Raising the Active Site of Factor VIIa above the Membrane Surface Reduces Its Procoagulant Activity but Not Factor VII Autoactivation*

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Tissue factor, the physiologic trigger of blood clotting, is the membrane-anchored protein cofactor for the plasma serine protease, factor VIIa. Tissue factor is hypothesized to position and align the active site of factor VIIa relative to the membrane surface for optimum proteolytic attack on the scissile bonds of membrane-bound protein substrates such as factor X. We tested this hypothesis by raising the factor VIIa binding site above the membrane surface by creating chimeras containing the tissue factor ectodomain linked to varying portions of the membrane-anchored protein, P-selectin. The tissue factor/P-selectin chimeras bound factor VIIa with high affinity and supported full allosteric activation of factor VIIa toward tripeptidyl-amide substrates. That the active site of factor VIIa was raised above the membrane surface when bound to tissue factor/P-selectin chimeras was confirmed using resonance energy transfer techniques in which appropriate fluorescent dyes were placed in the active site of factor VIIa and at the membrane surface. The chimeras were deficient in supporting factor X activation by factor VIIa due to decreased \( k_{cat} \). The chimeras were also markedly deficient in clotting plasma, although incubating factor VII or VIIa with the chimeras prior to the addition of plasma restored much of their procoagulant activity. Interestingly, all chimeras fully supported tissue factor-dependent factor VII autoactivation. These studies indicate that proper positioning of the factor VII/VIIa binding site on tissue factor above the membrane surface is important for efficient rates of activation of factor X by this membrane-bound enzyme/cofactor complex.

Tissue factor (TF) is the cell surface, type I integral membrane protein that triggers blood clotting in normal hemostasis and many thrombotic diseases (1, 2). TF initiates blood coagulation by binding the plasma serine protease, factor VIIa (fVIIa). The membrane-bound complex of fVIIa and TF can therefore be considered a two-subunit enzyme, with fVIIa as the catalytic subunit and TF as the regulatory subunit. The major substrates for the fVIIa-TF complex are factors X (fX) and IX (fIX), which are converted to active serine proteases by limited proteolysis. Activation of fX and fIX leads to propagation of the clotting cascade and, ultimately, clotted fibrin and activated platelets. Binding of fVIIa to TF embedded in a suitable phospholipid membrane increases its proteolytic activity many thousandfold (3, 4). For optimal fVIIa-TF enzymatic activity, the phospholipid bilayer must contain negatively charged phospholipid, most especially phosphatidylserine (PS) (5, 6). How PS augments the enzymatic activity of fVIIa-TF is not completely understood, but one role is to promote interaction of vitamin K-dependent clotting factors such as fVII, fIX, and fX with phospholipid surfaces (7). These proteins all have an N-terminal domain containing multiple \( \gamma \)-carboxyglutamate residues (Gla domain) that confers reversible binding to membranes containing PS. Incorporating PS into TF-containing membrane vesicles decreases the apparent \( K_m \) of fVIIa-TF for fIX and fX (3, 4). This may be due, at least in part, to increasing the local concentration of these substrates on the membrane surface.

TF also promotes the “autoactivation” of fVII (8). Substrate presentation in fVII autoactivation is unusual in that the enzyme is fVIIa bound to one TF molecule, whereas the substrate is fVII bound to a separate TF molecule (9). The fVIIa-TF and fVII-TF complexes must therefore diffuse within the plane of the membrane to encounter each other. Consequently, fVII autoactivation follows unusual kinetics in which the reaction rates are governed by the surface densities of fVII-TF and fVIIa-TF complexes on the membrane rather than the molar concentrations of any of the reactants (9).

Tethering both enzyme and substrate to the membrane surface has other consequences for catalysis in addition to increasing their local concentrations. It has been hypothesized that an important role for membrane-bound cofactors such as TF is to...
fix the active site of their cognate protease at the optimal position and orientation relative to the membrane surface for attack on the scissile bonds of membrane-bound protein substrates (10, 11). Using fluorescence resonance energy transfer (RET) techniques, we previously showed that when fVIIa binds to phospholipid membranes, its active site is located 83 Å above the membrane surface, but this distance shortens to about 75 Å when fVIIa binds to TF (11). This was true even when the fVIIa Gla domain was removed, demonstrating that TF alone is sufficient to dictate the location of the fVIIa active site above the membrane surface (12). In the crystal structure of the complex of fVIIa with a soluble, truncated form of TF (sTF), the distance from the active site of fVIIa to the portion of the Gla domain suspected to be in contact with the membrane surface is about 80 Å (13). Taken together, these results indicate that the elongated fVIIa molecule must bind nearly perpendicular to the membrane surface and that the active site is realigned relative to the membrane when fVIIa binds to TF, presumably to facilitate optimal attack on the scissile bond(s) of membrane-bound substrates.

The proteolytic activity of the serine protease, activated protein C, toward its natural substrate, factor V, is enhanced when this enzyme binds to its protein cofactor, protein S, on the membrane. Binding of activated protein C to protein S has been shown to relocate the active site of this enzyme relative to the membrane surface (14). Fascinatingly, when the distance from the active site of activated protein C to the membrane surface was altered by the formation of a chimeric enzyme, it exhibited full proteolytic activity toward factor V even in the absence of protein S (15).

In this study, we altered the distance of TF above the membrane through the formation of chimeras between the TF ectodomain and portions of another membrane-anchored protein, P-selectin. We studied the enzymatic activity of the resulting fVIIa-TF complexes to investigate the consequences to enzyme kinetics of changing the alignment of the active site of fVIIa relative to the membrane surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were purchased from the following suppliers: pooled normal plasma, George King Bio-Medical (Overland Park, KS); chicken egg phosphatidylcholine (PC), porcine brain PS, and 1, 2 dioleoyl-sn-glycero-3-phosphocholine (DOPC), Avanti Polar Lipids (Alabaster, AL); Chromozym® t-PA, Roche Applied Science; S-2765, DiaPharma (West Chester, OH); Bio-Beads® SM-2 adsorbent, Bio-Rad; fluorescein-Phe-Pro-Arg chloromethylketone (Fl-FPR-ck), Hematologic Technologies (Essex Junction, VT); octadecyl rhodamine (OR), Invitrogen; octaethylen glycol monododecyl ether (C12E8), Fluka, and octyl-β-D-glucopyranoside, Sigma; recombinant human fVIIa, American Diagnostica (Greenwich, CT); plasma-derived human fVII, fX, and fXa, Enzyme Research Laboratories (South Bend, IN); and HisTrap™ HP columns, GE Healthcare. Recombinant human sTF was expressed in *Escherichia coli* cells and purified as described previously (16). The P-selectin cDNA clone was kindly supplied by Dr. Rodger McEver.

**Production of TF and TF/P-selectin Chimeras**—PCR-based mutagenesis was used to create cDNA constructs encoding three chimeric proteins containing the TF ectodomain linked to varying portions of P-selectin, which were then subcloned into the *E. coli* expression vector, pET26b(+)(Novagen/EMD Biosciences, San Diego, CA). The construct for recombinant, membrane-anchored TF (memTF) encoded the following amino acid sequences (N-to-C-termina): 1) a bacterial leader peptide (pelB) for targeting expression to the periplasmic space; 2) a short peptide epitope (AEDQVDPRLIDGKS) for affinity purification using immobilized HPC4 antibody (16); 3) the extracellular and membrane-anchoring domains of human TF, consisting of amino acids 1–244 numbered according to Morrissey et al. (17); and 4) a hexahistidine tag. The coding sequences for TFCR9 and TFCR8.9 encoded the same pelB leader peptide and HPC4 epitope followed by the TF ectodomain (amino acids 1–219), varying portions of P-selectin sequence, and a hexahistidine tag. The P-selectin amino acid sequences are as follows (numbered according to Johnston et al. (18)): TFCR9, the ninth consensus repeat through the transmembrane domain (amino acids 663–756) and TFCR8.9, the eight and ninth consensus repeats through the transmembrane domain (amino acids 593–756). The coding sequence for TFLCR8.9 was identical to that of TFCR8.9 except that a spacer sequence consisting of (G2S3)QF was inserted between the TF residues 217 and 218 at the C-terminal end of the TF ectodomain (see Fig. 1). All three chimeras and membTF were expressed in *E. coli* strain BL21(DE3) and purified to apparent homogeneity using immunoaffinity chromatography on immobilized HPC4 antibody as described previously (16) followed by Ni3+-affinity chromatography using HisTrap HP columns according to the manufacturer’s instructions.

**Relipidation**—Purified membTF and chimeras were incorporated into phospholipid vesicles containing 80 mol % PC and 20 mol % PS (PCPS vesicles) using the rapid Bio-Bead method and 6 mM C12E8 as the detergent (19). Molar ratios of phospholipid to effective TF ranged from 7303:1 to 130,000:1. Blank PCPS vesicles were prepared in the same way but in the absence of TF.

For RET measurements, membTF or TF/P-selectin chimeras were incorporated into DOPC vesicles to avoid complications from Fl-FPR-fVIIa binding to PCPS vesicles in a TF-independent manner. TF in octyl-β-D-glucopyranoside detergent was mixed with DOPC containing or lacking OR, and then the detergent was removed using either exhaustive dialysis (11) or the rapid Bio-Bead method (19). OR acceptor concentrations ranged between 10 and 50 μM in the lipid preparations, measured using a molar extinction coefficient of rhodamine of 95,400 M⁻¹ cm⁻¹ at 564 nm. OR acceptor density (σ) was determined as described previously (11).

**Preparation of Active Site-labeled fVIIa**—fVIIa (0.5 mg) in 0.5 ml 50 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM EDTA was incubated with 1 mM Fl-FPR-ck inhibitor at 37 °C for 1.5 h. Aliquots of the reaction mixture were withdrawn every 15 min and tested for amidolytic activity. After the fVIIa activity was inhibited >99.9%, samples were dialyzed extensively at 4 °C to remove excess inhibitor, and the labeled fVIIa was subsequently purified over a MonoQ HR5 (Amersham Biosciences) column.
using a NaCl gradient, as described (11). Fl-FPR-fVIIa eluted as a sharp peak at ~0.45 M NaCl. Fractions were pooled and dialyzed into buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl₂. The dye:protein ratio was estimated to be 1:1 from measurements of A405 and A280, as described by Bock (20).

**RET Measurements**—RET measurements were performed essentially as described (11) except that initial concentrations of Fl-FPR-fVIIa in the donor-only (D) and the donor-acceptor (DA) cuvettes were 6 nM in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl₂. Data were normalized for the relatively small decrease in fluorescein emission in membTF vesicles lacking OR, to calculate quantum yields in the presence and absence of acceptor (QDA/QD). To obtain the extent of energy transfer between the fluorescein dye on fVIIa and OR on the membrane surface in the absence of binding to TF, samples were treated extensively with proteinase K as described (12). Distance measurements were calculated using (QDA/QD) values obtained when Fl-FPR-fVIIa was completely bound to membTF/DOPC and when Fl-FPR-fVIIa was completely free above the membrane surface, as described previously (11).

**fVIIa Amidolytic Activity**—Initial rates of fVIIa-catalyzed hydrolysis of Chromozym t-PA substrate were quantified in flat bottom 96-well plates by monitoring change in A405 using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). Reaction conditions were: 10 nM fVIIa, 0–40 nM relipidated membTF (or chimera), 1 mM Chromozym t-PA in HBSAC (25 mM Hepes buffer pH 7.4, 100 mM NaCl, 0.02% NaN₃, 0.1% bovine serum albumin, 5 mM CaCl₂) in a final volume of 0.1 ml. The effective (available) concentration of active TF in liposomes was quantified by titration with fVIIa as described previously (21). Unless otherwise stated, concentrations of TF or TF/P-selectin chimeras are given as effective concentrations.

**fX Activation**—Activation of fX by the fVIIa-TF complex was measured by adapting the continuous chromogenic assay of Fiore et al. (22). Reaction mixtures containing fVIIa and TF/PCPS in HBSAC were initiated by adding a mixture of fX and S-2765 substrate; change in A405 was monitored at ambient temperature, and initial rates of fX activation were determined by fitting a second-order polynomial to the A405 data as described (22). Typical reaction mixtures contained 5 μM relipidated TF, 20 nM fVIIa, 0–200 nM fX, and 0.5 mM S-2765 in 0.2 ml.

**Binding of fVIIa to TF**—Binding of fVIIa to various forms of TF was quantified using the TF-dependent increase in the rate of fX activation as the readout for fVIIa-TF complex formation, as described (23).

**Clotting Assays**—Clotting assays were carried out in a model ST4 coagulometer (Dianostica Stago, Parsippany, NJ). Briefly, 50 μl of 25 mM CaCl₂ was mixed in a solution containing 50 mM Tris-HCl, pH7.4, 100 mM NaCl, 0.02% NaN₃, 0.1% bovine serumalbumin, and 50 μM PCPS vesicles. A 50-μl aliquot of prewarmed, pooled normal plasma was added, and the time to clot formation was measured. One unit of TF procoagulant activity was defined as the amount of TF yielding a 50-s clot time with pooled normal plasma.

**fVII Autoactivation**—Rates of fVII-dependent fVII autoactivation were quantified as described (9, 23). Surface densities of membTF and TF/P-selectin molecules on the phospholipid vesicles varied from 3.7 x 10⁻¹¹ to 6.5 x 10⁻¹⁰ mol m⁻².

**RESULTS**

**Cofactor Activity of TF Chimeras**—Three chimeric TF/P-selectin proteins were produced in which the TF ectodomain was replaced by a variety of P-selectin chimeras (Fig. 1). These chimeras were designed to retain fVIIa binding but to dramatically alter the position of the active site of TF-bound fVIIa relative to the membrane surface. Both membTF and the chimeras were incorporated into PCPS vesicles for study of their properties. Binding of fVIIa to TF increases its amidolytic activity (mOD/min) was quantified. All four forms of TF were incorporated into PCPS vesicles. Effective concentrations of TF are plotted.

**Binding Affinities for fVIIa**—The Kₐ for binding of fVIIa to membTF in PCPS vesicles is ~50 pm, whereas the Kₐ for binding to sTF is ~5 nM (21). The tighter binding to membTF in PCPS vesicles may be attributed to additional binding energy afforded by interactions between the Glu domain of fVIIa and
PS in the membrane as the $K_d$ for binding of fVIIa to membTF in PC vesicles is $\sim 3$ nm (21). Because the TF/P-selectin chimeras are designed to raise the fVIIa binding site above the membrane surface, we predicted that fVIIa should bind with lower affinity to the chimeras than to membTF in PCPS vesicles. fVIIa bound to all three chimeras with somewhat lower affinity than to membTF, although with substantially higher affinity than the binding observed between fVIIa and sTF (Fig. 3 and Table 1).

**Location of the fVIIa Active Site**—RET was employed to determine the distance of closest approach of the active site of fVIIa to the membrane surface. When Fl-FPR-fVIIa was titrated with membTF in DOPC vesicles and containing OR acceptor, a relatively large decrease in fluorescent emission was observed due to energy transfer between the fluorescein donor dye on fVIIa and the OR acceptor dye at the membrane surface (Fig. 4, inverted triangles). The magnitude of RET was dependent on the surface density of OR acceptor dyes (not shown) as observed previously (11, 12). When Fl-FPR-fVIIa was titrated with TFCR9 or TFCR8.9 in DOPC vesicles, essentially no energy transfer was observed (Fig. 4, open squares and filled circles, respectively). The gradual decrease observed in QDA/QD represents an inner filter effect in the cuvettes due to direct absorption of light by acceptor dyes and was not reversed by proteinase K digestion.

From the extent of energy transfer quantified at three different OR surface densities, we measured an average distance of closest approach of $76 \pm 3$ Å between the fluorescein donor in the active site of Fl-fVIIa and OR acceptors in vesicles containing membTF. However, when Fl-fVIIa bound to the TF/P-selectin chimeras in OR-containing vesicles, energy transfer was essentially undetectable, indicating that the fluorescein and OR dyes were too far apart for measurable RET. Given the $R_0$ value for this dye pair ($\sim 54$ Å) and the fact that the efficiency of RET decreases as the inverse fourth power of the distance between the dyes under our experimental conditions (12), RET will be essentially undetectable when the dyes are separated by more than 100 Å. The active site of Fl-FPR-fVIIa was therefore located more than 100 Å above the membrane surface when bound to these TF/P-selectin chimeras.

**Activation of FX**—We hypothesized that raising TF, and therefore the fVIIa binding site, above the membrane surface should misalign the active site of fVIIa with the scissile bond in FX, thereby decreasing the rate of FX activation. The catalytic efficiencies ($k_{cat}/K_m$) of fVIIa bound to chimeras TFCR9 or TFLCR8.9 were decreased 4–5-fold relative to fVIIa bound to membTF in PCPS vesicles (Fig. 5 and Table 1). The catalytic efficiency of fVIIa bound to TFCR8.9 was reduced $\sim 10$-fold. These reductions were due to lower $k_{cat}$ values as the apparent distance of closest approach of the active site of Fl-fVIIa was reduced about 50-fold when fVIIa bound to membTF involves several steps prior to activation of FX by the fVIIa-TF complex. This includes rapid formation of fVII-TF complexes and rapid conversion of fVII to fVIIa, either by fVII autoactivation or by back-activation through downstream proteases such as FXa and thrombin. Preforming the fVII-TF or fVIIa-TF complexes therefore bypasses these early steps in initiating the clotting cascade. When complexed with fVII or fVIIa, the specific procoagulant activities of TFCR9 and TFCR8.9 were reduced only 3–6-fold as compared with membTF, which is less severe than the 11-fold reduction when tested without preforming the fVII-TF or fVIIa-TF complexes. The specific procoagulant activity of TFCR8.9 was reduced about 50-fold as compared with membTF when preincubated with fVII or fVIIa.
The acceptor density (triangles) for the titrations illustrated here were 1.5 × 10⁻⁴ dyes/Å². The ratio of the donor quantum yields in the presence and absence of acceptor, Q₀/D/Q₀, were determined for each titration as before (11).

Procoagulant activities of TF chimeras

Procoagulant activities of various forms of TF in PCPS vesicles were measured in the absence of FVIIa (TF alone) or with 10 nM FVII or FVIIa added to TF prior to the addition of pooled normal plasma. Specific procoagulant activities are given as percent of the specific activity of membTF. Data are mean ± S.E. (n = 3).

| Protein | TF alone | TF + FVII | TF + FVIIa |
|---------|----------|-----------|------------|
| membTF | 100      | 100       | 100        |
| TFCR9  | 8.7 ± 1.2| 18 ± 3.2  | 30 ± 12    |
| TFCR8.9| 0.24 ± 0.14| 2.0 ± 0.58| 1.9 ± 0.63 |
| TFLCR8.9| 8.7 ± 1.7  | 26 ± 1.5  | 16 ± 3.5  |
| sTF    | <0.01    | 0.02      | 0.4        |

* Clotting times with sTF concentrations (up to 300 nM).

We investigated the importance of the role of TF in aligning the active site of FVIIa with the scissile bond of its membrane-bound macromolecular substrates by creating chimeric molecules in which the TF ectodomain (the FVIIa binding domain) was attached to a heterologous type I integral membrane protein, P-selectin. Three chimeras were created, TFCR9, TFCR8.9, and TFLCR8.9, that contain varying portions of P-selectin consisting of either one or two of the consensus repeat domains followed by the transmembrane domain. TFLCR8.9 included a 17-amino-acid-long linker sequence designed to allow the resulting FVIIa-TF complexes to engage the membrane surface via interactions between the FVIIa Gla domain and PS residues in the bilayer. X-ray crystal structures of consensus repeat domains in other proteins indicate that they are roughly 35 Å in length (26). Chimera TFCR9 should therefore raise TF roughly 35 Å above the membrane, or about half the length of the TF ectodomain (27), whereas the longer chimera, TFCR8.9, should raise TF about 70 Å above the membrane surface. This was tested directly using RET techniques. The distance of closest approach of the active site to the membrane surface was calculated to be 76 ± 3 Å when bound to membTF, in excellent agreement with the value of ~75 Å we previously measured for the FVIIa-TF complex (11). We predicted that the active site of FVIIa should be located about 105 Å above the membrane when bound to TFCR9 and some 140 Å above the membrane when bound to the longer chimera (TFCR8.9). RET analysis demonstrated undetectable energy transfer between the fluorescein dye in FI-FPR-VIIa and OR in the membrane when FVIIa was bound to either chimera. Given the limitations of RET with this dye pair, this indicates that the distance of closest approach of the active site to the membrane was greater than 100 Å, confirming the elevated location of FVIIa when bound to the chimeras.
fVIIa bound to the chimeras with $K_m$ values that were intermediate between those for binding to repurposed membTF and sTF. The chimeras all fully allosterically activated fVIIa, as measured by increased amidolytic activity. When bound to the chimeras, fVIIa exhibited reduced rates of fX activation as compared with when it was bound to membTF, although the degree of reduction was, surprisingly, 10-fold or less. The long chimera, TFLCR8.9, was more deficient than the short chimera, TFCR9, and the long chimera with the linker sequence, TFLCR8.9, supported rates of fX activation that were similar to the short chimera.

Previous studies showed that including PS in TF vesicles increases the catalytic activity of fVIIa-TF complexes by decreasing the apparent $K_m$ and having essentially no impact on $k_{cat}$ (6). fVIIa bound to the TF-selecin chimeras should not gain the positive benefits of PS in the vesicles. We therefore hypothesized that we should see an increase in apparent $K_m$ with no change in $k_{cat}$. We were surprised to find that the deficiency in fX activation was almost exclusively due to a decrease in $k_{cat}$. It is possible that the local concentration of fX near the membrane surface is increased relative to the bulk fX in solution, such that there is a local "shell" of higher fX concentration near the membrane surface, which could explain why we do not see an increase in the apparent $K_m$. A possible explanation for the lower $k_{cat}$ values is that the rate at which product (fXa) dissociates from the fVIIa-TF complex may be slower if the fVIIa and/or fX Gla domains are not engaged with the phospholipid surface. Indeed, Hatchcock et al. (28) have suggested that product (fXa) egress from the fVIIa-TF complex via "skating" along the membrane surface may be rate-limiting for fX activation by this protease complex. Further work is needed to fully explain the catalytic defect in fVIIa-TF complexes when they are held above the membrane surface.

The plasma clotting activities of all three chimeras were reduced to a much greater extent than could readily be explained by the decreased catalytic efficiency of the resulting fVIIa-TF complexes toward fX. Preforming fVII-TF or fVIIa-TF complexes restored partial procoagulant activity of the chimeras such that their relative clotting deficiencies were more in line with their relative deficiencies in rates of fX activation. This is consistent with the notion that the reduced affinity of the chimeras for fVIIa slows the formation of the fVIIa-TF complexes, thereby reducing their specific activity in clotting assays.

Chimera TFLCR8.9 was included because several studies have tethered the TF ectodomain to targeting molecules such as monoclonal antibodies against cell surface proteins to target TF to the vasculature of tumors (29–31). The goal of that approach is to use the tethered TF ectodomain as a coaguloligand to trigger the blood clotting cascade locally within the tumor vasculature, causing tumor death through infarction and ischemia. Some studies have employed this same $G_4S_3$ linker in the hopes that it will allow the tethered TF moiety access to the membrane surface once it binds fVIIa (31). We therefore employed the TF/P-selectin chimeras to test the cofactor activity of TF when tethered near, but not directly on, the phospholipid membrane. Attaching the $G_4S_3$ linker sequence between the TF ectodomain and the P-selectin sequence in the longer chimera (TFLCR8.9) restored some enzymatic and procoagulant activity, yielding a molecule with activity that was essentially equal to that of the shorter chimera (TFCR9). This indicates that tethering TF close to the membrane surface via a flexible linker sequence confers substantially more procoagulant activity than untethered sTF, but it fails to completely restore wild-type procoagulant activity. Thus, the tether did not allow the TF ectodomain to gain the full benefits that wild-type TF has of being positioned directly on the membrane surface.

The TF/P-selectin chimeras, although supporting substantially lower rates of fX activation and procoagulant activity, were not uniformly deficient as cofactors for fVIIa. fVIIa exhibited full amidolytic activity when bound to all three chimeras, indicating that they faithfully functioned as allosteric activators of this enzyme. This argues that the overall folding of the proteins is correct. In addition, the chimeras supported wild-type rates of fVII autoactivation. We previously showed that in TF-dependent fVII autoactivation, both fVII and fVIIa are bound to separate molecules of TF and that these fVII-TF and fVIIa-TF complexes diffuse within the plane of the membrane in order for enzyme and substrate to encounter each other (9). The reaction is strictly two-dimensional, with reaction rates dependent upon the membrane surface density of fVII-TF and fVIIa-TF complexes and essentially independent of the molar concentrations of the reactants. Because of this, sTF is essentially incapable of supporting fVII autoactivation (22, 32). The chimeras bind both fVII and fVIIa and should raise these proteins identical distances above the membrane surface (Fig. 7). Because of the unique mechanism of substrate presentation in fVII autoactivation, these should compensate for each other, thereby explaining why fVII autoactivation rates for wild-type and chimeric TF were the same. This also argues strongly that the protein-protein interactions necessary for catalytic competence of the fVIIa-TF complexes are intact in the chimeras.

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FIGURE 7. Schematic diagram of fVII/fVIIa bound to TF-chimeras. A, the active site of fVIIa bound to chimera TFCR8.9 is located more than 100 Å above the membrane surface and therefore should not be aligned with the scissile bond of membrane-bound fX. B, autoactivation of fVII is thought to require binding of both enzyme (fVIIa) and substrate (fVII) to separate TF molecules. Binding of fVIIa and fVII to the chimeras (TFCR8.9 in this diagram) therefore raises enzyme and substrate by an identical amount above the membrane, explaining why the chimeras support normal rates of fVII autoactivation.

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