The Roles of Factor VII's Structural Domains in Tissue Factor Binding†

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ABSTRACT: Factor VIIa binds to tissue factor in one of the initial steps of blood clotting. In order to determine the role of the various domains of the factor VII molecule in this interaction, we made several chimeric factor VII proteins using recombinant DNA techniques. The molecules have factor IX domains substituted into factor VII and vice versa. The domains exchanged were the 4-carboxyglutamic acid plus aromatic stack domain (gla), the first epidermal growth factor-like domain (Egf-1), the second epidermal growth factor-like domian (Egf-2), and the catalytic domain. Using tissue factor-coated microtiter wells, competition binding studies with ^{125}I-labeled factor VIIa indicated factor VIIa's K_d is 4.2 nM. Employing the same microtiter plate assay, k_{off} and k_{on} were determined and yielded a K_d of 1.5 nM. The results of competitive binding experiments and activation assays using chimeric proteins indicated the interaction between factor VIIa and tissue factor involves direct contact between tissue factor and factor VIIa's Egf-1 domain and catalytic domain. On the other hand, the gla and Egf-2 domains, while necessary for optimal binding, may merely impart structure to the rest of the molecule. However, either one or both of the latter domains might contribute a relatively small amount of energy to direct binding.

Factor VII−tissue factor association is one of the first events in the cascade of blood clotting reactions. Factor VII is a single-chain serine protease zymogen which is post-translationally modified to produce 10 gla residues among the first 40 residues of the N-terminus. This modification is vitamin K-dependent. Like other gla-containing coagulation proteins, factor VII has several distinct structural domains. They are the gla domain, the aromatic stack, two Egf-like[1] domains, and the catalytic domain which contains the catalytic residues of the serine protease (Hagen et al., 1986). Factor VII is activated by proteolytic cleavage at a single site (Radcliffe & Nemerson, 1975). Although the physiologically important activator is not known, factor VIIa (Pederson et al., 1989) and factor Xa (Radcliffe & Nemerson, 1976) seem to be the most likely candidates. Once activated, factor VIIa can in turn activate factors IX and X (Bom & Bertina, 1990; Ruf et al., 1991a).

Tissue factor is an integral membrane protein found in most tissues other than the normal endothelium (Nemerson & Bach, 1982). The molecule has three domains; one extracellular, one transmembrane, and one cytoplasmic (Spicer et al., 1987). Regions in the extracellular domain are involved in factor VII binding (Ruf & Edgington, 1991; Schullek et al., 1994). Tissue factor functions not only as a cell surface binding protein but also as a cofactor for factor VIIa. Factor VIIa's 1:1 complex with tissue factor activates factor X 4000-fold faster than factor VIIa alone (Bach et al., 1986; Silverberg et al., 1977).

Various parts of factor VII have been implicated in binding to tissue factor including the gla, Egf-like, and catalytic domains (Sakai et al., 1990; Ruf et al., 1991b; Higashi et al., 1992, 1994; Toomey et al., 1991; Clarke et al., 1992; Chaing et al., 1994; Kazama et al., 1993; Wildgoose et al., 1990; Kumar et al., 1991; O'Brien et al., 1991; Matsushita et al., 1994). Our previous results suggested that one or both of the Egf domains were predominantly responsible for high-affinity binding to tissue factor (Toomey et al., 1991).

In our present study, we used recombinant DNA techniques to effect further protein domain exchanges between factors IX and VII. On the basis of the results, we submit a model in which the major energetic components of binding are the Egf-1 and catalytic domains.

EXPERIMENTAL PROCEDURES

Materials. Removable microtiter wells (Immulon 1) were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). The recombinant tissue factor (from residue 1 to residue 243) was a gift from Dr. G. Vehar (Genentech Inc., South San Francisco, CA). Geneticin (G418) was purchased from Gibco-BRL (Gaithersberg, MD). Iodobeads were purchased from Pierce (Rockford, IL). Na^{125}I was purchased from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin was purchased from United States Biochemical (Cleveland, OH).

Immobilon-P transfer membrane was purchased from the Millipore Corp. (Bedford, MA). Restriction enzymes and all the enzymes used in molecular cloning were purchased from United States Biochemical. The [α-^{35}S]dATP was purchased from Amersham Corp. Plasma factors XIa, VII, and IX were purchased from Enzyme Research Laboratories (South Bend, IN). The two monoclonal antibodies to factor VII, one Ca^{2+}-dependent (CDVII) and the other Ca^{2+}-independent (CIVII), were gifts from Dr. U. Hedner (Novo Nordisk, Copenhagen) and Dr. W. Kisiel (University of New Mexico School of Medicine, Albuquerque), respectively. Monoclonal antibodies to factor IX (A2 and 2D5) were previously described (Frazier et al., 1989) and were gifts from Dr. K. Smith (University of New Mexico School of

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[1] Abbreviations: Egf-1, amino-terminal epidermal growth factor-like domain; Egf-2, carboxy-terminal epidermal growth factor-like domain; TBS, 20 mM Tris, pH 7.5, 150 mM NaCl.

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Medicine, Albuquerque) and Dr. H. Reisner (University of North Carolina at Chapel Hill), respectively. Oligonucleotides were purchased from Oligos Etc (Guilford, CT). The anion exchange resin Q-Sepharose Fast Flow was purchased from Pharmacia LKB Biotechnology (Mechanicsburg, PA). Vitamin K (Aquamephyton) was purchased from Merk Sharp & Dohme (West Point, PA). Bovine serum albumin was purchased from United States Biochemical.

Chimeric DNA Constructions. The cDNAs of both human factor VII and human factor IX are available in our laboratory. We have created several unique restriction enzyme digestion sites in those DNAs by site-directed mutagenesis (Kramer & Fitz, 1988). Each mutated molecule was sequenced by the dideoxy-chain termination method (Sanger et al., 1977) to confirm that the sequence was as expected and no extra mutations were introduced. The unique restriction sites in the cDNA of factor VII and factor IX for domain exchange are as follows: a *Bst*E2 site is at the 5′-end of the Egf-1 domain (residues 47−49 of factor VII; residues 48−50 of factor IX); a *Sac*I site is at the domain junction of Egf-1 and Egf-2 (residues 82−83 of factor VII; residues 83−84 of factor IX); a *Not*I site is at the 3′-end of the Egf-2 domain (residues 135−137 of factor VII; residues 132−134 of factor IX). In addition constructions #1213, 1193, 1203, and 1223 (Table 1) have the A-7 monoclonal antibody site of factor IX substituted for the factor VII sequence in the gla domain (residues 32−40). This substitution did not affect either tissue factor binding or clotting activity in otherwise wild-type factor VII. All the recombinant DNAs were subcloned in the expression vector pCMV5 (Andersson et al., 1989) for mammalian cell transfection.

Expression and Purification of the Chimeric Protein. Each one of these constructions together with pSV_2neo (the antibiotic-resistant gene) (Southern & Berg, 1982) and pCMVhGC+ (human carboxylase gene) (Wu et al., 1991) was transfected into the expression host, human kidney cell line 293 (ATCC CRL 1573), by the calcium phosphate coprecipitation method (Graham & van der Eb, 1973). A clone expressing a high level of each construction was selected and screened as described previously (Toomey et al., 1991). Each screened clone was expanded to 900 cm^2 roller bottles for large-scale protein production. All the recombinant proteins were purified by a pseudo-affinity chromatographic method using Fast Flow Q-Sepharose and elution with a calcium gradient, followed by a NaCl gradient (Yan et al., 1990; Zhang et al., 1992).

Characterization of the Purified Proteins. The gla content of each recombinant protein was determined according to Kuwada and Katayama (1981). This analysis was generously provided by Dr. P. A. Friedman of Merck Sharp & Dohme. Protein concentrations were determined by the Bradford assay using human plasma factor IX as a standard.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blot analyses were performed as described (Laemmli, 1970; Towbin et al., 1979).

The calcium-induced intrinsic fluorescence quenching of all the recombinant proteins was determined. The chimeras were analyzed in TBS with 0.1% poly(ethylene glycol) 8000 with increasing concentrations of $CaCl_2$. The measurements were made in a Shimadzu RF-5000 spectrofluorometer with an excitation wavelength of 280 nm (slit width = 1.5 nm) and an emission wavelength of 340 nm (slit width = 10 nm).

The clotting activity of the proteins was measured in the prothrombin time assay according to the manufacturer's instructions (Sigma, St. Louis, MO).

Chimeras #5602 and #1193 were activated using human plasma factor XIa (1:50 molar ratio) at 37 °C for 1−2 h.

The interaction of certain chimeric proteins with tissue factor was evaluated by measuring tissue factor-dependent activation of the chimera. The protein (3 μM in TBS + 4.0 mM $CaCl_2$, 0.1% PEG) was incubated at room temperature in the presence or absence of tissue factor (0.2 μM). Portions were removed at different times, denatured, and analyzed by gel electrophoresis under reducing conditions. In addition, the proteolytic activity of the tissue factor−chimera complex was measured using synthetic substrate S-2288 (Kabi).

Immobilization of Tissue Factor. Tissue factor was immobilized on polystyrene plates in a manner similar to that previously described (Clarke et al., 1992). The recombinant tissue factor was diluted to 5 μg/mL with TBS, 5 mM $CaCl_2$−1 mM $MgCl_2$. One hundred microliters of this diluted tissue factor was put into a polystyrene Immulon 1 microtiter well and allowed to sit at 4 °C overnight. After removal of unbound tissue factor, the nonspecific binding sites were blocked by treating the wells with 300 μL of 3% bovine serum albumin in TBS, 5 mM Ca^{2+}−1 mM Mg^{2+} overnight at 4 °C.

Iodination of Factor VII(a). Recombinant factor VII was radiolabeled using Iodobeads following the manufacturer's protocol (Pierce). The iodinated protein was separated from free ^{125}I by gel filtration through a 10 mL G-25 (Sigma) column.

Kinetics of Factor VII−Tissue Factor Binding. The factor VIIa−tissue factor association and dissociation rates were studied essentially as described before (Bennett & Yamamura, 1985; Cheng et al., 1986). In the association study, ^{125}I-factor VIIa (20 nM in TBS, 5 mM Ca^{2+}−1 mM Mg^{2+}, and 1% bovine serum albumin) was in excess compared to the receptor (the effectively immobilized tissue factor, about 1.8 nM per well). At different incubation times, the well was washed with TBS, 5 mM Ca^{2+}−1 mM Mg^{2+}, and the total bound radioactivity was determined in an LKB minigamma counter (Model 1275). The nonspecific binding of each incubation time is the radioactivity remaining in the microtiter well coated only with bovine serum albumin. The specific binding data were derived from the difference between total binding counts and nonspecific counts.

The dissociation study was performed by incubating ^{125}I-labeled factor VIIa (20 nM) in tissue factor-coated wells at room temperature for 2 h. The wells were washed with TBS, 5 mM Ca^{2+}−1 mM Mg^{2+} 3 times, and 3000 nM nonlabeled factor VIIa (more than 1000-fold the bound ^{125}I-factor VIIa) was added to each well. Samples were removed at different times, and labeled factor VIIa released into solution was measured in a gamma counter.

Competition Binding Study. The competition mixture was composed of 2 nM ^{125}I-factor VIIa plus increasing concentrations of the competing protein in 100 μL of 1% bovine serum albumin−TBS, 5 mM Ca^{2+}−1 mM Mg^{2+}. Each competition mixture was added to a tissue factor-coated microtiter well and incubated at room temperature for 2 h. At the end of this incubation, each well was washed by TBS, 5 mM Ca^{2+}−1 mM Mg^{2+} 3 times, and the bound radioactivity was determined in a gamma counter. The binding data were

Table 1: Chimeric Proteins and Their Gla Content and K_d's[d]

Proteins	Domain Compositions	Gla[a]	Kd[b] (nM)
Factor VII	Gla A EGF1 EGF2 Catalytic Region	8.8 ±0.1	4.2± 0.3
#5401	Gla A EGF1 EGF2 Catalytic Region	9.1 ±0.2	21± 3
#1213	Gla A EGF1 EGF2 Catalytic Region	10.8 ±0.3	426± 21
#1063	Gla A EGF1 EGF2 Catalytic Region	9.7 ± 0.1	-----
#1343	Gla A EGF1 EGF2 Catalytic Region	6.2 ± 0.2	-----
Factor IX	Gla A EGF1 EGF2 AP Catalytic Region	11.1 ± 0.1	-----
#5602	Gla A EGF1 EGF2 AP Catalytic Region	7.8 ± 0.1	177± 3
#5602a	Gla A EGF1 EGF2 Catalytic Region	7.8 ± 0.1	88[c]
#1193	Gla A EGF1 EGF2 AP Catalytic Region	7.2 ± 0.2	477± 82
#1193a	Gla A EGF1 EGF2 Catalytic Region	7.2 ± 0.2	131± 6
#1203	Gla A EGF1 EGF2 AP Catalytic Region	9.7 ± 0.1	-----
#5501	Gla A EGF1 EGF2 AP Catalytic Region	8.3 ± 0.1	-----
#1023	Gla A EGF1 EGF2 AP Catalytic Region	8.8 ± 0.3	-----

[a] Values represent the total number of Gla residues per molecule. [b] Values represent the average of three to six experiments. [c] Value represents only one experiment. [d] K_d values were determined by competition binding experiments and nonlinear regression analyses as described uner Experimental Procedures. A dashed line means the protein did not compete for tissue factor binding.

evaluated and the dissociation constants (K_d) calculated using the nonlinear regression program MK model (Biosoft, Milltown, NJ).

RESULTS

Expression, Purification, and Characterization of the Proteins. Recombinant proteins were produced at 0.1−0.25 mg/L of cell culture media. The total yield of purified protein was in the range of 2.5−5.0 mg/preparation. Wild-type recombinant factor VII had normal clotting activity compared to plasma factor VII in a prothrombin time assay. Results of gla analyses for all proteins are shown in Table 1. Western blot analyses employing antibodies to various parts of factors VII and IX confirmed the presence of the expected domains (results not shown).

Under normal circumstances, after purification and concentration, the chimeric proteins with factor VII's activation site are activated according to gel analysis. Those with factor IX's activation sites are in the zymogen form. Factor VII zymogen could be isolated after calcium elution from Q-Sepharose if immediately stored at −80 °C.

The extent of calcium protein fluorescence quenching varied between 20 and 50%. However, the shapes of all the curves were similar, suggesting that the calcium−protein interaction had the same affinity, and the conformational change occurred at the same calcium concentration for all the proteins (data not shown).

Recombinant wild-type factor VII had normal clotting activity compared to normal pooled plasma. Chimeric proteins #1213 and #5401 had 3% and 11% of normal activity, respectively. While chimeric protein #1063 had no measurable clotting activity, in the presence of tissue factor it was cleaved to a two-chain form similar to wild-type factor VIIa. Also in the presence of tissue factor after activation, this protein had proteolytic activity toward synthetic substrate S-2288.

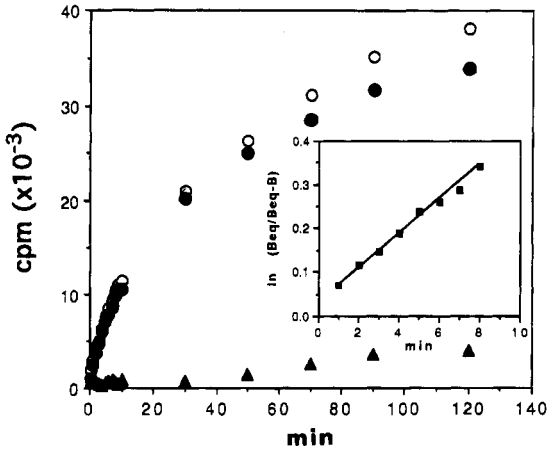

FIGURE 1: Association kinetics of ^{125}I-factor VIIa binding to tissue factor. Iodinated factor VIIa (20 nM) was aliquoted into tissue factor-coated microtiter wells at room temperature and incubated for the indicated period of time. The binding was measured as described under Experimental Procedures. The figure shows the data for total binding (○), specific binding (●), and nonspecific binding (▲). The inset shows an association rate plot that gives a k_{on} of 2.8×10^4 M^{-1} s^{-1}. B is the amount bound at the indicated time, and Beq is the amount bound at saturation. Each point represents the mean of triplicate measurements, the standard deviation of each point is less than 2% of the mean value.

Kinetic Binding Study. Previous studies showed that recombinant tissue factor interacts with factor VII specifically in the absence of phospholipid (Bom & Bertina, 1990; Ruf et al., 1991; Toomey et al., 1991). We took advantage of this observation to set up a binding assay to examine the affinity between factor VII chimeric proteins and recombinant tissue factor immobilized on the surface of microtiter wells. It allowed us to use purified components to study the interaction, thus eliminating features of nonspecific phospholipid binding in relipidated tissue factor assays or internalization of ligand characteristic of cell binding assays. Initial experiments showed (1) the binding of ^{125}I-factor VIIa to the coated tissue factor was specific and saturable, and (2) the iodinated factor VIIa still retains normal clotting activity in a prothrombin time assay (data are not shown). Bach et al. (1986) have previously shown that the complex contains one molecule of factor VIIa and one molecule of tissue factor.

The kinetics of ^{125}I-factor VIIa binding to tissue factor were examined. Measurements were done at room temperature with 20 nM ^{125}I-factor VIIa. Figure 1 shows the association time course. The binding reaches equilibrium after 2 h incubation. The association rate data from the early time points (Figure 1, inset) give the second-order association rate constant, k_{on}, of 2.8×10^4 M^{-1} s^{-1}. The first-order dissociation rate plot (Figure 2) provides an estimate of the dissociation rate constant, k_{off}, of 4.2×10^{-5} s^{-1}. The ratio k_{off}/k_{on} can be used to obtain the dissociation constant of 1.5 nM, which is similar to the K_d of 4.2 nM obtained in competition studies (Table 1).

Competition Binding Studies. The binding constants of the recombinant proteins determined by competition with ^{125}I-factor VIIa for tissue factor are shown in Table 1. Interestingly, the chimeras with factor VII's catalytic domain that bound to tissue factor, activated factor X at rates proportional to their K_d's (Chang et al., manuscript in preparation).

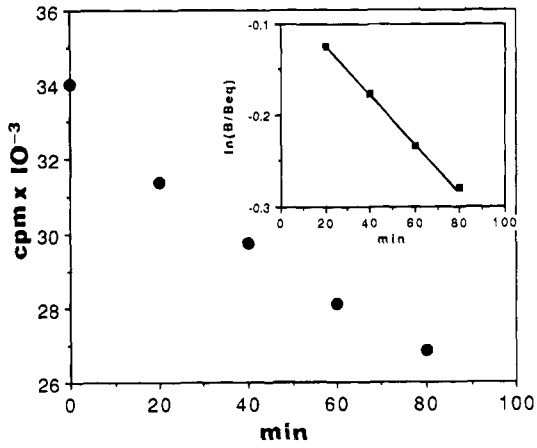

FIGURE 2: Dissociation kinetics of ^{125}I-factor VIIa binding to tissue factor. ^{125}I-factor VIIa (20 nM) was incubated in tissue factor-coated microplate wells at room temperature for 2 h. After the incubation, ^{125}I-factor VIIa was replaced with nonlabeled factor VIIa and incubated for the indicated time. The amount of radioactivity released into solution was measured as described under Experimental Procedures. The first-order dissociation rate plot gives a k_{off} of 4.2×10^{-5} s^{-1}. *B* is the amount bound at the indicated time, and *B*eq is the amount bound at saturation. Each point represents the mean of triplicate measurements; the standard deviation of each point is less than 5% of the mean value.

DISCUSSION

Our goal in this investigation was to study factor VII domain by domain to see which parts of the molecule are involved in tissue factor binding. We used domain exchanges between factors VII and IX to create proteins with various combinations of domains. Others in our laboratory have previously described the logic of this approach, and described results supporting its validity (Lin et al., 1990). In that study, it was shown that substitution of factor X's Egf-1 domain in factor IX did not affect clotting activity, indicating that domain substitution does not inherently disrupt the protein's overall structure. In addition, in the present study we found that all the chimeric proteins exhibited the calcium-dependent fluorescence change characteristic of wild-type factors VII and IX. Finally, the clotting activities of the chimeras containing factor VII's catalytic domain were close to those predicted from their affinities for tissue factor (#1213 and #5401). That is, the enzyme's reduced catalytic activity was due to decreased affinity for the cofactor, and not to disruption of the overall structure affecting the active site or substrate binding site.

To evaluate tissue factor binding, we wanted to use the simplest assay possible, with the minimum number of functional components. That is one without phospholipids and cell surface proteins other than tissue factor. Yet, we needed an assay that resulted in a functional factor VII–tissue factor complex at equilibrium. The assay we used was similar to one previously described, but our system employed direct measurement of radiolabeled tissue factor-bound factor VIIa, as opposed to immunodetection of the complex (Clarke et al., 1992). Characterization of the tissue factor–factor VII interaction on the microtiter plates indicated that the K_d derived from off/on rates was similar to that derived from competition binding (Figures 1 and 2 and Table 1), and the activation of factor X by the formed complex occurred over the concentrations at which binding occurred (Chang et al., manuscript in preparation). These results indicate the assay represents a functional factor VIIa–tissue factor complex at equilibrium.

Thus, we employed this assay to clarify the roles of the various factor VII domains in tissue factor binding. Some previous studies indicate that the gla domain of factor VII is directly involved (Sakai et al., 1990; Ruf et al., 1991; Higashi et al., 1992), while others suggest that the gla domain is not directly involved in binding (Toomey et al., 1991; Wildgoose et al., 1992; Neuenschwander & Morrissey, 1994; Martin et al., 1993); likewise, some reports suggest that the catalytic domain is directly involved in binding (Wildgoose et al., 1990; Kumar et al., 1991; O'Brien et al., 1991; Higashi et al., 1994; Matsushita et al., 1994). We had previously thought that the primary contact was between one or both Egf-like domains and tissue factor (Toomey et al., 1991). Subsequently, Kazama et al. (1993) concluded that the gla through Egf-2 domain includes factor VII's tissue factor binding site. Clarke et al. (1992) showed that an antibody to the first Egf-like domain inhibited factor VII binding to tissue factor, Chaing et al. (1994) showed factor VII which had a point mutation in the Egf-1 domain bound with reduced affinity to tissue factor, and Higashi et al. (1994) showed the gla-Egf-1 fragment of bovine factor VII binds to bovine tissue factor.

We constructed factor IX proteins containing factor VII's Egf-1, Egf-2, or both Egf-like domains to explore the roles of these domains. Perhaps surprisingly, none of these proteins bound to tissue factor (data not shown). In addition, as we previously reported, the molecule with the gla domain of factor VII in factor IX did not bind, nor did the one with the catalytic domain of factor VII and the light chain of factor IX (Toomey et al., 1991). So no single domain of factor VII is adequate for binding to tissue factor.

For that reason, we constructed a family of chimeric proteins containing various combinations of the domains of factors IX and VII (Table 1). To begin, we combined each of the Egf domains of factor VII with another domain of factor VII in factor IX. Factor IX with the gla domain and Egf-1 of factor VII (1193) was the only protein that bound to tissue factor; thus, the Egf-1 domain is essential for binding. This result extends our previous observation that one or both Egf domains are involved in binding and confirms the prediction of Clarke et al. (1992), Chaing et al. (1994), and Higashi et al. (1994) that the Egf-1 domain is involved. The chimera with the gla domain and Egf-2 (1203) did not bind, nor did the ones with the Egf-1 or Egf-2 plus the catalytic domain (1343, 1063). Thus, the roles of the gla, Egf-2, and catalytic domains were unclear at this point.

To further clarify the roles of these domains, we created proteins with three domains of factor VII and one of factor IX. First of all, the gla domain of factor IX may be substituted in factor VII with only a 5-fold increase in K_d (5401). This suggests the gla domain probably is not directly involved in binding. It may impart optimum structure to the rest of the protein through calcium binding as suggested by Wildgoose et al. (1992). Our results, however, do not completely resolve the role of the gla domain.

It does not seem likely that the Egf-2 domain is involved in binding since the difference in K_d between the protein containing the gla through Egf-2 domain and the one with gla through Egf-1 is only 2.4-fold. The activated forms of the same proteins (5602a, 1193a) show only a 1.5-fold

difference. On the other hand, when the Egf-2 domain is added to the Egf-1 and catalytic domains (1343 and 5401), the affinity goes from unmeasurable to 20 nM. Our results do not rule out the possibility that the Egf-2 domain binds directly to tissue factor, but we think it is more likely that this domain is essential for maintaining the correct tertiary structure of the molecule as a whole including the tissue factor recognition site. This result is in line with results from X-ray crystallographic studies on factor X that indicate there is a close interaction between the catalytic domain and the region including the Egf-2 through the C-terminus of the light chain (Padmanabhan et al., 1993). This interpretation is also supported by results from thermodynamic studies, showing there is minimal interaction between the Egf-1 and Egf-2 domains at least in factor IX, but a strong interaction between the Egf-2 and catalytic domains (Vysotchin et al., 1993).

This raises the issue of the role of the catalytic domain in binding. We had previously thought that the catalytic domain was not involved (Toomey et al., 1991); however, results from this study suggest otherwise. If one considers the protein with the Egf-1 domain of factor IX in factor VII (1213), this molecule still binds to tissue factor, although with reduced affinity. In addition, the protein with factor VII's Egf-1 in factor IX does not bind. These results suggest that there is another part of factor VII that binds to tissue factor in addition to the Egf-1 domain. As discussed above, we believe the gla domain and/or the Egf-2 domain plays at most a minor role in direct binding, so based on the results in this study and those of others, we believe the second major site must be in the catalytic domain (Wildgoose et al., 1990; Kumar et al., 1991: O'Brien et al., 1991; Higashi et al., 1994; Matsushita et al., 1994). All the proteins containing factor VII's activation site were activated during purification, while those with factor IX's activation sites were not. Activation by proteolytic cleavage probably causes a conformational change in the light chain and/or the catalytic domain of these proteins and perhaps in the interdomain contacts. Thus, it was of interest to measure tissue factor binding of activated forms of the proteins with factor IX's catalytic domain. When chimeras with gla through Egf-1 or Egf-2 of factor VII combined with the catalytic domain of factor IX were activated by factor XIa (1193a and 5602a), their K_d's decreased from 477 to 131 nM and from 177 to 88 nM, respectively. This indicates there is a conformational change recognized by tissue factor on activation, but in the absence of the factor VIIa catalytic domain, normal binding still does not occur. Our results agree with those obtained using proteolytic fragments of factor VII to investigate tissue factor binding (Kazama et al., 1993). However, to gather more direct evidence about the role of the catalytic domain, we further examined the protein with the Egf-2 through the catalytic domain of factor VII (1063). As described earlier, this protein does not compete with plasma factor VII for the tissue factor site at the concentrations we used in our studies (10 μM). However, if this protein does bind to tissue factor, but with relatively low affinity, our competition binding assay might not be sensitive enough to detect binding. So we incubated the protein with tissue factor and analyzed the reaction mixture on polyacrylamide gels. In the absence of tissue factor, the protein was not activated even after a 15 h incubation. On the other hand, with tissue factor the protein was completely activated after the same time. That activation occurred was confirmed by the fact that the cleaved form, in the presence of tissue factor, had activity toward the synthetic substrate S-2288. This result indicates that the chimera containing only the Egf-2 and catalytic domains of factor VII binds to tissue factor. Since the Egf-2 domain does not appear to participate directly in binding, this result offers perhaps the strongest and most persuasive evidence that the catalytic domain is directly involved in tissue factor binding.

Some interesting albeit speculative points can be made by comparing the free energies of binding (ΔG) from some earlier studies to those calculated from our chimeric proteins. Higashi et al. (1994) produced proteolytic fragments of bovine factor VII, and measured the fragments' inhibition of factor VIIa–tissue factor activation of factor X. They derived K_i's from these data from which one can calculate ΔG's for binding. In turn, one can calculate $\Delta\Delta G$'s from the difference between the values of K_i for intact factor VII and the fragment. For the light-chain fragment, the $\Delta\Delta G$ is 2.1 kcal/mol. Kazama et al. (1993) using similar methods, but with human factor VII, employed a fragment consisting of the light chain and a small part of the catalytic domain. Their data yield a $\Delta\Delta G$ of 1.6 kcal/mol. Our results give $\Delta\Delta G$'s for the protein with the light chain of factor VII and the proteolytic domain of factor IX (5602) of 1.8 or 2.2 kcal/mol depending on activation or not, respectively. In addition, using the results of Higashi et al. (1994) with a fragment containing the gla-Egf-1 domain of bovine factor VII, one may calculate a $\Delta\Delta G$ of 2.8 kcal/mol. Our results yield a $\Delta\Delta G$ for the protein with the gla-Egf-1 of factor VII of 2.1 or 2.8 kcal/mol depending on activation or not, respectively. Thus, factor VII with domains deleted by proteolysis or factor VII with factor IX domain substitutions gives similar $\Delta\Delta G$'s. This indicates that neither factor IX's Egf-2 or catalytic domain can substitute for the corresponding factor VII domain to support tissue factor binding. This confirms one of our basic assumptions: that even similar domains from similar proteins have specific functions and cannot substitute for one another. The results from the three studies also lend support to each other, in that two rather different approaches lead to similar conclusions.

Several groups have studied tissue factor–gla-domainless factor VII binding (Sakai et al., 1990; Ruf et al., 1991b; Higashi et al., 1994; Neuenschwander & Morrissey, 1994). $\Delta\Delta G$'s calculated from these data range from essentially zero to infinity. That is, gla-domainless factor VII binds the same as native factor VII (Ruf et al., 1991b) or not at all (Sakai et al., 1990) depending on the study. The affinities of factor VII and gla-domainless factor VII for tissue factor seem to vary depending on the form of tissue factor used, and whether the tissue factor was associated with negatively charged phospholipids in the reaction mixture. If one considers the results from seven experiments (some studies have more than one experiment), not including the extreme values (0 and infinity), the $\Delta\Delta G$ for gla-domainless factor VII is 1.6 $\pm$ 0.3 kcal/mol (Ruf et al., 1991b; Higashi et al., 1994; Neuenschwander & Morrissey, 1994). Our $\Delta\Delta G$ was 1.0 kcal/mol for the molecule with factor IX's gla domain in factor VII. At the risk of overinterpretation, this comparison suggests that removing the gla domain and substituting for it may have different effects. That is, as we suggested above, the gla domain may impart structure to the rest of the factor VII molecule necessary for optimal tissue factor binding, and

even factor IX's gla domain may partially fill this role. As described above, our chimera with the gla domain of factor IX undergoes a calcium-dependent fluorescence change similar to that of native factor VII. While these numbers make for an interesting comparison, the actual physical significance of them remains to be seen. Obviously, the $\Delta\Delta G$'s for the domains derived from the binding constants of the various chimeras are less than the binding energy of native factor VII. As suggested above in the case of the gla domain, this may mean that the corresponding factor IX domain may partially fulfill the structural requirements for binding. In addition, while our K_d values are consistent both internally and with values from other groups, our estimates of K_d's especially for the chimeras with low affinity for tissue factor are highly dependent on the analysis method. On the other hand, for the Egf-2 and catalytic domains our $\Delta\Delta G$'s are similar to those derived from experiments using proteolytic fragments, so it is unlikely that factor IX domains can partially substitute for these factor VII domains.

Our present model for the factor VIIa−tissue factor interaction accounts for the role of each of the four regions in this interaction. The gla domain is important for calcium binding, and in turn the structure of the rest of the factor VII molecule. The gla domain may also be involved directly in binding to tissue factor. The Egf-1 and catalytic domains directly contact tissue factor. However, the catalytic domain seems to require the Egf-2 interdomain contacts to maintain the structure necessary for optimal binding. Information on the three-dimensional structure of related proteins (factors IX and X), combined with computer modeling of factor VII's structure, may offer information on likely residues involved in Egf-2−catalytic domain contacts. This might allow creation of chimeras to investigate the importance of interdomain contacts in function. In addition, studies to elucidate the specific residues in the Egf-1 and catalytic domains involved in binding are underway in our laboratory.

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