

Characterization of the interaction between the light chain of factor VIIa and tissue factor

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Abstract Factor VIIa (fVIIa) consists of a heavy chain (serine protease domain) and a light chain (γ -carboxyglutamic acid (Gla)-rich and epidermal growth factor (EGF)-like domains). The light chain, primarily the first EGF-like domain, appears to provide most of the binding energy in the interaction with tissue factor (TF). The Ca^{2+} -binding sites in the protease domain and in the first EGF-like domain influence activity and interaction with TF, but the contribution from the Ca^{2+} -binding sites in the Gla domain has not been established. We have compared the soluble TF (sTF)-binding properties of intact fVIIa to those of a fragment comprising almost the entire light chain and a small disulphide-linked peptide from the protease domain. Half-maximal binding of fVIIa and the light chain to sTF occurred around 0.3 and 1 mM Ca^{2+} , respectively. The Ca^{2+} dependence of light-chain binding indicates an influence of Ca^{2+} binding to the Gla domain on the interaction between fVIIa and sTF. Comparison of the sTF-binding properties of fVIIa and a truncated variant lacking the Gla domain suggests that this domain interferes with sTF association at suboptimal Ca^{2+} concentrations. The light chain of fVIIa associated 5-fold slower with sTF than did fVIIa at saturating Ca^{2+} concentrations, whereas the dissociation of its complex with sTF was at least 100-fold faster than that of fVIIa:sTF. This gave a dissociation constant of 1–2 μM for the interaction between the light chain and sTF compared to about 3 nM for the fVIIa:sTF interaction.

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Key words: Ca^{2+} binding; Factor VIIa; Gla domain; Light chain; Surface plasmon resonance; Tissue factor

1. Introduction

Exposure of the integral transmembrane protein tissue factor (TF) to blood results in initiation of the coagulation cascade through the formation of a factor VIIa (fVIIa):TF complex [1–4]. The binding to TF potentiates the fVIIa-catalyzed activation of the macromolecular substrates factors IX [5] and X [6] as well as the fVIIa amidolytic activity [7,8]. The degree of amidolytic activity enhancement depends on pH with a minimum around the physiological value and, interestingly, the pH optimum of fVIIa differs from that of the fVIIa:soluble TF (sTF) complex [9].

Biochemical studies and the structure of the fVIIa:sTF

complex have shown that the interaction between fVIIa and TF involves several areas on both molecules [10,11]. The major contact surfaces on fVIIa are located in the first epidermal growth factor (EGF)-like domain and in the protease domain. Results from a mutagenesis study [12] and the hydrophobic nature of the interface [11] suggest that the interaction between the first EGF-like domain of fVIIa and TF tethers the enzyme to the cofactor. In a physiological setting, fVIIa presumably interacts both with TF and, via the γ -carboxyglutamic acid (Gla) domain, with the phospholipid surface surrounding TF. Both interactions are important for high affinity between fVIIa and membrane-embedded TF. It is not known whether fVIIa in solution binds TF directly or first binds to the phospholipid, but the initial contact most likely involves the Gla and first EGF-like domains close to the membrane surface.

The presence of Ca^{2+} ions facilitates fVIIa binding to sTF as measured by functional titration [13–16] or by biophysical techniques [17,18]. The Ca^{2+} -loaded form of fVIIa has a dramatically higher affinity for sTF (dissociation constant K_d) = 1–5 nM) than has the apo form ($K_d > 1 \mu\text{M}$) [15,19]. The Ca^{2+} dependence of fVIIa:sTF complex formation, assessed by sTF-induced enhancement of fVIIa amidolytic activity, is characterized by a half-maximum around 0.2–0.3 mM [14,19–21]. TF does not bind Ca^{2+} [22], whereas fVIIa binds a total of nine Ca^{2+} ions, seven of which are found in the N-terminal Gla domain [11,19]. The remaining two Ca^{2+} ions are found in sites of relatively higher affinity in the first EGF-like domain [19,23] and in the protease domain [13,19], respectively, with K_d values around 0.1 mM. A fVIIa molecule mutated at a putative Ca^{2+} -coordinating residue (Glu²²⁰) in the protease domain appears to bind slightly weaker to sTF and requires a significantly higher Ca^{2+} concentration for maximal activity than does wild-type fVIIa [13]. Abolishment of the Ca^{2+} site in the first EGF-like domain results in a decreased affinity for sTF [24]. It has been suggested that low-affinity Ca^{2+} sites are important for high-affinity interaction between fVIIa and TF [22], but no clear evidence of an involvement of Gla-dependent Ca^{2+} -binding sites has been presented. In addition, intact and N-terminally truncated (Gla-domainless) fVIIa bind sTF with similar Ca^{2+} dependences [21]. Once the fVIIa:sTF complex is formed, Ca^{2+} appears not to be essential for its activity [15,25].

In this paper, surface plasmon resonance is used to characterize the sTF-binding properties of the light chain of fVIIa (fVII-GlaEGF_{NC}), a region containing eight out of the nine Ca^{2+} -binding sites present in intact fVIIa. The results show that considerable binding energy is involved in the light chain:sTF interaction and demonstrate a role for Gla-dependent Ca^{2+} -binding sites in optimal sTF binding. A comparison of the sTF-binding kinetics of fVIIa and Gla-domainless

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Abbreviations: fVIIa, factor VIIa; des(1–38)-fVIIa, fVIIa lacking the N-terminal 38 amino acid residues; fVII-GlaEGF_{NC}, residues 1–144 plus 248–266 of fVIIa; Gla, γ -carboxyglutamic acid; EGF, epidermal growth factor; TF, tissue factor; sTF, the soluble, extracellular part of TF (residues 1–219); K_d , equilibrium dissociation constant

fVIIa (des(1–38)-fVIIa) reveals an obstructive effect of the Gla domain on the association of fVIIa with sTF at suboptimal Ca^{2+} concentrations, at which the protease and first EGF-like domains, but not the Gla domain, are virtually saturated with Ca^{2+} .

2. Materials and methods

2.1. Proteins

Recombinant fVIIa was produced in baby hamster kidney cells and purified as described [26]. Des(1–38)-fVIIa was obtained by fVIIa autodigestion under Ca^{2+} -free conditions [27] and fVII-GlaEGF_{NC} was isolated after limited tryptic cleavage of fVIIa [21]. sTF was expressed in *E. coli*, refolded and purified according to published procedures [28,29].

2.2. Tissue factor-binding experiments

The binding of fVIIa, des(1–38)-fVIIa and fVII-GlaEGF_{NC} to sTF was measured in a BIAcore instrument (Biacore AB, Uppsala, Sweden) essentially as described [30,31]. The analytes, the concentrations of which are given in the figure legends together with the amounts of sTF coupled to the sensor chip, were in 20 mM HEPES, pH 7.4, containing 0.1 M NaCl, 0.02% Tween 80 and various concentrations of CaCl_2 or 2 mM EDTA. All injections in a series of experiments were made over the same sTF-coated surface which retained its binding capacity over the experimental time frame. In the experiments designed to determine the on- and off-rates (Figs. 2 and 3), the association and dissociation phases lasted for 7 and 6 min, respectively. In the Ca^{2+} -dependence experiments (Fig. 1), association was allowed to proceed for 12 min. The data was evaluated employing a one-site model and the BIAevaluation 2.1 software.

3. Results

The binding of fVIIa, des(1–38)-fVIIa, and fVII-GlaEGF_{NC} to sTF was measured at different Ca^{2+} concentrations. The analyte concentrations in this set of experiments were chosen to rapidly reach equilibrium levels of binding to the sTF sites on the sensor chip under optimal conditions. Half-maximal binding of fVIIa was achieved around 0.3 mM Ca^{2+} and of des(1–38)-fVIIa between 0.1 and 0.2 mM (Fig. 1). This is in agreement with the Ca^{2+} dependence of the sTF-dependent amidolytic activity of fVIIa:sTF and des(1–38)-fVIIa:sTF [14,19–21]. Both proteins bound maximally at Ca^{2+} concentrations above 1 mM. The fVII-GlaEGF_{NC} fragment required higher Ca^{2+} levels in order to bind sTF. No significant binding was observed at 0.2 mM and half-maximum occurred around 1 mM, roughly corresponding to the average affinity of the Ca^{2+} -binding sites in the Gla domain [19,21]. This suggests that saturation of Gla-dependent Ca^{2+} -binding sites is necessary for optimal sTF binding. The thorough characterization [21] and unique Ca^{2+} dependence of fVII-GlaEGF_{NC} excludes the possibility that contaminating fVIIa contributed to the observed sTF binding.

Because it could be inferred from the Ca^{2+} dependence of fVII-GlaEGF_{NC} binding to sTF that an optimal interaction requires saturation of Ca^{2+} -binding sites in the Gla domain, it was investigated whether this is true also for the interaction of fVIIa with sTF. The kinetics at optimal (5 mM) and suboptimal (0.5 mM) Ca^{2+} concentrations were analyzed in the presence (fVIIa) and absence (des(1–38)-fVIIa) of the Gla domain. A profound effect was observed on the interaction of fVIIa with sTF upon lowering of the Ca^{2+} concentration (Fig. 2A). This could be attributed to a 6-fold decrease in the rate of association, being $3.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at 5 mM Ca^{2+} and $5.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ at 0.5 mM Ca^{2+} (and even lower at 0.2

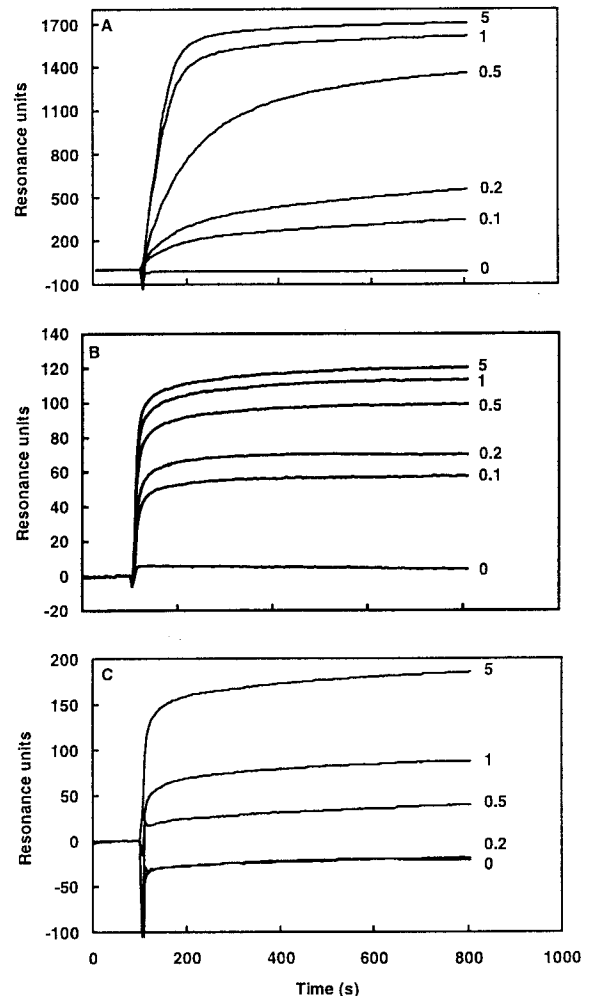


Fig. 1. Ca^{2+} dependence of the interactions between sTF and fVIIa, des(1–38)-fVIIa and fVII-GlaEGF_{NC}. Overlays of sensorgrams of the interactions of fVIIa (100 nM, A), des(1–38)-fVIIa (300 nM, B), and fVII-GlaEGF_{NC} (5 μM , C) with sTF at the indicated Ca^{2+} concentrations (mM) are shown. The curves indicated by '0' were acquired in the presence of 2 mM EDTA. 910 (A and C) or 330 (B) resonance units of sTF were immobilized on the sensor chip. The sensorgrams were corrected by subtracting the signal obtained when injecting the samples over a blank surface.

mM Ca^{2+} ; see Fig. 1A). The dissociation rate remained the same ($1.3 \times 10^{-3} \text{ s}^{-1}$ and $1.2 \times 10^{-3} \text{ s}^{-1}$, respectively). This yields an increase in K_d from 4 nM at 5 mM Ca^{2+} to 23 nM at 0.5 mM Ca^{2+} . Dramatic effects on fVII-GlaEGF_{NC} binding to sTF was also evident upon lowering of the Ca^{2+} concentration (Fig. 1C). In contrast, when the experiment was performed with des(1–38)-fVIIa, only marginal changes in the association (10% slower) and dissociation (30% faster) rates were observed upon lowering the Ca^{2+} concentration (Fig. 2B). It can also be deduced from Fig. 1 that, although the steady-state level of both fVIIa and des(1–38)-fVIIa binding to sTF dropped with decreasing Ca^{2+} concentrations, the time to reach steady-state level increased significantly for fVIIa but was much less affected for des(1–38)-fVIIa. To exclude the possibility that increased electrostatic repulsion between the non-saturated Gla domain and the dextran matrix in which sTF is immobilized causes the decreased association rate,

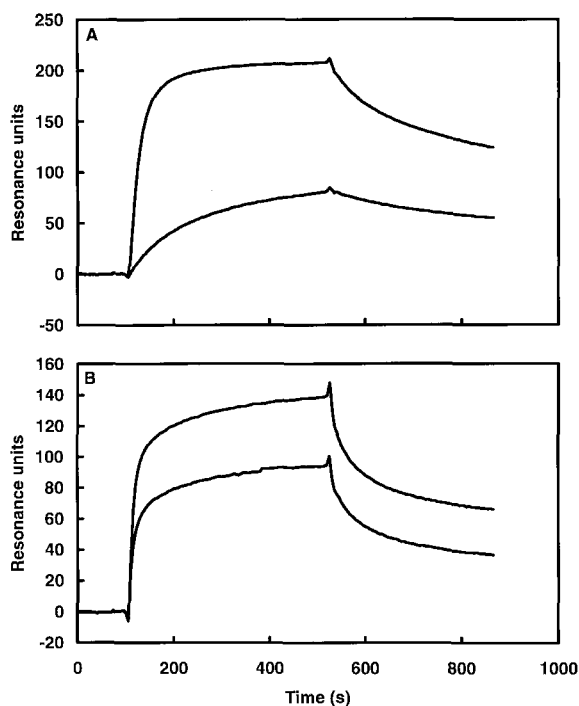


Fig. 2. The interactions of fVIIa and des(1–38)-fVIIa with sTF at optimal and suboptimal Ca^{2+} concentrations. The corrected sensorgrams show the binding of fVIIa (60 nM, A) and des(1–38)-fVIIa (120 nM, B) to sTF at 5 mM (top curves) and 0.5 mM Ca^{2+} (bottom curves). 330 resonance units of sTF had been coupled to the sensor chip.

fVIIa binding to a Ca^{2+} -independent monoclonal antibody was measured at 0.5 and 5 mM Ca^{2+} . There was no visible difference in binding kinetics showing that fVIIa has the same access to sTF at both Ca^{2+} concentrations (not shown). These results demonstrate that the decreased rate of association of fVIIa with sTF at suboptimal Ca^{2+} concentrations is indeed an effect of the Gla domain being (only) partially saturated and, as a consequence thereof, structurally disordered. Des(1–44)-fVIIa has been found to have slightly higher affinity than fVIIa for sTF in the absence of Ca^{2+} [15]. Our results suggest that fVIIa in fact has a lower (or similar) affinity for sTF compared to that of des(1–38)-fVIIa at Ca^{2+} concentrations below 0.5 mM due to what appears to be steric hindrance by the non-saturated Gla domain.

The binding of GlaEGF fragments of fVIIa to TF has been demonstrated by their ability to competitively inhibit fVIIa:TF activity [32,33], but the kinetics have not been investigated. The kinetics of the fVII-GlaEGF_{NC}:sTF interaction (Fig. 3) were found to be strikingly different from those of the interaction between intact fVIIa and sTF. The estimated rate of fVII-GlaEGF_{NC} association with sTF, $4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, is about 5-fold slower than that of fVIIa association with sTF [18,30,31,34]. The most dramatic difference was seen in the rate of dissociation, which was approximately 100-fold faster for the fVII-GlaEGF_{NC}:sTF complex, or $0.05\text{--}0.1 \text{ s}^{-1}$, than for fVIIa:sTF. The resulting K_d for the interaction between fVII-GlaEGF_{NC} and sTF is between 1 and 2 μM .

4. Discussion

Under physiological circumstances, fVIIa interacts both

with TF and the phospholipid surface in which TF is anchored. Both interactions are Ca^{2+} -dependent (occur optimally at plasma concentration) and important for high-affinity binding of fVIIa to membrane-associated TF. The interactions are sensitive to changes in the Ca^{2+} concentration in approximately the same interval and studies of the Ca^{2+} dependence of either event using lipidated TF is complicated by a simultaneous effect on the other. However, sTF has been used to characterize the protein–protein interaction between fVIIa and TF and we have used it for instance to study the role of the membrane-binding Gla domain of fVIIa in the interaction with TF. The biosensor technique employed in this study has proven suitable for the study of the fVIIa:sTF interaction and generates binding data in agreement with those obtained in solution phase assays [15,30]. It allows real-time monitoring of binding to sTF, not only of the enzymatically active fVIIa and des(1–38)-fVIIa but also of fragments such as fVII-GlaEGF_{NC}. It has been inferred from X-ray structural [11] and alanine scanning mutagenesis [12] data that contacts mediated by the light chain, or rather the first EGF-like domain, glues fVIIa to sTF. We have directly shown that the interaction between fVII-GlaEGF_{NC} and sTF, based on the estimated binding constants, retains 2/3 of the binding energy of the fVIIa:sTF interaction. The affinity of fVII-GlaEGF_{NC} for sTF was 500–1000-fold lower than that of fVIIa mainly due to an increased rate of dissociation from sTF. The slower formation and very rapid dissociation of the fVII-GlaEGF_{NC}:sTF complex compared to fVIIa:sTF could be anticipated based on the reduced number of potential docking sites present on fVII-GlaEGF_{NC} and the reduced number of stabilizing interactions between the fragment and sTF. Effects, albeit less pronounced, on both association and dissociation rates have been observed with a fVIIa mutant where Gln replaced the TF-interactive residue Arg⁷⁹ in the first EGF-like domain [18]. Thus, fVII-GlaEGF_{NC}, which lacks a major contact area (the protease domain), and the fVIIa R79Q mutant, which is defective in a major contact area, both exhibit decreased association and increased dissociation rates. Increased dissociation rates have also been observed for Gla-domainless forms of fVIIa [30] but the effects of the N-terminal truncations (residues 1–38 or 1–44) were smaller than that of deleting the protease domain (as in fVII-GlaEGF_{NC}), conceivably explained by the relative contributions of these regions of fVIIa to the interaction with sTF

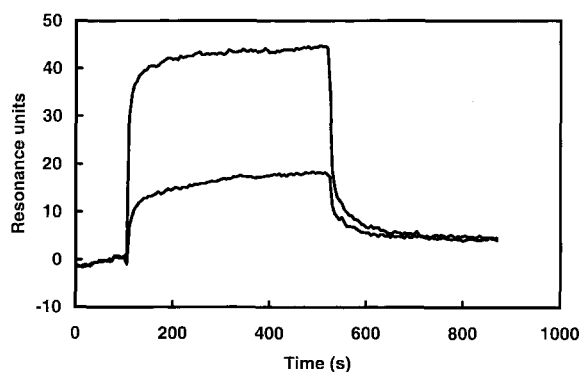


Fig. 3. Corrected sensorgrams for the interaction between fVII-GlaEGF_{NC} and sTF. The concentration of fVII-GlaEGF_{NC} during the association phase was 4 μM (top curve) or 1 μM (bottom curve). The Ca^{2+} concentration was 5 mM and 640 resonance units of sTF were coupled to the sensor chip.

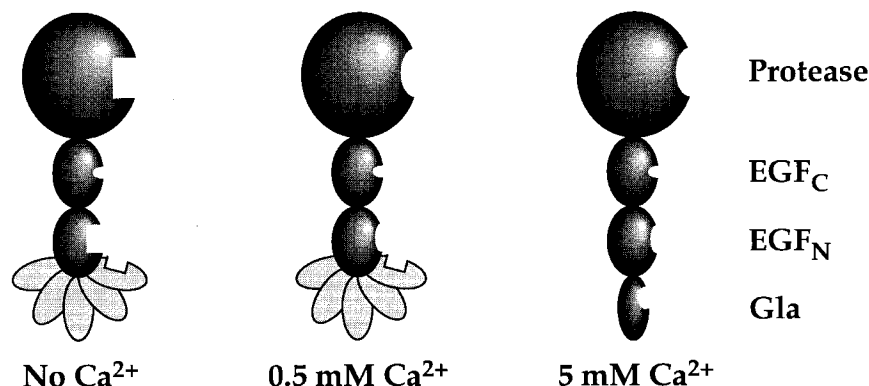


Fig. 4. Schematic model of the TF-interactive regions of fVIIa at different degrees of Ca^{2+} saturation. Left: In the absence of Ca^{2+} , the areas on fVIIa interacting in a Ca^{2+} -dependent manner with sTF are not complementary to the cofactor and the low-affinity interaction is mediated by Ca^{2+} -independent contacts. The contact between the second EGF-like domain and sTF is assumed to be Ca^{2+} -independent. The Gla domain is internally flexible. Middle: At the suboptimal Ca^{2+} concentration used in this study, the Ca^{2+} -binding sites in the protease and first EGF-like domains are nearly ($\geq 75\%$) saturated, whereas the Gla domain requires more Ca^{2+} to be fully ordered. Right: At 5 mM Ca^{2+} , the Gla domain is properly ordered and oriented resulting in an optimal fit between fVIIa and sTF.

[11]. Regions within the first EGF-like and protease domains of fVIIa are likely to function as sTF docking sites [11] and have to be available for rapid association to occur. This implies that fVIIa and des(1–38)-fVIIa should associate equally fast with sTF, which they do at optimal Ca^{2+} concentrations [30], and that fVII-GlaEGF_{NC} should associate slower with sTF since the protease domain is missing, which is also true. Lowering of the Ca^{2+} concentration to 0.5 mM results in a fVIIa conformation which associates slower to sTF, kinetically resembling fVIIa mutated at the C-terminal Gla residue [31]. On the other hand, des(1–38)-fVIIa associates at an almost optimal rate at 0.5 mM Ca^{2+} , illustrating a functional difference between fVIIa and Gla-domainless fVIIa in their modes of interacting with sTF not demonstrable in earlier studies. The observation that the rate of dissociation of the fVIIa:sTF complex is unaffected by lowering of the Ca^{2+} concentration is in agreement with previous studies showing that after complex formation Ca^{2+} is not necessary for its integrity and activity [15,25]. Furthermore, it suggests that sTF establishes and stabilizes the correct contacts with the flexible Gla domain of fVIIa in the complex at 0.5 mM Ca^{2+} although the spacing of the relevant Phe residues [11] in free fVIIa is not optimal. The structures of the apo and Ca^{2+} -loaded forms of the Gla domain of factor IX infer that the sTF-interactive Phe residues in the Gla domain of fVIIa are solvent-exposed at both 0.5 and 5 mM Ca^{2+} [35,36].

The fVIIa:TF interaction and amidolytic activity are Ca^{2+} -dependent [13–15,19–21]. Site-directed mutagenesis has revealed the importance of the Ca^{2+} -binding sites in the first EGF-like and protease domains for the expression of fVIIa activity and sTF-binding epitopes [13,24]. Although the true value of the Ca^{2+} concentration at which fVIIa attains half-maximal sTF-binding capability is debatable, it appears to be higher than the K_{d} s reported for the two Ca^{2+} -binding sites mentioned above [19,23]. A conceivable explanation is that Ca^{2+} -binding sites in the Gla domain play a role in the Ca^{2+} -dependent fVIIa activity in the presence of sTF, i.e. in sTF binding, considering the broad range of affinities represented within this group of sites [37]. These sites have been hypothesized to be important for the expression of TF-interactive epitopes [38]. However, the Ca^{2+} concentrations required for half-maximal sTF binding of intact and Gla-do-

mainless fVIIa were almost identical [21]. The results obtained with fVIIa and des(1–38)-fVIIa using the biosensor are in agreement with these observations but also clearly demonstrate different sTF-binding kinetics, especially at suboptimal Ca^{2+} concentrations. They provide the first direct demonstration of the need for a Ca^{2+} -loaded Gla domain for optimal sTF binding. I hypothesize that the unsaturated Gla domain interferes with the interaction between the neighbouring EGF-like domain and sTF, although more long-range effects are possible, and thereby precludes rapid association. We have shown by CD that Ca^{2+} binding to the Gla domain brings about ordering of structural elements [29], and it is conceivable that the flexible apo form causes steric problems in the interaction with sTF. In addition, our results with fVII-GlaEGF_{NC} unambiguously show that Gla-dependent Ca^{2+} sites of relatively low affinity are pivotal for sTF binding. It is important to remember that the Gla and first EGF-like domains bind Ca^{2+} with the same affinity in the fragment as they do in intact fVIIa [21], shown also to be the case for corresponding fragments from protein C [39], factor IX [40] and factor X [41]. Hence, the removal of the protease domain per se is not the reason for the Ca^{2+} dependence of fVII-GlaEGF_{NC}:sTF complex formation, but the binding experiments with the fragment provide valuable insights into the role of the Ca^{2+} -binding sites in the Gla domain in the interaction between fVIIa and sTF.

The Ca^{2+} dependence of the fVIIa:sTF interaction appears to be a complex function of the affinities of several Ca^{2+} -binding sites. The importance of the Ca^{2+} -binding site in the protease domain for activity has been demonstrated [13], although none of the residues in the Ca^{2+} -binding loop interact directly with sTF [11]. Perhaps this site is most important for fVIIa activity and less for sTF binding. The Ca^{2+} conformer of the first EGF-like domain is required for optimal recognition of sTF/TF [24], although the only residue that contacts sTF and is likely to be affected by Ca^{2+} binding to this domain is Gln⁶⁴ [11,42]. The present study demonstrates that a certain extent of saturation of the Gla domain appears to be essential to avoid steric hindrance. On this basis, a schematic model of the Ca^{2+} -induced changes in fVIIa that facilitate its interaction with sTF can be put forward (Fig. 4). It should be kept in mind that fVIIa binds sTF in the absence

of Ca^{2+} and the binding energy is about 2/3 of that at optimal Ca^{2+} concentrations [15,19].

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