative experiment with the identical phospholipid preparation for all ligands.](image)

![Fig. 3. Substrate cleavage by TF1-219-VIIa in solution. A, hydrolysis of the peptidyl substrate Spectrozyme FXa (1.25 mM) by 100 nM VIIa bound to increasing concentrations of detergent (1% Triton X-100)-solubilized TF (1.6 μM) diluted in TBS or TF1-219 in the absence of detergent, 5 mM CaCl$_2$ was present. B, cleavage of factor X (5 μM) by TF-VIIa or TF1-219-VIIa in solution. Conditions were identical to those in A. A representative experiment with identical dilutions of the reactants in A and B is shown.](image)
Soluble Tissue Factor

Proteolytic Activation of Factor X in the Presence of Phospholipid—The proteolytic activation of factor X is dependent on the presence of phospholipid vesicles. Although a 12-fold increase in the catalytic rate was observed, the 230-fold decrease in the rate observed for intact, phospholipid-reconstituted TF (Table I) contributing most to the enhancement of the catalytic efficiency. These data therefore suggest facilitated substrate access to the binary complex if the reaction is localized on a phospholipid surface. The lack of membrane insertion of TF1-219, indicating that TF1-219 contains all relevant structures for catalytic enhancement of VIIa.

Addition of 100 μM phosphatidylcholine/phosphatidylserine vesicles at fixed activator, enzyme and substrate concentrations enhanced the rate more than 100-fold (Fig. 4) suggesting that factor X is preferentially cleaved when associated with the phospholipid surface. If this is the case, the reaction rate should reach saturation with increasing concentrations of factor X at a fixed phospholipid concentration. Indeed, the rate of factor Xa cleavage did not decrease at high factor X concentrations (Fig. 4B). With membrane-anchored TF the rate of factor Xa cleavage did not decrease at high factor X concentrations. In addition, lower VIIa concentrations were required in the presence of TF in comparison to TF1-219 to obtain similar rates (Fig. 5B), suggesting an affinity difference which becomes apparent at low VIIa concentrations.

The observed decrease in rate at high factor X concentrations in the case of TF1-219 VIIa can be explained in two ways. (i) Increasing concentrations of factor X might compete with TF1-219 VIIa for binding to a limited number of identical binding sites on the phospholipid surface. This would imply that the catalytic complex must be associated with phospholipid for enhanced recognition and cleavage of factor X as substrate. (ii) Alternatively, factor X bound to phospholipid represents a preferential substrate for TF1-219 VIIa. The inhibition at higher factor X concentrations could result from association of the complex with fluid-phase factor X which

**Figure 4.** Factor Xa formation by TF1-219 VIIa in the presence of various concentrations of mixed (phosphatidylcholine/phosphatidylserine, 70/30) phospholipid vesicles. 1 μM TF1-219, 10 nM VIIa, and 1 μM factor X were used. Mean and standard deviation for three experiments are given. In the absence of phospholipid (0 μM), the data point represents 2.0 ± 0.2 nM/min for the rate of factor Xa formation.

**Table I**

| TF        | VIIa | n  | $K_{m(app)}$ | $V_{max(app)}$ | [Enzyme] | $k_{cat}$ |
|-----------|------|----|--------------|---------------|----------|-----------|
| 0         | 250  | 3  | 13.6 ± 4.8   | 0.44 ± 0.04   | 250      | 2.9 × 10^{-5} |
| TF1-219   | 50   | 50 | 23.8 ± 10.0  | 18.8 ± 11.5   | 50       | 6.3 × 10^{-3} |
|           | 5000 | 50 | 24.9 ± 6.2   | 52.9 ± 8.7    | 50       | 1.8 × 10^{-2} |
|           | 5000 | 1000| 22.4 ± 11.3  | 93.2 ± 13.3   | 50       | 3.1 × 10^{-2} |
| TF in detergent | 50 | 50 | 39.3 ± 18.0  | 465.1 ± 229.6 | 50       | 0.155     |
|           | 5000 | 1000| 20.9 ± 4.9   | 483.4 ± 50.7  | 50       | 0.161     |
| Relipidated TF | 1 | 0.05 | 4  | 0.091 ± 0.01 | 6.1 ± 1.9  | 0.05      | 2.0   |
reaction, cleavage of factor X in solution is demonstrable (Fig.
6). Prothrombin fragment 1 at 2 μM effectively competed the
enhanced reaction at low factor X concentrations in the
presence of phospholipid. At 2 μM prothrombin fragment 1,
increasing the concentration of factor X accelerated the re-
action rate, approaching the rate observed in the absence of
prothrombin fragment 1 at higher factor X concentrations
(Fig. 6). These data demonstrate that association of factor X
with the phospholipid surface is the critical event which
accelerates the reaction rate. The initial increase of the re-
action rate excludes that TF1-219 VIIa associated with phospho-
lipid cleaves free factor X more efficiently, since no further
drop of the reaction rate was observed with increasing factor
X concentrations. However, the drop in reaction rate depicted
in Fig. 6 for the reaction with phospholipid, but without
prothrombin fragment 1 appears to be more pronounced than
expected from a simple competition of the fluid-phase reac-
tion. Based on a K_m(app) of 22 μM for the fluid-phase reaction
(Table I), a 21% reduction would be expected at 6 μM factor
X. This is observed in Fig. 5 at 1 nM VIIa. Since the VIIa
concentration in Fig. 6 was 10-fold higher, an effect of phos-
pholipid binding of free VIIa could influence the reaction rate.
Since VIIa binding to phospholipid was demonstrated at the
given concentration (Fig. 2), the binding of VIIa to phos-
pholipid vesicles could influence the equilibrium with TF1-219
by providing a higher local concentration of VIIa in the phos-
pholipid shell which could facilitate assembly of TF1-219-VIIa
with phospholipid-bound factor X. If the concentration of
VIIa is too low to significantly allow phospholipid interaction,
as in Fig. 5, the reaction rates reflect the simple competition
between two substrate species more adequately. Although the
data in Fig. 6 exclude that TF1-219-VIIa associated with factor
X binding sites during proteolysis of factor X, a transient
interaction of the binary complex at a site different from the
substrate binding site cannot be excluded from these experi-
ments. These data therefore do not exclude TF1-219-VIIa
interactions with phospholipid, but emphasize the signifi-
cance of phospholipid-bound substrate factor X.

These data therefore suggest a conformational transition of
factor X upon membrane association. Additional evidence for
an altered susceptibility of membrane-associated factor X for
recognition and proteolysis by a relevant protease is demon-
strated by the lack of cleavage of factor X by the activator
from Russell’s viper venom when factor X is bound to phos-
pholipid. In contrast, the venom rapidly activates factor X
when free in solution (34). We observed an increase in the
K_m(app) for cleavage of factor X by the venom enzyme upon

represent a less efficiently cleaved substrate. Since inhibition
was observed at factor X concentrations approximating the
K_m(app) of the fluid-phase reaction, the latter is plausible. To
distinguish these two hypotheses, we designed the following
experiment. Prothrombin fragment 1 is devoid of the second
kringle and serine protease domain of prothrombin. It is
devoid of proteolytic or amidolytic activity which might in-
terfere with the chromogenic assay used, and it is not a
substrate for factor Xa. Prothrombin fragment 1 contains the
entire Gla domain of prothrombin which competes with phos-
pholipid binding of factor X (34). Addition of prothrombin
fragment 1 should therefore decrease the rate of factor Xa
formation by TF1-219-VIIa on phospholipid either by restrict-
ing phospholipid binding sites for factor X or for the TF1-219-
VIIa complex. If assembly of TF1-219-VIIa with the phos-
pholipid surface at factor X and prothrombin fragment 1 binding
sites is required for efficient cleavage of factor X, then, in the
presence of prothrombin fragment 1, increasing concentra-
tions of factor X should further decrease the rate, since
binding sites would be further restricted. However, if the
binding of factor X to the phospholipid surface is critical, in
the presence of prothrombin fragment 1 increasing concentra-
tions of factor X should restore phospholipid-associated
factor X and thereby increase the reaction rate. At the con-
centrations of factor X which produced inhibition of the

FIG. 5. A, formation of factor Xa by TF-VIIa inserted into a
phospholipid vesicle (39 μM). 5 nM TF, 0.1 nM VIIa were assembled
for 10 min at 37 °C, followed by addition of various concentrations of
factor X. The initial rate of these reactions is given as mean and
standard deviation calculated from three experiments. The insert
depicts the substrate concentration range up to 10 times the K_m(app),
a concentration range which was typically used for calculation of
kinetic parameters. B, cleavage of factor X by TF1-219-VIIa was
studied as described above for membrane-inserted TF. 1 μM TF1-219,
100 μM mixed phospholipid vesicles, and a 10-fold higher VIIa con-
centrations (1 nM) as in A were assembled for 10 min at 37 °C,
followed by initiation with the substrate. Means and standard devia-
tions calculated from three experiments are given.
addition of 100 μM mixed phospholipid from 135 ± 16 nM (n = 3) to 981 ± 417 nM (n = 3), while the $V_{\text{max(app)}}$ was identical with (12.2 ± 5.6 nM/min) or without (12.6 ± 3.7 nM/min) phospholipid. These data are consistent with sequestration of factor X to phospholipid where it is not cleaved, and efficient cleavage of the substrate in solution, thus confirming the prior report (34).

Discussion

In order to gain insight into the functional properties of TF, independent of anchoring to the cell surface membrane and the known secondary membrane interactions of its ligand factor VIIa or substrate factor X, we produced a soluble recombinant TF mutant (TF$_{1-219}$). TF$_{1-219}$ embodies the complete surface-predicted primary structure of TF and was deleted of membrane spanning and intracellular domains. This was done by introducing a termination codon in the nucleotide sequence corresponding to amino acid 220. The DNA for this coding sequence and its natural leader sequence was cloned into a eukaryotic shuttle vector and stably expressed in mammalian CHO cells. The secreted TF$_{1-219}$ was isolated from culture supernatant by a single-step immunoaffinity purification.

TF$_{1-219}$ was highly glycosylated and the highest as well as less glycosylated forms of TF$_{1-219}$ interacted with polyvalent affinity purified antibodies to TF and also with VII as demonstrated by ligand blotting. TF$_{1-219}$ in solution prevented ligand blotting of VII to TF, consistent with association with VII in solution to prevent interaction with the immobilized native TF. The cell-surface domain of TF embedded in TF$_{1-219}$ therefore appeared to mediate association with the natural ligand in the absence of phospholipid. We were able to address three specific questions using TF$_{1-219}$. (i) Does the TF extracellular domain, free or complexed with VII, stably interact with phospholipid? (ii) Is the interaction of VIIa with TF in the absence of phospholipid sufficient to induce and fully support catalytic function of VIIa? (iii) How is the cleavage of factor X by the binary TF$_{1-219}$-VIIa complex affected by the presence of physically independent phospholipid surfaces?

Whereas association of VIIa with immobilized phospholipid was readily demonstrated, no association of TF$_{1-219}$ with phospholipid was observed under identical conditions. Addition of VIIa to form a binary TF$_{1-219}$-VIIa complex and addition of factor X to assemble a ternary complex also did not lead to observable association of TF$_{1-219}$ to phospholipid. The membrane anchoring of TF by its transmembrane domain thus represents the crucial mechanism to localize the catalytic initiation of the coagulation protease cascade in the two-dimensional array on the cell surface. Although the direct binding data and the functional characteristics clearly demonstrate that TF$_{1-219}$ associates with VIIa, and that VIIa alone associates with phospholipid, VIIa did not mediate relatively stable association of TF$_{1-219}$ to the phospholipid surface under conditions where it binds as a free molecule. The TF$_{1-219}$-VIIa complex may be less stable than the TF-VIIa complex which is characterized by a long half-life on cell surfaces (3, 4). A rapid dissociation of VIIa from TF$_{1-219}$ could account for the lack of association of TF$_{1-219}$-VIIa with phospholipid. Alternatively, TF$_{1-219}$-VIIa may dissociate faster from the phospholipid surface compared to free VIIa, or TF$_{1-219}$-VIIa might not interact with phospholipid due to an eclipse of VIIa binding function when in complex with TF. When considered in the context of the functional importance of the VIIa Gla domain for binding to TF expressing cells and generation of an active binary complex (35), the latter explanation suggests that the VIIa Gla domain might not function by association with charged phospholipid, but rather by association with TF itself. The availability of TF$_{1-219}$ will be useful to address this hypothesis in a more detailed study under equilibrium conditions.

TF$_{1-219}$ association with VIIa enables function of the catalytic domain as evidenced by hydrolysis of peptidyl substrate. In comparison to nonionic detergent-solubilized native TF, the truncated TF$_{1-219}$ formed a catalytically equivalent binary complex with VIIa. These data demonstrate that the protein-protein interactions of VIIa and the surface domain of TF alone are sufficient for both binding and formation of a catalytically functional protease complex. Therefore, TF$_{1-219}$ is a valid model for analysis of the TF-VIIa binary complex. The association of VIIa with TF or TF$_{1-219}$ was sufficient to mediate an enhancement in the proteolytic activation of factor X, extending the observations with the peptidyl substrate to the natural protein substrate. The rate-enhancing effect of TF on the catalytic domain of VIIa is reflected by a 5,550-fold increase in the $K_{cat}$, if compared with free VIIa. There is less than a 2-fold change in the $K_{cat(app)}$ between VIIa and TF-VIIa, suggesting that substrate association with VIIa is not significantly influenced by the binding of VIIa to TF. Previous results have indicated a 10-fold decrease in the $K_{cat(app)}$ for cleavage of factor X upon VIIa complex formation with TF (7, 36), although the $K_{cat(app)}$ (13.6 μM) in the study for factor X by free VIIa is consistent with the reported value (11.6 μM) (36). The higher range of substrate concentration (up to 32 μM) used in our analyses may explain our estimate of a higher $K_{cat(app)}$. In addition, we determined the initial rates immediately upon mixing reactants, whereas Bom and Bertina (36) determined rates starting 10 min after substrate addition. They also observed changes in the rate of factor Xa generation between 10 and 20 min after substrate addition with certain TF preparations, suggesting that the catalytic efficiency of the TF-VIIa complex might vary with time after the addition of substrate. Our initial measurements exhibited a linear rate of factor Xa formation during the first 3 min, suggesting that slow changes of the catalytic efficiency did not influence the rate determinations. We are aware of the limitations of the estimates for the $K_{cat(app)}$ presented in this study, since the substrate concentrations used for the calculations did not exceed the $K_{cat(app)}$ more than 2-fold for the fluid-phase reaction. Much larger quantities of recombinant protein, which are not available to us, would be required to obtain a more reliable estimate. Our study, however, does not support the reported decrease in the $K_{cat(app)}$ established with an even lower substrate concentration range.

Native TF in the absence of phospholipid association was more effective than TF$_{1-219}$ as a cofactor for VIIa cleavage of factor X, although no difference was observed in respect to peptidyl substrate hydrolysis. Minor changes in the extended substrate recognition embodied in secondary protein-protein interactions essential for substrate association and catalysis might account for the observed difference. This may be consistent with the finding that loci on the catalytic domain of factor X, which appear not to be in proximity to the Arg-Ile cleavage site have been implicated in the limited proteolytic activation of factor X by TF-VIIa (37). TF$_{1-219}$-VIIa-mediated proteolytic activation of factor X associated with phospholipid seemed to occur with a catalytic efficiency similar to TF-VIIa, demonstrating that the alterations in the activator function of TF$_{1-219}$ are minor and restricted to the fluid-phase reaction. Our data indicate that TF$_{1-219}$ is not only able to induce requisite conformational transitions for VIIa catalytic triad recognition and cleavage of a peptidyl substrate, but also that protein-protein interactions are sufficient for the effec-
tive cleavage of the natural substrate factor X.

To analyze the effect of membrane anchoring of TF and substrate interaction with the phospholipid surface, we compared factor Xa formation by membrane-anchored TF-VIIa complex with the fluid-phase reaction and observed a 230-fold decrease in the $K_{\text{m(app)}}$, which significantly contributed to the catalytic enhancement. This effect could be attributed to concentrating or uniformly orienting the substrate in vicinity of the proteolytic complex on the phospholipid surface. However, characterization of the cleavage of factor X by soluble TF$_{1-219}$ VIIa showed a more than 100-fold enhanced reaction rate in the presence of TF, it appears that the catalytic step rather than the substrate assembly is essential for proper substrate presentation to the prothrombinase complex. We propose a similar conformational alteration of factor X upon assembly of its Gla domain with phospholipid resulting in a more efficient cleavage by TF-VIIa.

Our analysis demonstrated that the soluble TF$_{1-219}$ VIIa complex preferentially cleaved membrane bound factor X. Forman and Nemerson (41) presented evidence that the membrane anchored TF-VIIa complex preferentially cleaves free factor X, based on kinetic evaluation of the effect of interference with membrane association of factor X by prothrombin fragment 1 and the use of differently charged phospholipid vesicles which influence the affinity of factor X binding (42). We cannot exclude that membrane-anchored TF-VIIa allows more efficient cleavage of factor X associating from the fluid phase, thus marking a difference from the soluble TF$_{1-219}$ VIIa complex. However, the prominent decrease in the $K_{\text{m(app)}}$ upon membrane insertion of TF demonstrated here and by others (6, 41, 43-45) suggests to us that phospholipid interaction of factor X may facilitate substrate presentation to the membrane-anchored binary complex.

In this study we used the isolated TF extracellular domain to discriminate between protein-protein interactions versus protein-phospholipid interactions in the assembly of the TF-VIIa activator complex. The slow cleavage of factor X by VIIa was markedly enhanced by protein-protein interactions with the isolated TF extracellular domain. Since the $K_{\text{m(app)}}$ revealed only minor changes in the presence of TF, it appears that the catalytic step rather than the substrate assembly is favored by TF. This enhancement could result from a preferential interaction between TF and the catalytically active conformer of VIIa, which occurs with low frequency in the absence of cofactor. In this manner, TF would trap the favorable conformation of VIIa and utilize the binding energy to retain VIIa in this state rather than actively inducing a conformational transition in VIIa. An additional catalytic enhancement would be achieved by anchoring the TF-VIIa complex to the lipid surface. TF uniformly oriented due to its membrane anchor could align VIIa to assemble with the substrate factor X which bound to the phospholipid, was shown to be presented in a favorable conformation for cleavage by the soluble complex. The decrease in the $K_{\text{m(app)}}$ due to assembly of the binary complex on the phospholipid surface may be critically important for the functioning of the complex under physiological concentrations of the substrate factor X.

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