Factor VIIa's First Epidermal Growth Factor-like Domain's Role in Catalytic Activity†

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ABSTRACT: Factor VIIa−tissue factor complex formation initiates the extrinsic blood coagulation pathway. We investigated factor VIIa's first epidermal growth factor-like (egf1) domain's role in the catalytic activity increase caused when factor VIIa binds tissue factor. Starting with a factor VIIa with factor IX's egf1 domain (factor $VII_{(IXegf1)}a$), we made 4 proteins with egf1 residues changed to those in factor VIIa, including E51A, D64Q, FG74−75PA, and K79R. We measured each enzyme's affinity for tissue factor and determined the enzymes' kinetic constants with and without tissue factor. The K_d for factor $VII_{(IXegf1)}a$ binding to tissue factor was 60−200-fold higher than that of factor VIIa depending on the assay employed. Only factor $VII_{(IXegf1)}a$ with the K79R (K79Ra) mutation, among all the mutants, had an effect on binding with a K_d 3−8-fold lower than that of factor $VII_{(IXegf1)}a$. In kinetic analyses with a small peptide substrate, in the absence of tissue factor, factor VIIa, factor $VII_{(IXegf1)}a$, and K79Ra had similar k_{cat}'s and K_m's. With tissue factor, due to a k_{cat} decrease, factor $VII_{(IXegf1)}a$'s catalytic efficiency (k_{cat}/K_m) was 2-fold lower than factor VIIa's. K79Ra's catalytic efficiency was intermediate between those of factor VIIa and factor $VII_{(IXegf1)}a$. With factor X as substrate, in the absence of tissue factor, K79Ra and factor $VII_{(IXegf1)}a$ had catalytic efficiencies 1.5-fold and 2-fold lower than that of factor VIIa. In contrast, with tissue factor and with factor X as substrate, due to higher K_m's, factor $VII_{(IXegf1)}a$ and K79Ra had only 9% and 33% of factor VIIa's catalytic efficiency. Our results suggest the egf1 domain's role in tissue factor binding involves critical alignment of tissue factor with factor VIIa's catalytic domain. Proper alignment in turn promotes optimal catalytic activities.

The interaction between factor VIIa and its cofactor, tissue factor, is the initial step in the extrinsic blood coagulation pathway. Characterizing this event's mechanism is important for understanding how coagulation is controlled. Such characterization may also lead to basic understanding of how cofactors and enzymes interact to increase catalytic activity. A significant step in this process was the publication of the crystal structure of the factor VIIa-subtilisin-cleaved soluble tissue factor complex (*1*). This structure confirms the results from studies using a variety of approaches that suggested the interaction's complexity. Residues in the egf1[1] domain (*1*−*5*) and the catalytic domain (*1*, *4*−*9*) are the main contact points, but residues in both the egf2 domain and the gla domain also play roles (*1*, *5*, *10*−*12*).

However, the understanding of the mechanism by which factor VIIa, on binding tissue factor, becomes a better enzyme is still far from being complete. Functionally important residues have been identified by alanine scanning mutagenesis (*13*), which indicates that egf1 residues bind tissue factor, but do not cause the increase in catalytic activity effected by tissue factor binding (cofactor activity). According to this report only residues in the catalytic domain participate in this process. In another study, however, the gla-egf1 domains' high-affinity calcium-binding site was implicated in cofactor activity. Mutation of residues at this site, while having no effect on amidolytic activity, causes a small decrease in k_{cat} for factor X activation (*14*). The authors

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[1] Abbreviations: Gla, the γ-carboxyglutamic acid rich region; egf1, the first epidermal growth factor-like domain; factor $VII_{(IXgla)}$, factor VII with its gla domain substituted by that of factor IX; factor $VII_{(IXegf1)}$, factor VII with its egf1 domain substituted by that of factor IX; factor $VII_{(IXegf1)}a$, activated factor $VII_{(IXegf1)}$; K79R, factor $VII_{(IXegf1)}$ with a point mutation of lysine at position 79 to arginine; K79Ra, activated K79R; E51A, factor $VII_{(IXegf1)}$ with a point mutation of glutamic acid to alanine at residue 51; D64Q, factor $VII_{(IXegf1)}$ with a point mutation of aspartic acid to glutamine at residue 64; FG74-75PA, factor $VII_{(IXegf1)}$ with point mutations of phenylalanine and glycine at positions 74 and 75 to proline and alanine, respectively; TBS, 20 mM Tris pH 7.4−150 mM NaCl; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate); EDTA, [ethylenedinitrilo]-tetraacetic acid.

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suggest that calcium binding supports either a direct interaction of the egf1 domain with factor X or alignment of the gla domain so that domain can interact with factor X. In another study of calcium site mutants, the authors report a 2−3-fold reduction in amidolytic activity, but normal factor X kinetics (*15*). In both studies the effects of mutations at the calcium-binding site are manifested with or without tissue factor. Studies with tissue factor mutated at residues contacting factor VIIa's egf1 domain indicate that some egf1 domain residues affect cofactor activity (*16*−*18*). Thus, it appears there may be at least two sites in the egf1 domain that affect catalytic activity: one (the calcium site) is tissue factor-independent and the other is tissue factor-dependent.

Replacing factor VII's egf1 domain with that of factor IX (factor $VII_{(IXegf1)}$) causes a 100-fold tissue factor affinity decrease and a 90% clotting activity decrease (*5*). Using this chimeric protein as a starting point, in the present study we investigated the egf1 domain and some of its residues' roles in factor VIIa's catalytic activity. We changed residues in the egf1 domain of factor $VII_{(IXegf1)}$ back to those in factor VII. Our hypothesis was that factor $VII_{(IXegf1)}$a should lack tissue factor-dependent functions related to binding and cofactor activity, and that changing amino acid residues in this chimera to functionally important ones found in factor VIIa might cause an increase in activity. We made four proteins with mutations to factor $VII_{(IXegf1)}$a including the following residues that are different in factors VII and IX (*19*, *20*): E51A, D64Q, FG74−75PA, and K79R. Q64 and R79 bind tissue factor (*1*, *21*, *22*). We measured tissue factor binding and catalytic activity of the purified, activated chimeric proteins. The results suggest that the egf1 domain's primary role is binding tissue factor, but in addition, it is indirectly involved in factor VIIa's interaction with factor X.

EXPERIMENTAL PROCEDURES

Materials. Human plasma factors VII, VIIa, X, and Xa were products of Enzyme Research Laboratories, Inc. (South Bend, IN). The recombinant tissue factor apoprotein (residues 1−243) was a generous gift from Dr. Gordon Vehar (Genentech, Inc., South San Francisco, CA). The factor VII calcium-dependent monoclonal antibody, CDVII, was a kind gift from Dr. U. Hedner (Novo Nordisk, Copenhagen). Chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroaniline-dihydrochloride) and S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroaniline hydrochloride) were purchased from Chromogenix (Franklin, OH). $Na^{125}I$ was purchased from Dupont and Iodobeads from Pierce Chemical (Rockford IL). Immulon 1 plates for competitive binding assays were purchased from Dynatech Laboratories, Inc. (Chantilly, VA), and the EIA/RIA plates for amidolytic assays from Costar Corporation (Cambridge, MA). Centriprep-30's were obtained from Amicon, Inc. (Beverly, MA). Q Sepharose Fast Flow was purchased from Pharmacia Biotech (Piscataway, NJ). The reagents for the Bio-Rad protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The Enhanced Chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science (Arlington Heights, IL). Restriction enzymes and ligase were purchased from New England Biolabs (Beverly, MA). Sequenase 7-deaza-dGTP DNA sequencing kit was purchased from USB (Cleveland, OH). Coagulation control level 1, factor VII deficient plasma, thromboplastin, and benzamidine-HCl were obtained from Sigma Chemical Company (St. Louis, MO). Vitamin K was from Merck Sharp and Dohme (West Point, PA). Insulin-transferrin-sodium selenite tissue culture supplement was from Boehringer Mannheim (Indianapolis, IN).

Site-Directed Mutagenesis. A chimeric cDNA with factor IX's egf1 domain in factor VII was used as the starting material to create mutant cDNA's as described previously (*5*). Eight residues in this construct which were from factor IX's gla domain substituted for purposes of purification using a monoclonal antibody were changed back to those of factor VII. Site-directed mutagenesis was performed using the Kunkel method (*23*), and the expected mutations were confirmed by nucleotide sequencing (*24*), and then the complete cDNA sequences were confirmed again with Applied Biosystems 373A DNA Sequencers.

Expression, Purification, and Characterization of Proteins. Proteins were expressed and purified essentially as described previously (*2*, *5*, *25*) with some modifications. Briefly, each of the mutants was cloned into the mammalian expression vector pCMV5 and then cotransfected with pSV2neo into the human kidney cell line 293. The antibiotic G418 resistant cell clones were subcloned and expanded. The supernatants of each clone were collected and assayed for expression using the ECL dot blotting method with the antibody CDVII. ECL analysis was performed according to manufacturer's instruction. The supernatant collected from expression medium was directly blotted onto a poly(vinylidene difluoride) microporous membrane under weak vacuum using a Minifold I dot blot apparatus as described (*2*). The clones with high levels of protein expression were selected, expanded, and transferred to 850 cm^2 roller bottles for larger scale protein expression. The expression medium was serum free DMEM/F12 supplemented with L-glutamine, penicillin, streptomycin, 5 μg/mL vitamin K, and 10 mg of insulin-transferrin-sodium selenite supplement/L. Culture medium was collected every day for 15−20 days and stored at −20 °C. The proteins were purified by a modification of the method of Yan et al. (*25*). The frozen media were thawed, and EDTA and benzamidine-HCl were added (4 and 5 mM, respectively). Culture media were filtered through a 0.45 μm Millipore filter and incubated at 4 °C while shaking overnight with Q Sepharose (5.0 mL/L of supernatant). The resin was equilibrated in 0.02 M Tris, pH 7.4, 0.15 M NaCl, 2 mM benzamidine-HCl. After incubation the supernatant was removed, and the resin was loaded onto a column and washed with equilibration buffer containing 2 mM EDTA, then with equilibration buffer without EDTA. Protein was eluted from the column with a calcium gradient from 0 to 60 mM in 0.02 M Tris, pH 7.4, 0.15 M NaCl, 2 mM benzamidine. The proteins were concentrated in a Centriprep-30, characterized by gel electrophoresis (*26*), and quantitated using absorbance at 280 nm ($\epsilon^{1\%} = 13.9$) (*27*) or the Bio-Rad protein assay kit using plasma factor VII as a standard. Gla analyses were kindly performed by Dr. Cindy Payne at Lilly Research Laboratories (*28*), and all proteins were essentially fully carboxylated.

Iodination of Factor VII. Factor VII was iodinated with $Na^{125}I$ using Iodobeads following the instructions of the

Table 1: Summary of the Results of Tissue Factor Binding Experiments[a]

	competitive binding		amidolytic assay[b]		amidolytic assay[c]		factor Xa formation[c]	
enzyme	K_d (nM)	$\Delta\Delta G$ (kcal/mol)	K_d (nM)	$\Delta\Delta G$ (kcal/mol)	K_d (nM)	$\Delta\Delta G$ (kcal/mol)	K_d (nM)	$\Delta\Delta G$ (kcal/mol)
factor VIIa	20.8 ± 2.7		0.23 ± 0.04		0.52 ± 0.13		0.49 ± 0.07	
factor VII(IXegf1)a	1239 ± 216	2.41	46.0 ± 6.5	3.12	46.4 ± 4.3	2.68	44.4 ± 11.1	2.66
K79Ra	245 ± 35	1.46	14.7 ± 1.7	2.42	5.4 ± 0.7	1.41	9.1 ± 1.0	0.72

[a] The results represent 3−5 experiments. [b] Varying tissue factor concentration.. [c] Varying enzyme concentration.

manufacturer (Pierce). The specific activity of labeled factor VII was $2.3-3.6 \times 10^6$ cpm/μg of protein.

Competitive Binding Assay. The competitive binding assay was performed as previously described (*5*). Data analysis was performed using MK model (Biosoft, Ferguson, MO) as recommended by the manufacturer. These analyses were performed slightly differently than the ones in our previous study. Briefly, we used the power of the software by combining all of the binding data from experiments with one protein and allowing the program to determine a single set of binding parameters. In addition we set the parameters for nonspecific binding to be equal for radio-labeled and unlabeled proteins in a given analysis. When we analyzed data from our previous work by using this method, the results were consistent with the results presented in the present study.

Activation of Factor VII. The wild-type or mutant factor VII (10 μM) was activated by incubation in TBS with 0.1% PEG and 5 mM $CaCl_2$ at 37 °C overnight. The activation was confirmed through polyacrylamide gel electrophoretic analysis (*26*). Activated protein concentrations were determined by antithrombin III plus heparin active site titration (*29*).

Measurement of Factor VIIa−Tissue Factor Binding by Amidolytic Activity and factor X Activation. Tissue factor at various concentrations (0−400 nM) was distributed into 96 well plates in duplicate. Factor VIIa or activated mutant proteins were added to the wells at a final concentration of 10 nM. Conversely, varying concentrations of factor VIIa proteins (0−128 nM) were added to a fixed concentration of tissue factor at 10 nM. The reactions were conducted in TNP (Tris-HCl 20 mM, pH7.4, NaCl 100 mM, and PEG 0.1%) with 5 mM $CaCl_2$ and 2 mM CHAPS. The mixtures of factor VIIa/activated mutant chimera and tissue factor were incubated at ambient temperature for 1 h. Synthetic substrate S-2288 (2 mM) was added and mixed well to initiate the reaction. The absorbance at 405 nm was measured on a microplate reader (THERMOmax, Molecular Devices) for 30 min using SOFTmax software for MAXlineII microplate reader version 2.0. The initial rate of hydrolysis of S-2288 of each reaction was used for analysis. The data were analyzed by a quadratic solution to the equilibrium equation as described (*30*). K_d's and maximum cleavage rates were estimated using Kaleidograph software (Synergy Software, Reading PA). For binding tissue factor studies using factor X activation, tissue factor (1 nM) with different concentrations of enzymes was incubated at ambient temperature for 30−60 min. Factor X was added at a concentration of 0.4 μM, and the mixture was incubated at ambient temperature for 10−15 min before stopping with EDTA solution. The concentration of factor Xa generated was determined using S-2222 (2mM final concentration).

Kinetics of Factor VIIa cleavage of S-2288. For kinetic studies, factor VIIa or activated mutant proteins (10 nM) were incubated with 100−125 nM tissue factor at room temperature for 30−60 min in the buffer described above. The same volume of synthetic substrate S-2288 (0−2 mM final concentration) was added to initiate the reaction. In data analyses to determine k_{cat} for these experiments and those with factor X as substrate, the concentrations of factor VIIa−tissue factor complex, $FVII_{(IXegf1)}a$−tissue factor complex, or K79Ra-tissue factor complex were calculated using K_d values deterimined in the amidolytic assay or in the factor Xa formation assay (Table 1).

Measurement of Kinetics of Factor X Acitivation. Factor VIIa or activated mutant enzyme (1 nM) was incubated with 100−125 nM tissue factor in TNP buffer with 5 mM $CaCl_2$ and 2 mM CHAPS at ambient temperature for 30−60 min. Then factor X at concentrations ranging from 0 to 20 μM final concentration was added and incubated at ambient temperature for 10−15 min before adding 5 mM EDTA to stop the reaction. In control experiments the rates of factor Xa generation were linear for at least 20 min. Factor Xa concentration was measured by the addition of 2 mM final concentration of synthetic substrate S-2222. Control experiments with factor X and tissue factor alone showed no significant contamination of factor VII or factor VIIa in the factor X preparation. For factor X activation in the absence of tissue factor, due to the slow reaction rate, enzyme (10 nM) was incubated with varying concentrations of factor X for 30 min. Then the factor Xa concentration was measured using S-2222. Velocities were converted to rates of factor Xa generation on the basis of a standard curve of factor Xa activity. These rates were then analyzed using nonlinear least-squares analyses to determine K_m and k_{cat}. Analyses were performed using Kaleidograph.

Molecular Modeling Analysis. Alignment of the egf1 domains of factors VIIa and IXa was performed using SYBYL Molecular Modeling Package version 6.4 (Tripos Associates, Inc, St. Louis, MO) on an SGI Octane workstation (Silicon Graphics, Mountain View, CA). The X-ray crystallographic coordinates of the human factor VIIa-tissue factor complex (*1*) and porcine factor IXa (*31*) were employed to build the alignment model of the egf1 domains of factor VIIa and factor IXa, and the weighted root-mean-square distance was calculated by the software. The comparison was of residues 50−82 (factor VII numbering).

RESULTS

Competitive Tissue Factor Binding Studies of Wild-type and Mutant Factor VIIa. The major function of the egf1 domain of factor VII is binding to tissue factor. In competition binding studies using ^{125}I-labeled factor VIIa, factor

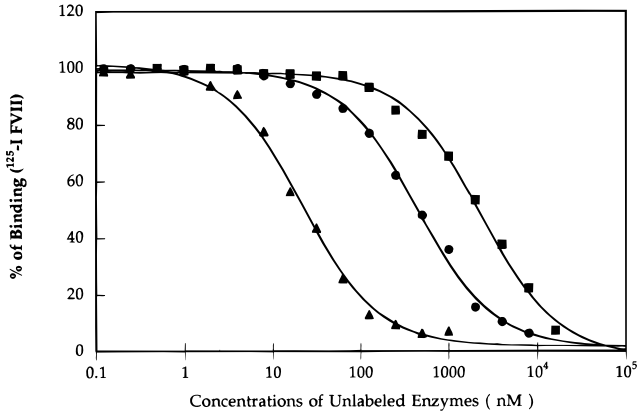

FIGURE 1: Competitive tissue factor binding studies of factor VIIa, factor VII$_{(IXegf1)}$a, and K79Ra. Microtiter plates were coated with tissue factor as described in Expreimental Procedures. Factor VIIa or one of the mutant proteins at the indicated concentrations (TBS with Ca^{2+}, Mg^{2+}, and 0.3% ovalbumin) and^{125}I-factor VIIa (5.0 nM) were added to duplicate wells. The plate was incubated at ambient temprature for 2 h. After the wells were washed 3 times with TBS/Ca^{2+} Mg^{2+} buffer, the radioactivity in each well was determined in a gamma counter (1275 Minigamma, LKB): ▲ represents wild-type factor VIIa, ● K79Ra, and ■ factor VII$_{(IXegf1)}$a. The results are given as a percentage of the maximal binding of ^{125}I-factor VIIa. Each point is the average of 3−5 separate experiments, and the standard error of each point is less than 8%. The binding constants were determined using MKMODEL (Biosoft, Ferguson MO).

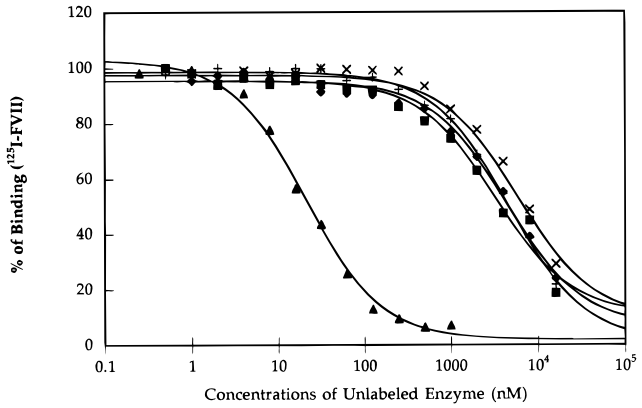

FIGURE 2: Competitive tissue factor binding studies with factor VIIa and factor VIIa mutants. Studies were performed as described in the legend for Figure 1: ▲ represents factor VIIa, ◆ E51Aa, + FG74−75PAa, ■ factor VII$_{(IXegf1)}$a, × D64Qa.

VII$_{(IXegf1)}$a's K_d for immobilized tissue factor was 60-fold higher than that of wild-type factor VIIa (Figure 1, Table 1). Residues Q64 and R79 have been implicated in tissue factor binding (*1, 13, 21*). The mutant K79Ra partially restored tissue factor binding affinity, and its K_d was only 12-fold higher than that of wild-type factor VIIa (Figure 1, Table 1). Surprisingly, D64Q exhibited no change in tissue factor binding. The other mutant proteins E51Aa and FG74−75PAa, which are not involved in tissue factor binding, had affinities similar to that of the parent enzyme, factor VII$_{(IXegf1)}$a (Figure 2).

Measurement of Factor VIIa−Tissue Factor Binding by Amidolytic Activity and Factor X Activation. The factor-VIIa−tissue factor binding affinity can also be measured employing functional assays, where the rate increase with increasing factor VIIa or tissue factor concentrations reflects formation of the complex (*30*). We studied factor VIIa, factor

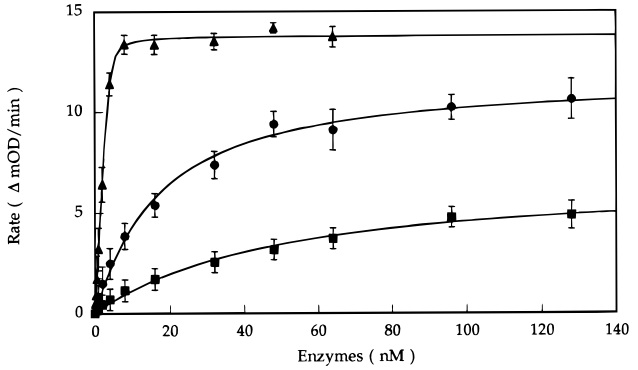

FIGURE 3: Dependence of factor VIIa−tissue factor complex amidolytic activity on factor VIIa concentration. Factor VIIa, factor VII$_{(IXegf1)}$a, or K79Ra (0−128 nM) was mixed with tissue factor (10 nM) in a 96 well microplate. The mixture was incubated for 60 min. Substrate (S-2288, 2 mM final concentration) was added and the reaction followed (absorbance at 405 nm) for 30 min: ▲ represents the data for Factor VIIa, ■ factor VII$_{(IXegf1)}$a, and ● K79Ra.

VII$_{(IXegf1)}$a, and K79Ra using either a fixed concentration of tissue factor with various enzyme concentrations (Figure 3 and Table 1) or fixed enzyme with various tissue factor concentrations (Table 1). In the fixed tissue factor experiment using amidolytic activity as the measure of complex formation, factor VII$_{(IXegf1)}$a's and K79Ra's K_d's were 200- and 60-fold higher than that of wild-type factor VIIa, respectively (Table 1). When we measured complex formation between factor VIIa and tissue factor using factor X activation, factor VII$_{(IXegf1)}$a and K79Ra had 90 and 18.5-fold higher K_d's than that for wild-type factor VIIa, respectively (Table 1). When we kept factor VIIa or the mutant enzyme concentration constant and varied tissue factor, using amidolytic activity to measure complex formation, the K_d's were 93- and 11-fold higher for factor VII$_{(IXegf1)}$a and K79Ra, respectively (Table 1).

Although the determined K_d's were different between the functional assays and the competitive binding assys, the results showed a similar relationship among the K_d's and $\Delta\Delta G$'s of the three enzymes (Table 1). The total $\Delta\Delta G$ for replacement of the egf1 domain ranged from 2.41 to 3.12 kcal/mol, and the mutation to arginine at residue 79 decreased the $\Delta\Delta G$ by 0.7−0.95 kcal/mol. The different K_d's determined using different methods are consistent with previously reported results (*5, 13, 30, 32*).

Kinetics Studies. To address the question of whether the egf1 domain plays a role in the catalytic activity of factor VIIa, we performed kinetic analyses using a small peptide (S-2288) or factor X as substrates for factor VIIa and the mutant enzymes. The results (Figure 4 and Table 2), with S-2288 in the presence of tissue factor, showed a 2.2-fold reduction of k_{cat}/K_m (catalytic efficiency) for factor VII$_{(IXegf1)}$a, while the catalytic efficiency of K79Ra was decreased 1.8-fold relative to factor VIIa. The difference was due to a lower k_{cat}, which was 15.3 for factor VII$_{(IXegf1)}$a, 25.7 for K79Ra, and 41.7 for wild-type FVIIa. The K_m's were similar (Figure 4). Without tissue factor, factors VIIa, VII$_{(IXegf1)}$a and K79Ra, had similar k_{cat} and K_m values toward S-2288 (Table 2).

With factor X as substrate, the k_{cat} and K_m values for the three enzymes were similar in the absence of tissue factor (Table 2 and Figure 5). The catalytic activities (k_{cat}) were 95% and 70% of wild-type for K79Ra and factor VII$_{(IXegf1)}$a,

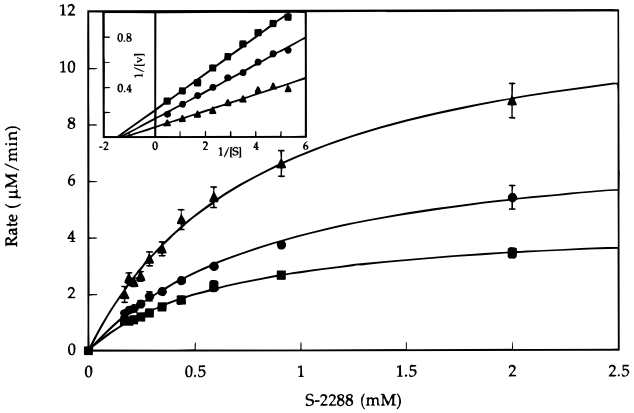

FIGURE 4: Kinetics of factor VIIa−tissue factor complex cleavage of S-2288. Factor VIIa, factor $VII_{(IXegf1)}a$, or K79Ra (10 nM) was incubated with tissue factor (100−125 nM, 30−60 min, at ambient temperature) in a 96 well microplate. Activity was measured with various concentrations of S-2288 (0.1−2 mM). Experiments were performed 3−6 times in duplicate. Factor VIIa ▲, factor $VII_{(IXegf1)}a$ ■, K79Ra ●.

respectively. In the presence of tissue factor, the three enzymes' k_{cat}'s for factor X activation were also similar (Table 2). The K_m*'s* for factor VIIa, factor $VII_{(IXegf1)}a$, and K79Ra, however, were significantly different when these enzymes were bound to tissue factor (Table 2 and Figure 6). We found 11 and 3-fold differences in K_m between wild-type factor VIIa and factor $VII_{(IXegf1)}a$ and K79Ra, respectively. Factor $VII_{(IXegf1)}a$ and K79Ra, therefore, had catalytic efficiencies of only 9% and 33% of that of wild-type factor VIIa, respectively. To summarize, the kinetic behavior of factors VIIa, $VII_{(IXegf1)}a$, and K79Ra were similar in the absence of tissue factor. The kinetic differences, especially the effect on K_m in factor X activation, were manifested only after binding to tissue factor. Results for other mutants with both S-2288 and factor X as substrates were similar to those of factor $VII_{(IXegf1)}a$ and are not shown.

DISCUSSION

We and others have demonstrated the importance of factor VII's egf1 domain for tissue factor binding (*3*−*5*). The crystal structure of the complex confirms the central role of egf1 in the interaction (*1*) but discloses little about the functional significance of the individual contact residues or the domain as a whole. For example, do egf1 residues participate in tissue factor binding only, or do residues in the egf1 domain have an effect on factor VIIa's catalytic activity either directly through substrate binding, or through an allosteric effect on the active site or macromolecular substrate-binding site? Studies with factor VIIa point mutations in the gla-egf1 domain high-affinity calcium-binding site show that this site is important for catalysis whether the mutant factor VIIa is bound to tissue factor or not *(14*, *15*). Likewise, mutations of tissue factor residues contacting the egf1 domain cause a decrease in activity relative to the wild-type tissue factor−factor VIIa complex (*18*). However, the results from alanine scanning mutagenesis showed that the egf1 domain seems to be involved only in tissue factor binding (*13*). In our previous study we found that the two chimeric molecules, factor $VII_{(IXgla)}a$ and factor $VII_{(IXegf1)}a$, had significant, but reduced, clotting activity (*5*). On the basis of estimates using our derived K_d's we concluded that the decreased clotting activity could be attributed to the reduction in affinity for tissue factor and not to a loss in catalytic efficiency of the enzymes. So whether the egf1 domain plays a major role in cofactor activity (the activity increase caused by tissue factor binding) is still unclear.

We designed this study to further address egf1's role in cofactor activity. Our hypothesis was that replacement of factor VIIa's egf1 domain with the corresponding domain in factor IX should eliminate the factor VIIa egf1-specific functions and allow investigation of the effect on tissue factor-dependent and -independent activity. In turn changing a residue in the egf1 domain of factor $VII_{(IXegf1)}a$ back to the residue in factor VIIa might cause an increase in binding and/or catalytic activity. The chimera's overall tertiary structure is apparently not disrupted since substitution of factor IX's egf1 domain causes only a 3 kcal/mol binding energy change. Point (alanine) mutations in factor VII's egf1 domain show at least a total of 5 kcal/mol binding contribution by this domain (*13*). This result also suggests that, since certain tissue factor contact residues in factor VII (e.g. C70, C72, E77, and G78) are conserved in factor IX, these residues might participate in tissue factor binding in the chimeric protein. This conclusion is supported by the model shown in Figure 7, which depicts a comparison of the polypeptide backbone structures of factors VIIa and IXa. The factor VIIa structure comes from the crystal structure of the factor VIIa-tissue factor complex (*1*), but there is evidence that this structure differs little from that of free factor VIIa (*33*). The factor IXa structure is that of porcine factor IXa (*31*), which bears 89% identity with human factor IX from residue 46 to residue 82. The residues in contact with tissue factor are conserved in both species. The result of the comparison showed that the weighted root-mean-square distance deviation between the two peptide backbones is 0.91 Å. The similarity between the two structures indicates the chimera's overall tertiary structure is intact and suggests that residues identical in factor IXa to those in factor VIIa may be available for tissue factor binding.

Major residues different in factors IX and VII egf1 domains, that contact tissue factor, are 64, 69, 71, and 79 (Figure 7). Residue 69 is I in factor VII and E in factor IX. Residue 71 is F in factor VII and W in factor IX. Along with the aliphatic part of R79's side chain, I69 and F71 form a hydrophobic core that is one of the major interaction sites of factor VIIa with tissue factor. Therefore, these changes, from a nonpolar to a negatively charged group (I to E) and to a larger nonpolar group (F to W) on substitution of factor IX's egf1 in factor VII, should cause a significant change in the abilities of the residues to bind tissue factor. R79 is also involved in at least one other hydrophobic interaction and two hydrogen bonds. On the other hand, since the backbone structures of factor IXa and factor VIIa are quite similar, the side chains of the residues are in similar positions in factors VII and IX and are, therefore, available to make contact with tissue factor (Figure 7). This is consistent with our results with K79Ra showing that it has increased affinity for tissue factor. In addition, on the basis of these observations, one might predict that changing residues 69 and 71 in K79R to those in factor VII might regain most of the tissue factor binding affinity.

Perhaps surprisingly, D64Q which is a tissue factor binding residue, does not have increased binding or activity. Q64 is

Table 2: Kinetic Constants of Factor VIIa and Its Chimeras with S-2288 and Factor X[a]

substrate	TF	enzyme	k_{cat} (s^{-1})	K_m	k_{cat}/K_m (M^{-1} s^{-1})
S-2288	+	factor VIIa	41.7 ± 4.0	0.81 ± 0.1 mM	5.15×10^4
		factor $VII_{(IXegf1)}a$	15.3 ± 0.3	0.65 ± 0.1 mM	2.35×10^4
		K79Ra	25.7 ± 4.0	0.89 ± 0.1 mM	2.89×10^4
	−	facotr VIIa	2.26 ± 0.234	1.71 ± 0.1 mM	1.32×10^3
		factor $VII_{(IXegf1)}a$	1.83 ± 0.07	1.73 ± 0.2 mM	1.06×10^3
		K79R	1.81 ± 0.10	1.47 ± 0.3 mM	1.23×10^3
factor X	+	factor VIIa	0.39 ± 0.01	0.87 ± 0.44 μM	4.48×10^5
		factor $VII_{(IXegf1)}a$	0.40 ± 0.01	9.55 ± 0.95 μM	0.42×10^5
		K79Ra	0.39 ± 0.03	2.68 ± 1.20 μM	1.46×10^5
	−	factor VIIa	2.0×10^{-4} [b]	0.57 ± 0.07 μM	3.51×10^2
		factor $VII_{(IXegf1)}a$	1.4×10^{-4}	0.78 ± 0.14 μM	1.79×10^2
		K79Ra	1.9×10^{-4}	0.79 ± 0.09 μM	2.41×10^2

[a] The results represent 4−5 experiments. [b] The SE values for all three enzymes are less than 2.4×10^{-5}.

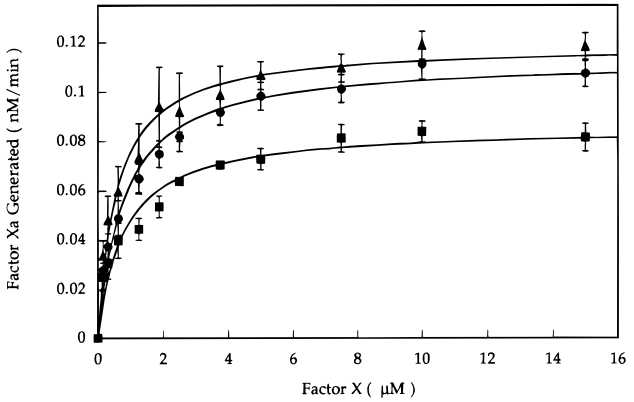

FIGURE 5: Kinetics of factor VIIa and mutants cleavage of factor X without tissue factor. Factor VIIa, factor $VII_{(IXegf1)}a$, or K79Ra (10 nM) was incubated with various concentrations of factor X (0−15 μM) for 30 min. Factor Xa generation was determined as described in Experimental Procedures. Experiments were performed 3 times: factor VIIa ▲, factor $VII_{(IXegf1)}a$ ■, K79Ra ●.

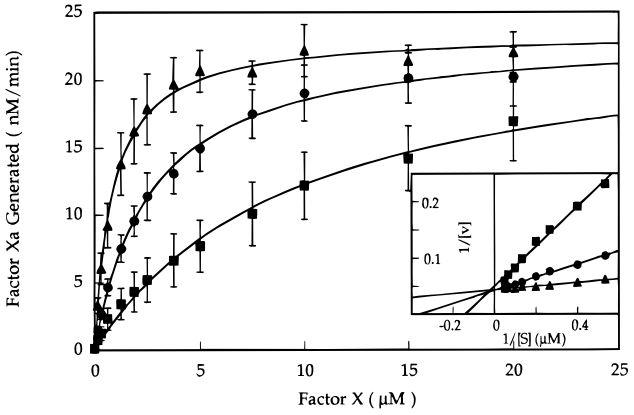

FIGURE 6: Kinetics of factor VIIa−tissue factor complex cleavage of factor X. Factor VIIa, factor $VII_{(IXegf1)}a$, or K79Ra (1 nM) was incubated with tissue factor (100−125 nM, 40−60 min). Factor X was added (0−20 μM) and the mixture incubated for 10−15 min at room temperature. EDTA was added to stop the reaction and factor Xa activity measured using S-2222 (2 mM final concentration). The results represent 6 separate experiments: factor VIIa ▲, factor $VII_{(IXegf1)}a$ ■, K79Ra ●.

involved in both calcium coordination and tissue factor binding. The calcium-binding residues in the egf1 domain are identical in factor VII and factor IX, except at the position 64, which is glutamine in factor VII and aspartic acid in factor IX (65 in factor IX numbering sequence) (*1, 34*). This

FIGURE 7: Structure comparison of the egf1 domains of factors VIIa and IXa. Modeling was performed as described in Experimental Procedures. The backbone of factor VIIa is in blue, and that of factor IX is in red. The side chains of four factor VII residues (factor VII numbering) that bind tissue factor are also shown in blue. The corresponding factor IX residues are in red (factor IX numbering). Residue 50 (C) is the N-terminus and residue 82 (E) is the C-terminus (factor VII numbering). The root-mean-square deviation for the backbone alignment of factors VII and IX is 0.91 Å.

suggests that the calcium-binding site in D64Q is intact. The effect caused by Q64's tissue factor contact may be too subtle to be detected or D64 may participate in tissue factor binding. This might explain why D64Q did not have increased function.

Our kinetic results show small (within 2-fold) differences in the catalytic efficiencies for factor X cleavage (k_{cat}/K_m) by factor VIIa, factor $VII_{(IXegf1)}a$, and K79Ra without tissue factor and essentially no difference for the peptide substrate. Similarly, with tissue factor the mutant enzymes' catalytic efficiencies for the small peptide were only slightly different (~2-fold) than wild-type factor VIIa, although factor $VII_{(IXegf1)}a$ had a 2.7-fold lower k_{cat}. On the other hand, in the presence of tissue factor, k_{cat}/K_m for factor X cleavage is inversely proportional to the enzyme's K_d, and is 9% and 33% of that for wild-type for factor $VII_{(IXegf1)}a$ and K79Ra, respectively. The difference in k_{cat}/K_m is due primarily to factor VIIa's lower K_m since its K_m is 0.87 μM, while those for factor

$VII_{(IXegf1)}a$ and K79Ra are 9.55 and 2.68 μM, respectively. An alternative way of looking at these data is to compare the relative increases in activity for the 3 enzymes when bound to tissue factor. With factor X as substrate the increases are 1276-, 606-, and 235-fold for factor VIIa, K79Ra, and factor $VII_{(IXegf1)}a$, respectively. This means that factor $VII_{(IXegf1)}a$ is essentially normal without tissue factor, but when bound to tissue factor, it yields 18% of the activity increase of that observed with factor VIIa. Factor X's interaction with factor VIIa−tissue factor depends on factor VIIa's gla domain (*10*, *12*). Thus, one explanation for these results would be that the gla-egf1 alignment is disrupted in the chimera and that is why it has a different K_m. While we observe no effect on factor X activation without tissue factor, it is still possible that there is an effect on the gla-egf1 orientation in the complex with tissue factor. Our results do not rule out this possibility. However, previous studies have concluded that disruption of the calcium-binding site in factor VIIa's egf1 domain causes misalignment of the gla and egf1 domains (*14*, *15*, *33*). This misalignment causes decreased activity versus factor X in the presence or absence of tissue factor and phospholipids. Since the defect we describe is tissue factor-dependent, we suggest that it is not due to the misalignment of the gla-egf1 domain.

Our results might be explained by a factor X-binding site in the egf1 domain, which is either carried by the egf1 domain itself or created in the factor VIIa−tissue factor complex. For the following reasons a binding site in egf1 seems unlikely: (1) without tissue factor all the enzymes have similar K_m's, and (2) there is no change in wild-type factor VIIa's K_m when it binds tissue factor. In addition, factor IX's egf1 in the chimeric molecule is probably not a factor X-binding site since earlier results indicate that factor IX with factor X's egf1 has normal activity (*35*).

Alternatively, our results may be explained by a model in which a site not in egf1 is affected by misalignment of tissue factor due to the substituted factor IX domain. In addition to contacts in the egf1 domain, the factor VIIa−tissue factor crystal structure shows contacts between tissue factor's N-terminal domain and factor VIIa's proteolytic domain. It appears that tissue factor contact with these proteolytic domain residues induces a conformational change or stabilizes an existing conformation of factor VIIa that has increased catalytic activity relative to free factor VIIa (*36*−*40*). When factor IX's egf1 domain is substituted into factor VIIa, perhaps tissue factor is misaligned with the proteolytic domain residues. This could cause a smaller than normal catalytic efficiency increase due to effects on the active site and/or a macromolecular substrate-binding site. Other studies have been interpreted to mean that two residues in factor VIIa's proteolytic domain, when contacting tissue factor, control allosteric effects on the primary binding site/active site (M306) and the macromolecular substrate-binding site (R277) *(13, 37, 41)*. If this interpretation is correct, then disruption of the tissue factor interaction with R277 may explain our results with factor X activation. Interestingly, a recent study using a soluble tissue factor mutated at two residues that contact factor VIIa's egf1 domain (K20A,-D58W) showed that this change, in contrast to our results, produces a factor VIIa−tissue factor complex with a decreased k_{cat} for factor X (*18*). However, its binding affinity with factor VIIa is normal. The previous study was performed with soluble tissue factor and phospholipid vesicles. When we analyzed our chimera with relipidated tissue factor, the relationships between k_{cat}'s and K_m'*s* were similar to those with detergent solubilized tissue factor which we used in most of our studies. Residues K20 and D58 contact residues I69, C70, and G78 in factor VIIa, the latter two of which are conserved in factor IX. This suggests that these contacts may affect factor VIIa differently than our chimera and, additionally, that tissue factor binding to egf1 may exercise strict control over factor VIIa−tissue factor complex activity through cofactor alignment with more than one site on factor VIIa's proteolytic domain. According to this model, factor VIIa's egf1 domain's primary role is binding tissue factor, but it is also necessary for proper contact between tissue factor and the proteolytic domain which is essential for optimal cofactor activity.

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