Tissue Factor Positions and Maintains the Factor VIIa Active Site Far above the Membrane Surface Even in the Absence of the Factor VIIa Gla Domain

A FLUORESCENCE RESONANCE ENERGY TRANSFER STUDY*

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Coagulation factor VIIa (fVIIa), a soluble serine protease, exhibits full proteolytic activity only when bound to its cofactor, tissue factor (TF). Both proteins interact with membranes; TF is an integral membrane protein, while fVIIa binds reversibly to phospholipid surfaces via its Gla domain. In this study, we examine the extent to which the location of the active site of fVIIa relative to the membrane surface is determined using fluorescence resonance energy transfer between the fluorophores bound to fVIIa and OR in vesicles composed of phosphatidylcholine/phosphatidylserine (PC/PS, 4:1) because the Gla domain is required for the binding of fVIIa to phospholipid. However, when Fl-FPR-GD-fVIIa was titrated with PC or PC/PS vesicles into which purified TF had been reconstituted, energy transfer was observed. Based on the dependence of fluorescence resonance energy transfer on OR density, the average distance of closest approach between fluorophores in the active site of Fl-FPR-GD-fVIIaTF and OR at the vesicle surface was determined to be 78 Å (k = 0.3). Since this value is nearly the same as that obtained with intact Fl-FPR-fVIIa bound to TF, the presence or absence of the fVIIa Gla domain has only a small effect on the location of the active site of fVIIa in the TF complex. The extracellular domain of tissue factor therefore must be fairly rigid and fixed relative to the surface to maintain the fVIIa active site far above the membrane even in the absence of the fVIIa Gla domain.

The binding of factor VIIa (fVIIa) to the integral membrane protein, tissue factor (TF), triggers the initiation of the extrinsic pathway of blood coagulation by activating factors IX and X (fIX and fX) (1). The proteolytic activity of fVIIa toward its substrates is increased several thousand-fold when fVIIa is complexed with TF, calcium ions, and phospholipid (2), but the mechanism by which TF increases fVIIa activity has not yet been elucidated.

Structurally, fVIIa consists of five domains: an amino-terminal domain that contains Gla residues, the aromatic stack (also called the helical or hydrophobic stack; some investigators consider it to be part of the Gla domain), two epidermal growth factor-like domains, and the protease domain (reviewed in Ref. 3). In the presence of anionic phospholipid, fVIIa binds to the membrane surface, and this binding is Gla domain-dependent (4). Fluorescence resonance energy transfer (FRET) experiments have shown that the active site of membrane-bound fVIIa is located far above the phospholipid surface (5). Membrane-bound fVIIa therefore projects approximately perpendicularly from the surface with its protease domain farthest from the bilayer, a topography that is similar to those of the other vitamin K-dependent enzymes (6–9).

Interestingly, when TF binds to fVIIa, the location of its active site above the membrane surface is altered (5). This cofactor-dependent structural change may be responsible, at least in part, for the TF-dependent increase in fVIIa activity, since this reorientation may serve to align the active site of fVIIa for optimal cleavage of its membrane-bound substrates (5). Similar cofactor-dependent changes in active site location have been observed when fVIIa binds to fXa (6) and protein S binds to activated protein C (9). Factor Va has also been shown to alter the topography of its membrane-bound substrate, meizothrombin, a result consistent with the proposed cofactor-dependent alignment of active sites and scissile bonds (8).

The crystal structure of fVIIa and a proteolyzed, soluble derivative of TF lacking its transmembrane domain (sTF) shows that the two proteins are aligned side by side in the fVIIa-sTF complex (10). In this crystal structure, the main areas of fVIIa that contact TF are in epidermal growth factor-like domain 1 and the protease domain, with some additional con-
The role of the FVIIa Gla domain in FVIIa-TF function is not entirely clear. While removal of the Gla domain (and, in some studies, the adjacent amino acid) from FVIIa decreases its affinity for TF, most of the binding energy for this interaction is preserved (11, 12). The amidolytic activity of Gla-domainless FVIIa (i.e. its ability to catalyze the hydrolysis of small, tripeptidyl-amide substrates) is indistinguishable from that of intact FVIIa, and this activity is enhanced to the same extent as is intact FVIIa upon binding to TF (11). Thus, it appears that allosteric activation of FVIIa occurs through protein-protein interactions involving TF and epidermal growth factor-like domain 1 and the protease domain of FVIIa, but not involving the FVIIa Gla domain. On the other hand, the ability of FVIIa to catalyze the proteolysis of FVIII (in the presence of membrane-bound TF-Gla) and the protease domain of FVIIa but not involving the FVIIa Gla domain proper and TF, although this is less clear (10).

The structure of the FVIIa-TF complex suggests that the FVIIa-TF complex stands on the membrane on two “feet,” one foot being the FVIIa Gla-domain interacting with phospholipid head groups and the other foot being the connection to the membrane-anchoring region of TF that spans the phospholipid bilayer. Interestingly, the transmembrane domain of TF is connected to the extracellular domain by a span of nine amino acids that are disordered in all three crystal structures of this protein (10, 30, 31). This suggests that the extracellular domain of TF is connected to its membrane anchor, by a flexible peptide, that may allow the protein some range of motion relative to the membrane surface. If so, then the FVIIa Gla domain may be required to restrict the mobility of the complex relative to the membrane surface. Thus, as suggested above, it is conceivable that phospholipid-Gla domain interactions are required to maintain the FVIIa active site at the proper location above the surface in the FVIIa-TF complex, just as the Gla domain is obviously required to position the active site of membrane-bound FVIIa in the absence of TF (5).

To determine how much influence the Gla domain has in specifying the topography of the protease domain of the FVIIa-TF complex, we have repeated our earlier FRET measurements using a Gla-domainless form of FVIIa instead of intact FVIIa. This allowed us to assess the role of FVIIa-phospholipid interactions in establishing the position of the active site in the complex as well as to evaluate the flexibility of the extracellular domain of TF and its junction with the membrane surface. Interestingly, the location of the active site above the bilayer surface in the FVIIa-TF complex is nearly the same whether or not the Gla domain is present. Thus, TF plays a major role in dictating the location of the active site above the membrane in this procoagulant complex, a structural contribution that may have the functional ramifications suggested by our earlier study (5).

**EXPERIMENTAL PROCEDURES**

Reagents—β-Phenylalanyl-l-prolyl-l-arginyl (FPR) chloromethyl-ketone was obtained from Calbiochem. Succinimidyl acetylthioacetate, octadecylrhodamine (OR), and 5-iodoacetamido-fluorescein were supplied by Molecular Probes (Eugene, OR). 1-Palmitoyl-2-oleoyl-phosphatidylcholine (PC) and 1-palmitoyl-2-oleoylphosphatidylserine (PS) were obtained from Avanti Polar Lipids (Pelham, AL). Chromozym t-PA was purchased from Boehringer Mannheim. 1-O-n-Octyl-β-D-glucopyranoside was obtained from Calbiochem.

**Proteins—** Human recombinant GD-FVIIa was prepared as described earlier (15). Recombinant membrane-anchored human TF lacking most of the C-terminal cytoplasmic tail (des-cytoplasmic TF, hereafter designated TF) was expressed and purified as described previously (16). sTF, a soluble truncated form of TF containing amino acids 1–219, was expressed and purified as described previously (17). The concentrations of GD-FVIIa and sTF were determined using ε_{380}^max values of 13.9 and 14.8 and molecular weights of 45,000 and 26,800, respectively (17, 18), while the concentration of TF was measured using the micro-BCA assay from Pierce and bovine serum albumin as a standard.

**Fluorescent Labeling of GD-FVIIa—** GD-FVIIa (300–500 µg) was modified with N^4-(acyethylamino)acetyl-FPR-chloromethylketone and reacted with 5-iodoacetamido-fluorescein as described previously (5). Unreacted reagent was then removed by chromatography on a Sephadex G-25 column (0.5 cm inner diameter x 8 cm) in buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl) at room temperature. Fluorescent GD-FVIIa eluted in the void volume; was dialyzed overnight into 10 mM Tris-HCl (pH 7.5), 1 mM CaCl_2 at 4 °C; and was then further purified from free dye by exchange chromatography at room temperature (Mono-Q HR 5/5, Pharmacia Biotech Inc.) using a linear NaCl gradient. The protein eluted in a single peak near 0.45 M NaCl in 10 mM Tris (pH 7.5), 1 mM CaCl_2. The purified fluorescent protein was designated F1-FPR-GD-FVIIa, dialyzed into buffer A, and stored in small aliquots at ~80 °C.

**Vesicle Preparation—** Vesicles composed of 100% PC or PC/PS (in a molar ratio of PC/PS of 4:1) containing or lacking either TF or OR were prepared by exhaustive detergent dialysis as described previously (5, 16), except that higher concentrations of TF (up to 1 µM) were used in the reconstitution. Vesicles containing TF were purified from unincorporated TF by gel filtration over a Superdex 200 2FPLC column (Pharmacia) in buffer A at room temperature. The amount of TF exposed on the outside of the vesicles was quantified by measuring the TF-dependent enhancement of FVIIa activity in a chromogenic assay using Chromozym t-PA (11). Phospholipid concentration was determined using a trace amount of [14C]PC (Amersham Corp.) in the samples as before (6). TF-containing vesicles (TF/PC or TF/PC/PS) were stored at 4 °C and used within 1 week.

**Spectral Measurements—** Steady-state fluorescence intensity measurements were made using the same instrumentation and procedures as described earlier (5, 7). The distance between donor and acceptor dyes at which singlet-singlet energy transfer is 50% efficient (R_0) is obtained by the subtraction of the signal of B from the signals of DA, A, and D. Samples D and B were then titrated with an equivalent amount of reconstituted TF-containing or TF/PC/PS-containing vesicles to obtain the net dilution-corrected emission intensity of a sample at the signal of B from the signals of DA, A, and D. Samples D and B were then titrated with TF reconstituted into phospholipid vesicles lacking OR (TF/PC or TF/PC/PS), while samples DA and B were then titrated with an equivalent amount of reconstituted TF-containing OR (TF/PC/PS or TF/PC/PS/ORG) containing 10 nM F1-FPR-GD-FVIIa, while samples B (blank) and A (acceptor-containing) contained 10 nM unmodified GD-FVIIa. The initial net fluorescence intensity (F_0) was obtained by the subtraction of the signal of B from the signals of DA, A, and D. Samples D and B were then titrated with reconstituted phospholipid vesicles lacking OR (TF/PC or TF/PC/PS), while samples DA and A were then titrated with an equivalent amount of reconstituted TF-containing OR (TF/PC/PS or TF/PC/PS/ORG). The emission intensity of a sample was measured 5 min after each addition, a time that was found to be sufficient to reach equilibrium (i.e. a stable signal), and the net intensity of D, DA, or A (F_0, F_{DA}, and F_{A}, respectively) was obtained by subtracting the background signal and then correcting for dilution. The blank signal never exceeded 1% of the fluorescent signal of the D or DA samples. To compensate for any signal in the DA sample caused by direct excitation of the acceptor, the net dilution-corrected emission intensity of the A sample was subtracted from that of the DA sample. The intensity of DA was then normalized by comparison with its own intensity, and the net emission intensity of the A sample was used to determine the potential intensity of the A sample was used to determine the net dilution-corrected emission intensity of a sample at some point in the titration and the subscript o is used to denote the initial intensity of the sample.

**Equation 1**

\[
\frac{Q_o}{Q_{DA}} = \frac{F_o(F_{DA} - F_{A})}{(F_o - F_{DA} - F_{A})}
\]

where F is the net dilution-corrected emission intensity of a sample at some point in the titration and the subscript o is used to denote the initial intensity of the sample.
At the end of a titration, each sample was incubated with proteinase K (0.1 mg/ml, 20 min, 22 °C) to digest Fl-FPR-GD-fVIIa and release the donor dyes from the vesicles. The spectral measurements were then repeated to determine what fraction of the OR-dependent reduction in donor emission intensity was due to Fl-FPR-GD-fVIIa binding to membrane-anchored TF. In some experiments, sTF was added instead of proteinase K to compete with TF-PC or TF-PC-OR for binding to Fl-FPR-GD-fVIIa (10 min, 22 °C) and thereby release the Fl-FPR-GD-fVIIa from the vesicles. Titrations with sTF showed that no further change in FRET efficiency was observed after the sTF concentration reached a 6-fold excess over TF. In other experiments, TF-PC (with or without OR) vesicles containing 200 nM TF were either preincubated with monoclonal antibody TF9–9C3 (61 nM, 15 min, 22 °C), which binds specifically to TF and blocks the binding of fVIIIa or GD-fVIIa to TF-PC (19), or TF-9C3 was added at the end of the titration to release the Fl-FPR-GD-fVIIa from the vesicles.

The normalized $Q_o/Q_{DA}$ value used to calculate $L$, the distance of closest approach between the fluorescein dye in the active site of TF-bound Fl-FPR-GD-fVIIa and rhodamine dyes localized at the phospholipid surface, was obtained by dividing the $Q_o/Q_{DA}$ value before proteinase K or sTF addition by the $Q_o/Q_{DA}$ value after exposure to proteinase K or sTF. $L$ was then calculated using the equation (20),

$$Q_o/Q_{DA} = 1 + \frac{(\pi \sigma R_o^2)/(2R_o L)}{\alpha} \quad (\text{Eq. 2})$$

where $\sigma$ is the density of OR molecules on the surface of the vesicles, and $R_o$ is the distance between donor and acceptor dyes at which singlet-singlet energy transfer is 50% efficient.

**RESULTS**

**Fluorescent Labeling of the Active Site of GD-fVIIa—** Human GD-fVIIa was inactivated with N"-[acetylthio]acetyl]-FPR-chloromethylketone and then reacted with 5-(iodoacetamido) fluorescein to yield, after purification, Fl-FPR-GD-fVIIa. While the reaction of GD-fVIIa with the chloromethylketone was complete, based on the nearly total (>99%) loss of amidolytic activity (7), the number of dyes/protein molecule in our Fl-FPR-GD-fVIIa preparations averaged 0.75 when calculated as described by Bock (21) because the reaction of the fluorophore with the activated thiol was not complete. For the experiments discussed in this paper, the presence of non-fluorescein-labeled FPR-GD-fVIIa molecules in a sample does not interfere with the interpretation of the spectroscopic data.

**Spectral Properties of Fl-FPR-GD-fVIIa—** The wavelength of maximum emission (corrected) and the average values for the quantum yield ($Q$) and steady-state anisotropy ($r$) of the fluorescein dye in Fl-FPR-GD-fVIIa were found to be 520 nm, 0.41, and 0.19, respectively, in buffer A plus 2 mM CaCl$_2$. The same values were obtained for Fl-FPR-fVIIa (5), so the spectral characteristics and environment of the active site probe are unaffected by the presence or absence of the Gla domain. These spectral quantities were also unchanged by Fl-FPR-GD-fVIIa binding to TF-PC except that the emission intensity decreased slightly (−OR in Fig. 1).

**Fluorescence Energy Transfer: Fl-FPR-GD-fVIIa Active Site to Membrane Surface—** Our FRET experiments utilized the fluorescein covalently tethered to the active site histidine and serine in Fl-FPR-GD-fVIIa as the donor dye and the rhodamine in OR as the acceptor dye. The partitioning of the long aliphatic hydrocarbon chain of OR into the nonpolar interior of the bilayer anchors the charged rhodamine moiety at the phospholipid surface (22).

When Fl-FPR-GD-fVIIa was titrated with TF-PC or TF-PC/PS vesicles lacking OR, a slight decrease in fluorescein emission intensity was observed (−OR in Fig. 1). However, when Fl-FPR-GD-fVIIa was titrated with either TF-PC-OR or TF-PC/PS-OR vesicles, a much larger decrease in the fluorescein emission intensity was observed that reached a plateau after sufficient TF-containing vesicles were present to bind all of the Fl-FPR-GD-fVIIa (+OR in Fig. 1). The OR-dependent decrease in fluorescein intensity results primarily from FRET from the donor dyes in TF-bound Fl-FPR-GD-fVIIa to the acceptor dyes at the membrane surface. To focus on OR-dependent changes in donor emission intensity, the data in Fig. 1 were normalized and expressed as the ratio of the donor quantum yields in the presence and absence of acceptor (Fig. 2). Fl-FPR-GD-fVIIa appears to bind to TF-PC with lower affinity than full-length Fl-FPR-fVIIa, since less TF-PC was required to achieve complete binding of Fl-FPR-fVIIa in our earlier study (5). This is in agreement with previous findings that GD-fVIIa has a lower affinity for TF than does full-length fVIIa (11, 12, 23, 24). The binding of all of the Fl-FPR-GD-fVIIa molecules in our sample to vesicles and the TF-dependence of this association were confirmed by gel filtration (see below).

**Reversibility of FRET—** The average separation between free Fl-FPR-GD-fVIIa molecules and vesicle-bound OR is too large for significant energy transfer to occur at the concentrations used in our experiments. Thus, if the OR-dependent decrease in fluorescein intensity (Fig. 2) was due solely to energy transfer that occurs when Fl-FPR-GD-fVIIa binds to TF anchored in the OR-containing membrane, then the dissociation of Fl-FPR-GD-fVIIa from TF should eliminate the OR-dependent spectral change. We previously used EDTA to release intact Fl-FPR-fVIIa from vesicles (5). However, the EDTA addition did not release all of the Fl-FPR-GD-fVIIa from TF-PC, possibly because enzyme-cofactor interactions were not destabilized as much in the absence of calcium-dependent conformational changes in the Gla domain. We therefore added proteinase K to the Fl-FPR-GD-fVIIa/TF-PC-OR complex at the end of the ti-
The absence of the acceptor, Q

TF9–9C3 (or without OR) vesicles containing TF that had been incubated with PC

was 1.8 unchanged by sTF addition. In all experiments, the acceptor density Fl-FPR-GD-fVIIa was prevented from binding to vesicles containing TF (19). In both cases where because the TF9–9C3 antibody binds to TF and then blocks fVIIa or GD-fVIIa association with TF (19). In both cases where because the TF9–9C3 antibody binds to TF and then blocks fVIIa or GD-fVIIa association with TF (19).

The existence of an OR-dependent decrease in donor emission intensity that does not result from membrane binding-dependent FRET was confirmed by titrating Fl-FPR-GD-fVIIa and thereby release the fluorescein donor dyes from the vesicles. In some experiments, excess sTF or monoclonal antibody TF9–9C3 was added to the Fl-FPR-GD-fVIIa. Although gel filtration confirmed that each of these treatments released all of the fluorescein dye from the vesicle surface under the conditions used. Furthermore, preincubation of TF with TF9–9C3 prevented Fl-FPR-GD-fVIIa from binding to and eluting with the vesicles (Fig. 3C). This result further demonstrates that Fl-FPR-GD-fVIIa interacts and elutes with TF-PC via binding to TF. Similarly, to confirm that the addition of excess sTF at the end of the titration in Fig. 2 released all of the Fl-FPR-GD-fVIIa from the vesicles, Fl-FPR-GD-fVIIa (10 nm), TF-PC (588 nm TF, 1.3 µM PC), and sTF (3530 nm), were incubated prior to separation over the gel filtration column. As shown in Fig. 3D, the presence of sTF was sufficient to block Fl-FPR-GD-fVIIa binding to the TF-PC, because no Fl-FPR-GD-fVIIa eluted with the vesicles.

Since proteinase K was used in some of the FRET experiments to determine the portion of the decrease in Q

DA/Q

D that resulted from TF binding-dependent FRET from Fl-FPR-GD-fVIIa to OR, it was necessary to confirm that proteinase K digested the Fl-FPR-GD-fVIIa and released all of the fluorescein dye from the vesicle surface under the conditions used. Fl-FPR-GD-fVIIa was therefore incubated with TF-PC and proteinase K as usual and then passed over the gel filtration column. No fluorescent protein eluted with the vesicles, and a single broad fluorescein-containing peak eluted significantly later than did intact free Fl-FPR-GD-fVIIa (Fig. 3E). The fluorescein dye therefore eluted as a much smaller species than intact Fl-FPR-GD-fVIIa, and hence we conclude that the conditions used in our experiments were sufficient to digest the Fl-FPR-GD-fVIIa and thereby release the fluorescein dye from the membrane surface. Since the sTF, TF9–9C3, and proteinase K additions all released fluorescein completely from the vesicles, it is valid to use the sTF-, TF9–9C3-, or proteinase K-reversible portion of the decrease in Q

DA/Q

D to calculate the distance of closest approach between fluorescein in the active site of GD-fVIIa and OR at the membrane surface.

Distance of Closest Approach of Active Site Probe to Mem-

![Figure 2](image-url)
brane Surface—The spectral overlap integral, $J_{DA}$, was $3.89 \times 10^{15} \text{ m}^{-1} \text{ cm}^{-1} \text{ nm}^3$ for the fluorescein-rhodamine donor-acceptor pair. $R_o$ was calculated to be 53.8 Å, assuming that the index of refraction, $n$, equals 1.4 and that the dyes are totally randomized in orientation during the excited state lifetime of the donor dye (i.e. $\kappa^2 = \frac{2}{3}$). Since the rhodamine dyes at the membrane surface and the fluorescein dyes in the active site of GD-fVIIa do not rotate with complete freedom, the actual relative orientation of the transition dipoles of the donor and acceptor during the lifetime of the donor dye excited state is uncertain. The maximum uncertainty in $R_o$ due to orientation effects ($\kappa^2$) is calculated (25) from the measured steady-state anisotropies of the fluorescein in Fl-FPR-GD-fVIIa and the rhodamine in PC-OR to range from −20 to +26% of the $R_o$ ($\kappa^2 = \frac{2}{3}$). However, since the OR acceptors are oriented randomly in the plane of the bilayer (26, 27) and the distance between the donor and acceptor dyes is significantly greater than $R_o$ (28), the uncertainty in $R_o$ can more reasonably be estimated to be ±10% (22, 26). In this regard, it is noteworthy that the active site to membrane surface distance in the Fl-FPR-GD-fVIIa complex was determined by FRET to be 75 Å after assuming that $\kappa^2$ equals $\frac{2}{3}$ (Table I), and the same distance was estimated to be about 80 Å from the crystal structure (10).

Since the magnitude of the observed energy transfer depends upon $\sigma$, the density of the OR molecules on the surface of the vesicles, it is important to measure the energy transfer at different $\sigma$ values. Fig. 4 shows the results of 18 different Fl-FPR-GD-fVIIa to TF-PC-OR (solid circles) or TF-PC/PS-OR (solid triangles) energy transfer experiments where the net

![Diagram](image-url)
Table I

| Complex          | Phospholipid | n | L^c |
|------------------|--------------|---|-----|
| GD-fVIIa         | PC/PS        | 3 | ^b  |
| GD-fVIIa         | PC           | 6 | ^b  |
| GD-fVIIa · TF    | PC/PS        | 4 | 77 ± 1 |
| GD-fVIIa · TF    | PC           | 14| 78 ± 2 |
| fVIIa            | PC/PS        | 19| 83.1 ± 3.3' |
| fVIIa · TF       | PC/PS or PC  | 14| 75.0 ± 1.8' |
| GD-fVIIa · TF    | PC/PS or PC  | 18| 78.0 ± 1.8' |

*a* L was calculated using Equation 2 and R_c = 53.8 Å and assuming that \( \kappa^2 = 2/3 \) and \( n = 1.4 \).

*^b* No energy transfer was observed in the absence of TF.

*^c* Data reported in McCallum et al. (5) were recalculated here and are expressed with an additional significant figure. This accounts for the 1-Å difference in average L values reported here and previously.

**DISCUSSION**

Our previous investigation of fVIIa topography and its sensitivity to TF revealed that the association of membrane-bound fVIIa with TF caused the enzyme’s active site to be relocated. The average distance of closest approach between the active site probe in the fVIIa-TF complex and the OR at the membrane surface was found to be 75 Å (assuming \( \kappa^2 = 2/3 \); Table I). Here we report that the same measurement using a fVIIa derivative that lacks the Gla domain yields an average distance of 78 Å (\( \kappa^2 = 2/3 \); Table I). Although these average L values are very similar, a statistical analysis reveals that they are not the same. Thus, the location of the fVIIa active site in its complex with TF depends, to a small extent, on the presence or absence of the fVIIa Gla domain. If all of the Gla-dependent movement is translational, then the height of the fVIIa-TF active site above the membrane is slightly lower in the presence of the Gla domain than in its absence.

Yet it is also clear that the intact and the Gla domainless fVIIa molecules are positioned at close to the same location relative to the membrane surface when they are bound to TF. This result suggests that the cofactor itself makes a major contribution to positioning the active site in the fVIIa-TF complex. Such a view is also supported by our previous observation that L is the same for fVIIa-TF reconstituted into either PC/PS or PC vesicles, even though the fVIIa Gla domain does not bind to the latter (5). Since fVIIa interactions with phospholipid have little effect on FRET efficiency, it is possible that fVIIa binding to the membrane is not a major determinant for the proper positioning of the active site in the fVIIa-TF complex. Instead, the height and orientation of the fVIIa active site above the membrane in the fVIIa-TF complex may be established primarily during the association of fVIIa with TF, while the Gla domain contributes to the final alignment and positioning of the active site groove.

The FRET results also allow us to draw some important conclusions about TF structure and function. We earlier suggested that the TF-dependent alteration in fVIIa topography may constitute a mechanism to regulate fVIIa activity via cofactor-mediated alignment of the enzyme active site to optimize cleavage of the membrane-bound substrate (5). If this were true, one would predict that cofactor-dependent positioning of the active site would override any other effects that influence the location of the fVIIa active site relative to the membrane. The discovery that the Gla domain is not required to position the active site in the fVIIa-TF complex far above the membrane surface is therefore quite consistent with the preeminence of the cofactor in establishing topography. Thus, these data provide further experimental support for the view (5, 6, 8, 9) that cofactor-dependent changes in active site height and/or orientation above the membrane constitute a mechanism to regulate coagulation enzyme activity.

Since TF is able to localize and maintain the active site of fVIIa far above the membrane even in the absence of the fVIIa Gla domain, it is clear that the Gla domain is not required to act as a “foot” to stabilize the fVIIa-TF complex on the membrane surface. Instead, the extracellular domain of TF must extend from the surface and be fairly rigid. Furthermore, its orientation with respect to the membrane surface must be fixed to create a stable template for binding GD-fVIIa. This further suggests that when TF is integrated into the bilayer, the stalk region of TF adjacent to the transmembrane domain is not as flexible as is suggested by the lack of electron density observed in the sTF and sTF-fVIIa crystals (10, 30, 31); we are currently investigating this possibility.

The FRET data also place the role of the Gla domain in fVIIa-TF structure and function in a new perspective. Based on the current body of evidence, it is likely that the Gla domain stabilizes the conformation of the enzyme active site relative to the membrane surface but is not required for optimal positioning. Future studies will be required to determine the precise mechanism by which the Gla domain contributes to the proper alignment and positioning of the active site on the membrane surface.
upon the energy transfer results, the positioning of the active site relative to the membrane surface in the fVIIa-TF complex is primarily effected by the cofactor and not the Gla domain. Thus, although the Gla domain interacts directly with a phospholipid surface to mediate fVIIa binding to PC/PS vesicles (4), it is conceivable that the fVIIa Gla domain has minimal or even no contact with the bilayer once fVIIa is bound to membrane-anchored TF. Based on the position of the Gla domain in the fVIIa-TF crystal structure (10), this possibility seems unlikely, but it cannot be ruled out. It will be interesting to determine through further experimentation the actual extent of Gla domain interaction with phospholipid in the intact fVIIa-TF complex, particularly since the measured L values were the same for intact fVIIa-TF complexes in both PC/PS and PC vesicles (5) even though fVIIa does not bind to PC vesicles in the absence of TF.

Since our FRET and gel filtration data reveal that the affinity of TF for GD-fVIIa is sufficient both to localize the enzyme at the membrane surface and also to position the active site of GD-fVIIa at a location similar to that of intact fVIIa, one might ask why fVIIa even requires a Gla domain. One possible reason is that the Gla domain participates in protein-protein interactions that play a critical role in fVIIa-TF structure and function. For example, although the Gla domain of fVIIa is not absolutely required for fVIIa proteolysis of fX or chromogenic substrates in the absence of TF and phospholipid, GD-fVIIa is much less efficient than fVIIa in fX activation when membrane-bound TF is included as the cofactor (11). This dependence on the Gla domain for TF enhancement of fVIIa activity toward its natural substrate, fX, may be mediated by protein-protein interactions that modulate the conformation of the substrate, TF, and/or fVIIa. In fact, it is possible that the small Gla-dependent change in topography discussed above is effected via protein-protein interactions rather than protein-phospholipid interactions. Such conformational changes would require contact between the proteins, and both direct TF-fVIIa Gla domain and fVIIa Gla domain-fX Gla domain interactions have been proposed previously (13, 14, 32, 33). Of course, Gla domain-phospholipid interactions may also be important physiologically. For example, binding of fVIIa to phospholipids may serve to increase the local concentration of fVIIa near its membrane-bound cofactor. It is also possible that the Gla domain is required primarily during activation, when fVII is a substrate and may require a topography similar to those of fX and fIX for activation. Finally, although the presence of the fVIIa Gla domain has a relatively small effect on the location of the fVIIa-TF active site relative to the membrane surface, changing the active site alignment by only a few Å may have profound effects on catalytic activity. Indeed, in our previous studies we found that removal of the Gla domain substantially altered the rate of fX activation only when GD-fVIIa was complexed with TF in PC/PS vesicles (11).

In summary, the binding of GD-fVIIa to TF localizes the enzyme at the membrane surface, and its active site is located at nearly the same position as in the intact fVIIa-TF complex.

The fVIIa Gla domain is therefore not required to position the active site of the fVIIa-TF complex far above the membrane surface. Instead, the location of the active site appears to be largely cofactor-regulated, dictated primarily by the binding of the fVIIa enzyme to a rigid tissue factor molecule that has a fixed orientation relative to the membrane surface.

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