Replacing the First Epidermal Growth Factor-like Domain of Factor IX with That of Factor VII Enhances Activity In Vitro and in Canine Hemophilia B

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Abstract

Using the techniques of molecular biology, we made a chimeric Factor IX by replacing the first epidermal growth factor–like domain with that of Factor VII. The resulting recombinant chimeric molecule, Factor $IX_{VIIEGF1}$, had at least a twofold increase in functional activity in the one-stage clotting assay when compared to recombinant wild-type Factor IX. The increased activity was not due to contamination with activated Factor IX, nor was it due to an increased rate of activation by Factor VIIa-tissue factor or by Factor XIa. Rather, the increased activity was due to a higher affinity of Factor $IX_{VIIEGF1}$ for Factor VIIIa with a K_d for Factor VIIIa about one order of magnitude lower than that of recombinant wild-type Factor IXa. In addition, results from animal studies show that this chimeric Factor IX, when infused into a dog with hemophilia B, exhibits a greater than threefold increase in clotting activity, and has a biological half-life equivalent to recombinant wild-type Factor IX. (*J. Clin. Invest.* 1997. 100:886–892.) Key words: Factor IX • chimeric proteins • sequence homology • blood coagulation • hemophilia B

Introduction

Coagulation Factor IX is a single-chain serine protease zymogen that can be activated to a two-chain serine protease (Factor IXa) by release of an activation peptide (1). The catalytic domain of Factor IXa is located in the heavy chain. The light chain consists of four domains which are, from the NH_2 terminus, the Gla (γ-carboxyglutamic acid) domain, the aromatic stack, and the two EGF-like domains.

In the coagulation cascade, Factor IXa activates Factor X to Factor Xa, leading to thrombin generation. To activate Factor X, Factor IXa requires activated platelets, Ca^{++}, and its cofactor, Factor VIIIa, for optimal activity (2–4). Factor VIIIa is a calcium-dependent heterotrimer that consists of two A domains (A1, A2), a third A domain (A3), and two C domains linked by a calcium bridge. Factor VIII circulates as an inactive cofactor (tightly bound to von Willebrand factor), and is activated by serine proteases like thrombin (5, 6) or Factor Xa (7).

Recombinant DNA techniques have led to the production of several chimeric Factor IX molecules (8–12). A chimeric Factor IX molecule containing the EGF-1 domain of Factor X or protein C was reported to have near normal or reduced in vitro clotting activity, respectively (8, 11). Here we report another observation, namely a chimeric Factor IX molecule containing the EGF-1 domain from Factor VII, that possesses increased clotting activity both in vitro and in vivo. The enhanced activity is due to an increased affinity of the chimeric Factor IX for Factor VIIIa, suggesting that the EGF-1–like domain is important in the function of the tenase complex.

Methods

Materials. Plasma Factor XIa was purchased from Enzyme Research Laboratories (South Bend, IN). Factor VIII was purified from Profilate (Alpha Therapeutic Corp., Los Angeles, CA) by gel filtration on Sepharose CL2B. Thrombin and antithrombin III were purified as previously described (13, 14). Factor VIII-, IX-, and X-deficient plasmas were obtained from HRF, Inc. (Raleigh, NC). Kontact aPTT reagent was purchased from Pacific Hemostasis (Huntersville, NC). Polylysine (poly-L-lysine, M_r 102,000) was purchased from Sigma Chemical Co., (St. Louis, MO). Phosphatidylcholine (1-palmitoyl-2-oleoyl lecithin) and phosphatidylserine (disodium salt) were obtained from Avanti Polar Lipids (Alabaster, AL). Hirudin was purchased from Accurate Chemical and Science Corp. (Westbury, NY). Spectrozyme fXa (methoxycarbonyl-D-cyclohexylglycyl-glycylarginine-*p*-nitroanilide acetate) was purchased from American Diagnostica, Inc. (Greenwich, CT). The monoclonal antibody, AHIX-5041, that recognizes human Factor IX heavy chain, was obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Rabbit anti–human Factor IX antibody was purchased from Dako Corp. (Carpinteria, CA). Biotin-coupled goat anti–rabbit antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Chimeric DNA construction, expression, and purification of the recombinant proteins. The chimeric and wild-type Factor IX DNA construct, protein expression, purification, and characterizations were as described previously (12). In brief, both normal Factor IX cDNA and chimeric Factor IX cDNA were cloned respectively into an expression vector pCMV5 (15) and transfected into a human kidney cell line 293 (ATCC CRL 1573) together with pSV2neo by the calcium phosphate coprecipitation method (16). The recombinant protein was expressed in serum-free media and purified by a pseudo-affinity chromatographic method using Fast Flow Q-Sepharose and elution with a calcium gradient, followed by a NaCl gradient (17, 18).

Phospholipid vesicles. Large unilamellar phospholipid vesicles containing a 30/70 molar ratio of phosphatidylserine to phosphatidylcholine were prepared by extrusion using a previously described procedure and stored at 4°C under argon (19).

Portions of this study were presented in abstract form at the XVth Congress of the International Society on Thrombosis and Haemostasis in June 1995 at Jerusalem, Israel, at the American Society of Hematology Meeting in December 1995 at Seattle, WA, and at the XXII International Congress of the World Federation of Hemophilia in June 1996 at Dublin, Ireland.

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Active site titration. The active site concentration of each activated Factor IX was determined as previously described by titration with antithrombin III (20).

Clotting assays. The one-stage clotting assay for Factor IX was performed according to the method previously described (21). In brief, a standard curve was constructed by performing partial thromboplastin times on serial dilutions of normal human plasma into human hemophilic B plasma. The clotting times of similar dilutions of test plasmas were compared to the standard curve, which was defined as 100%.

Activation of Factor IX. Activation of Factor IX by Factor XIa or Factor VIIa-tissue factor complex was performed as described by Zhong et al. (11).

Binding of Factor IXa to Factor VIIIa. To determine the affinity of both normal Factor IXa and chimeric Factor IXa for Factor VIIIa, we performed the analysis as follows: in a 100-μl reaction, the concentrations of each component were: 0.2 nM Factor VIII; 200 nM Factor X; 5 mM $CaCl_2$; 100 μM phospholipid; 0.5 mM Spectrozyme fXa; and Factor IXa ranging from 0.02 to 4 nM. Factor VIII was incubated with thrombin at room temperature for 5 min. Hirudin in molar excess over thrombin and phospholipid was then added to the reaction for another 5 min at room temperature. 25 μl of the Factor VIIIa, thrombin, hirudin, and phospholipid solution was aliquoted to a microtiter well that contains 25 μl of Factor IXa at various concentrations. Factor X, $CaCl_2$, and Spectrozyme fXa were then added to each well to initiate the reaction in a final volume of 100 μl. The absorbance change at 405 nm was monitored. The data were fitted to a second order polynomial $a0 + a1*t + a2*t^2$. The a2 coefficient gives the rate of change in the A405, and is equal to kSpec*kX*[IXa/VIIIa]/2 where kSpec is the rate that Factor Xa cleaves Spectrozyme fXa. The Factor Xa generation rate was plotted against the concentration of Factor IXa, and the affinity of Factor IXa for Factor VIIIa was determined by fitting the data to the equation:

$$v = V_{max}\frac{[\text{FIXa/VIIIa}]}{[\text{FVIIIa}]_{total}}$$

where [FIXa/VIIIa] is determined as described in Larson et al. (22) in equations 4–10 in a minor modification of the equations reported by Krishnaswamy (23).

Dansyl Glu-Gly-Arg Factor IXa (DEGR-IXa)[1] inhibition of intrinsic Xase activity. Both wild-type Factor IXa and chimeric Factor IXa were inhibited by incubating Factor IXa with dansyl Glu-Gly-Arg chloromethyl ketone (DEGR-CK) for 4 h. Free DEGR-CK was removed from the proteins by exhaustive dialysis. Both inhibited forms were used to show that DEGR-chimeric IXa was more effective than wild-type DEGR-IXa in inhibiting intrinsic Xase activity (Factor IXa/Factor VIIIa activity). Final concentrations of each component were as follows: 0.05 nM Factor VIIIa, 1 nM Factor IXa, 200 nM Factor X, 5 mM $CaCl_2$, 100 μM phospholipid, 0.5 mM Spectrozyme fXa, and DEGR-Factor IXa ranging from 0.01–20 nM. Factor VIII was activated as described above, and was added to a mixture of Factor IXa and DEGR-IXa. Factor X was added, and kinetic measurements were made as described above. Factor Xa generation was plotted against the concentration of DEGR-IXa, and the K_i for Factor VIIIa determined by fitting the data to the equation:

$$\text{relative rate} = \frac{K_i*(K_d + [\text{IXa}])}{K_i*K_d + K_i*[\text{IXa}] + K_d*[\text{DEGR-IXa}]}$$

This equation assumes that the concentration of the complex of Factor IXa with Factor VIIIa is small relative to the concentration of Factor IXa, and that the concentration of the complex of DEGR-IXa with Factor VIIIa is small relative to the concentration of DEGR-IXa.

Kinetics of Factor X activation by Factor IXa with and without Factor VIIIa. The K_m and k_{cat} of the Factor IXa/Factor VIIIa complex for Factor X were determined by measuring the Factor Xa generation in the reaction that contained 0.2 nM Factor VIII, 0.1 nM Factor IXa, 50 μM phospholipid, 5 mM $CaCl_2$; 0.5 mM Spectrozyme fXa, and Factor X ranging from 1–400 nM. The assay was performed as follows: Factor VIII was incubated with thrombin at room temperature for 5 min before adding hirudin, phospholipid, Factor IXa, and Spectrozyme fXa. 50 μl of this solution was aliquoted to a microtiter well, and then Factor X at various concentrations was added to each well. The changes in 405 nm were monitored and converted to the Xa generation rate as described above. To monitor Factor Xa generation in the absence of Factor VIIIa, Factor VIIIa was omitted from the reaction above, and the Factor IXa concentration was increased to 10 nM. K_m and V_{max} were determined by fitting data for the Factor Xa generation rate vs. Factor X concentration to the equation:

$$v = V_{max}\frac{[\text{FX}]}{K_m + [\text{FX}]}.$$

Animal studies. The hemophilia B dog came from the inbred colony maintained since 1966 at the Francis Owen Blood Research Laboratory at the University of North Carolina in Chapel Hill. The animal studies were performed in accordance with the institutional guidelines at the University of North Carolina. A hemophilia B dog was infused with a bolus of Factor $IX_{VIIEGF1}$ at a dose of 0.08 mg/kg. Blood samples were taken at 5, 15, and 30 min, and at 1, 2, 4, 8, 12, 22, 24, 46, 48, 70, and 72 h after injection. Recombinant wild-type (normal) Factor IX (0.08 mg/kg) was also infused into this dog 72 h after infusion of the chimeric Factor IX. Samples were taken from this dog at the same time points as for Factor $IX_{VIIEGF1}$. This time was adopted based on our previous experience in evaluating the functional activity and biological half-life of human Factor IX preparations in canine hemophilia B animal models. As we have previously shown on numerous occasions, no measurable canine antibodies against human Factor IX could be detected in the dog during the time period of the experiments using human Factor IX. Whole blood clotting time was measured at 15 min, and 4, 8, 24, and 48 h after each injection. ELISA and the one-stage clotting assay were used to determine the Factor IX antigen and activity levels of each sample.

Results

Factor IX concentration and characterization. Expression, purification, and characterization of recombinant proteins were as described in our previous publication (12). The chimeric and wild-type Factor IX had 8.8 and 11.1 Gla residues per molecule, respectively. Neither the wild-type nor the chimeric Factor IX bound to tissue factor, even though the EGF1 domain of Factor VII has been shown to be in direct contact with tissue factor (24). Protein concentrations were determined by the absorbance at 280 nm ($\epsilon 1\% = 13.3$) and by Bradford assay. Both showed that Factor $IX_{VIIEGF1}$ and wild-type Factor IX were equivalent as determined by using human plasma Factor IX as a standard. The protein concentrations were determined on at least ten different occasions. After full activation, the antithrombin III–titrated active site concentration for wild-type Factor IX and Factor $IX_{VIIEGF1}$ revealed that both concentrations were identical.

In vitro clotting activities. The in vitro coagulation activities of the chimeric factor $IX_{VIIEGF1}$ as compared to wild-type recombinant Factor IX molecules are listed in Fig. 1. The average activity of wild-type Factor IX zymogen was 92% of normal plasma Factor IX as measured by the one-stage assay. This

1. *Abbreviations used in this paper:* DEGR-IXa, dansyl Glu-Gly-Arg Factor IXa; DEGR-CK, dansyl Glu-Gly-Arg chloromethyl ketone.

Recombinant Proteins	Domain Compositions	Clotting Activity (%)	
		Zymogen	Activated
F IX_{normal}	Gla A EGF1 EGF2 AP Catalytic Region	100	100
F $IX_{VIIEGF1}$	Gla A EGF1 EGF2 AP Catalytic Region	210	172

Figure 1. Clotting activity of recombinant Factor IX. The domain structure of both Factor IX and Factor $IX_{VIIEGF1}$ are shown. The *filled region with white lettering* indicates that the domain has the sequence of Factor VII. Clotting activity for both the zymogen and activated forms was determined as described in Methods, and reported as a percent of the values seen for wild-type.

value is comparable to that obtained by others (8, 11). Interestingly, an equal concentration of the zymogen chimeric Factor IX had more than twofold higher activity than wild-type Factor IX. To show that the increased activity was not due to small amounts of activated Factor IX in the zymogen preparation, both the chimeric and wild-type preparations were fully activated by the addition of Factor XIa and calcium. Even when fully activated, chimeric Factor IXa still has twice the activity of the wild-type Factor IXa (Fig. 1). These data demonstrate that on a mole for mole basis, chimeric Factor IX had twice the clotting activity of the wild-type molecule, e.g., 0.63 μg/ml of the chimera had the same clotting activity as 1.25 μg/ml of the wild-type Factor IX.

To determine the mechanism responsible for the increased activity, the following possibilities were tested: (*a*) increased rate of activation by Factor XIa; (*b*) increased rate of activation rate by Factor VIIa tissue factor; (*c*) increased activation of Factor X independent of Factor VIIIa; (*d*) increased affinity for Factor VIIIa; and/or (*e*) increased catalytic activity toward Factor X when Factor VIIIa is present.

Factor XIa activation. Fig. 2 shows the activation pattern of both chimeric and wild-type Factor IX by Factor XIa. As can be seen on reduced SDS-PAGE, activation of chimeric and wild-type Factor IX occur at essentially the same rate, both being completely activated by 30 min under these conditions. These data suggest that the increased activity of the chimera is not due to an increased rate of activation by Factor XIa.

Factor VIIa-tissue factor activation. SDS-PAGE analysis of the activation of chimeric and wild-type Factor IX by Factor VIIa/tissue factor is shown in Fig. 3. The results indicate that the activation pattern of chimeric and wild-type Factor IX is essentially identical. From these data, it does not appear that differences in the rate of activation by Factor VIIa/tissue factor account for the differences in activity between wild-type and chimeric Factor IXa.

Factor Xa generation in the absence of Factor VIIIa. In a Factor Xa generation assay in which Factor VIIIa was omitted, the amount of Factor Xa generated by chimeric and wild-type Factor IXa was essentially the same (K_m of 147 nM with respect to Factor X for wild-type, and 149 nM for chimeric Factor IXa; data not shown). These data indicate that the increased activity of the chimera was not due to direct activation of Factor X independent of Factor VIIIa.

Factor IXa interaction with Factor VIIIa. The binding affinity of activated Factor IX for Factor VIIIa was determined as described in Methods. When the assay was controlled so that the concentrations of Factor VIIIa, phospholipid vesicles, Factor X, and calcium ions were constant, and the concentration of Factor IXa was varied, Factor Xa generation would reflect the interaction between Factor IXa and Factor VIIIa. The lower the concentration of Factor IXa required for Factor Xa generation to reach saturation, the tighter the binding of Factor IXa to Factor VIIIa. As shown in Fig. 4, activated Factor $IX_{VIIEGF1}$–Factor VIIIa complex activates more Factor X at

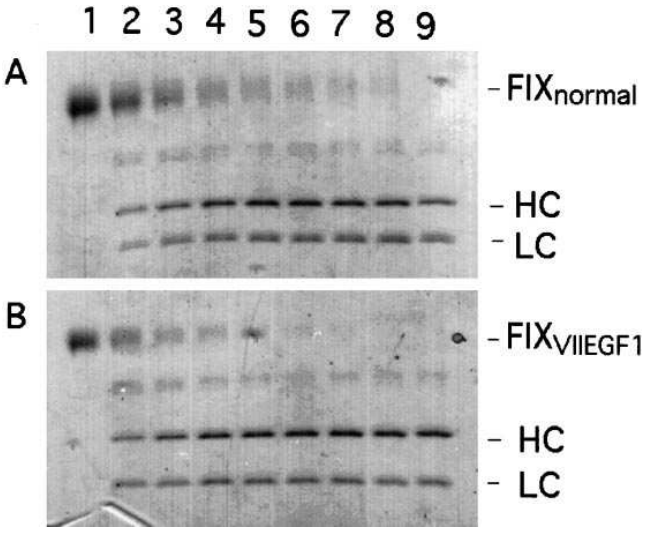

Figure 2. Activation of Factor IX by Factor XIa. Either normal Factor IX (*A*) or Factor $IX_{VIIEGF1}$ (*B*) was incubated with Factor XIa and calcium. At timed intervals, samples were removed for reduced SDS-PAGE on 10–27% acrylamide gels. Samples were visualized by Coomassie staining. Lanes *1–9* show time points at 0, 1, 2, 4, 8, 12, 16, 30, and 60 min, respectively.

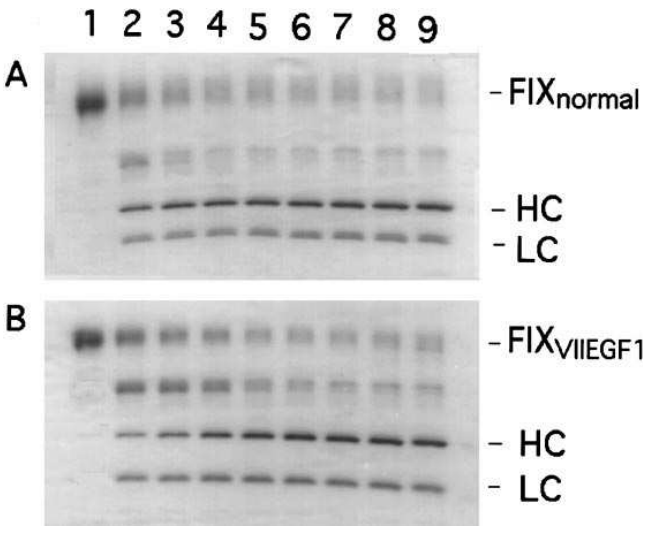

Figure 3. Activation of Factor IX by Factor VIIa/tissue factor. Either normal Factor IX (*A*) or Factor $IX_{VIIEGF1}$ (*B*) was incubated with tissue factor/Factor VIIa and calcium. At timed intervals, samples were removed for reduced SDS-PAGE on 10–27% acrylamide gels. Samples were visualized by Coomassie staining. Lanes *1–9* show time points at 0, 1, 2, 4, 8, 12, 16, 30, and 60 min, respectively.

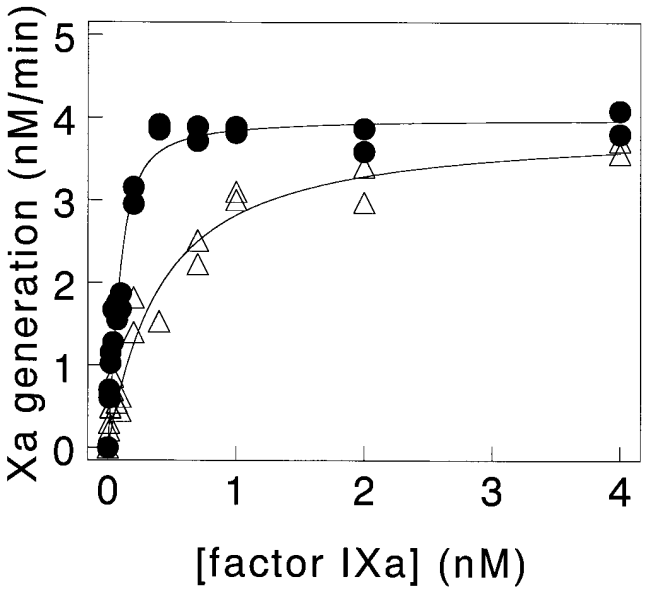

Figure 4. Factor IXa binding to Factor VIIIa. The rate of Factor X activation as a function of varied concentrations of normal Factor IX (*open triangles*) or Factor $IX_{VIIEGF1}$ (*closed circles*) with fixed concentrations of Factor VIIIa, phospholipid, and Factor X was assayed as described in Methods. Binding of Factor IXa to Factor VIIIa (K_d) was determined by fitting the data to the equations described in Larson et al. (22).

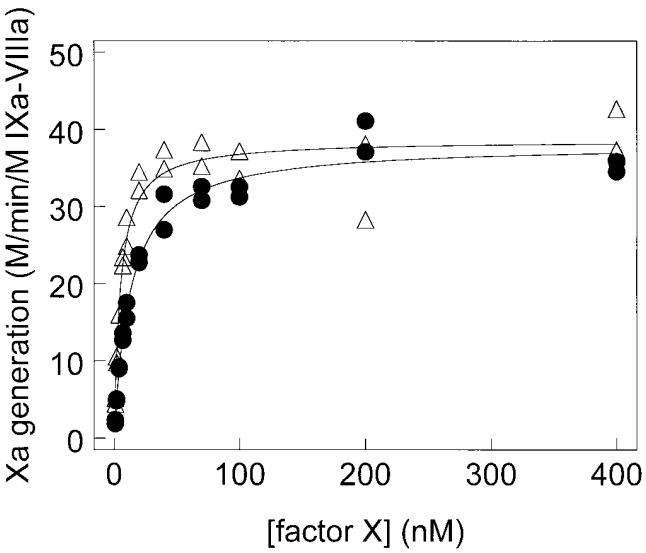

Figure 5. Factor IXa/VIIIa interaction with Factor X. The rate of Factor X activation with constant Factor IXa, Factor VIIIa, and phospholipid and varied Factor X concentrations was determined as described in Methods. *Open triangles* show the data for normal Factor IXa, and *closed circles* show the data for Factor $IXa_{VIIEGF1}$. The data is expressed as moles of Factor Xa per min per mole of the Factor IXa/VIIIa complex. The amount of Xase complex (which is different for chimeric and wild-type Factor IXa) was determined using the K_d values determined in Fig. 4 and shown in Table I using the equations described by Krishnaswamy (23) and Larson et al (22). The kinetic constants (K_m and k_{cat}) were calculated using the equation shown in Methods.

lower concentrations of Factor IXa than does wild-type Factor IXa–Factor VIIIa complex, though they both exhibit a similar V_{max}. The data were calculated according to the report of Krishnaswamy (23), and Larson et al. (22) to obtain the kinetic constants shown in Table I. These data show that the K_d of chimeric Factor IXa for Factor VIIIa is lower than the K_d for wild-type, indicating that the chimeric molecule has a higher affinity for Factor VIIIa than does the wild-type. Fig. 5 shows Factor X activation by both wild-type Factor IXa–Factor VIIIa and chimeric Factor IXa–Factor VIIIa. These data were normalized for the amount of Xase complex using the constants determined in Fig. 4 and shown in Table I. As can be seen, both chimeric Factor IXa and wild-type Factor IXa activate Factor X at similar rates (Table I). These data indicate that the primary difference between chimeric and wild-type Factor IXa is in their affinity for Factor VIIIa.

To confirm that chimeric Factor IXa has a higher affinity for Factor VIIIa than wild-type Factor IXa, we used DEGR-

Table I. Factor Xa Generation by Chimeric and Wild-type Factor IXa with Factor VIIIa

Recombinant proteins	K_d*	V_{max}‡	K_m§	k_{cat}‡
	nM	*nM/min*	*nM*	*M Xa/min/M IXa-VIIIa*
Factor IX_{normal}	0.35±0.07	3.9±0.2	5.0±0.7	38.6±1.1
Factor $IX_{VIIEGF1}$	0.04±0.01	4.0±0.1	13.1±1.3	38.1±0.8

*The affinity of Factor IXa for Factor VIIIa; ‡maximum Factor Xa generation at saturation of Factor VIIIa with Factor IXa; §the affinity of the Factor IXa/Factor VIIIa complex for Factor X; ‡rate constant for activation when Factor X is saturating. Raw data in the experiment are corrected for the amount of Factor IXa-Factor VIIIa complex.

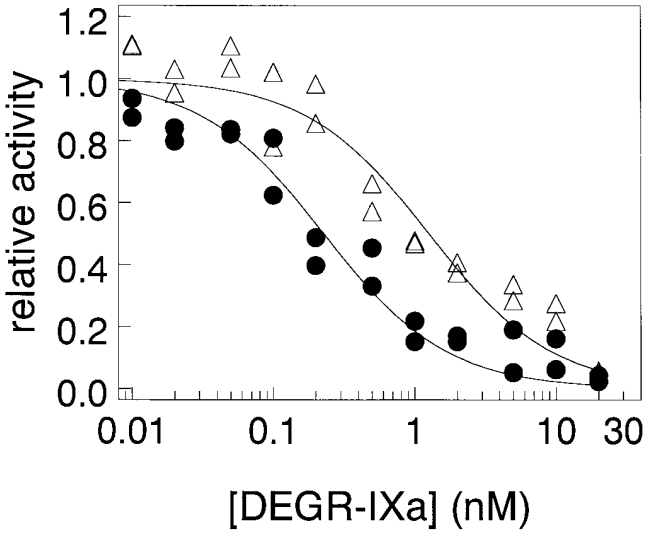

Figure 6. DEGR-IXa inhibition of intrinsic Xase activity. The ability of varied concentrations of DEGR-IXa to inihibit Factor X activation by Factor IXa, Factor VIIIa, and phospholipid was measured as described in Methods. The rate of Factor X activation in the presence of DEGR-IXa was normalized relative to rate seen in the absence of DEGR-IXa. *Open triangles* show the data for normal Factor IXa, and *closed circles* show the data for Factor $IXa_{VIIEGF1}$. Binding of DEGR-IXa to Factor VIIIa (K_i) was determined using the equation shown in Methods.

Table II. Inhibition of Factor Xa Generation by Factor IXa/VIIIa Using DEGR–Chimeric Factor IXa and DEGR–Wild-type Factor IXa with Factor VIIIa

	DEGR–Wild-type Factor IXa	DEGR–Chimeric Factor IXa
	K_i (nM)	K_i (nM)
Wild-type Factor IXa	0.36±0.05	0.065±0.008
Chimeric Factor IXa	0.16±0.01	0.038±0.002

CK–inhibited Factor IXa to show that DEGR chimeric Factor IXa was more effective than DEGR wild-type Factor IXa in inhibiting Factor IXa/Factor VIIIa interaction. These data are shown in Fig. 6. The Xase activity of either wild-type Factor IXa/Factor VIIIa (Fig. 6) or chimeric Factor IXa/Factor VIIIa (data not shown) is more effectively inhibited by DEGR chimeric Factor IXa than by DEGR wild-type Factor IXa. As shown in Table II, the K_i for Factor VIIIa is five to sixfold higher for DEGR wild-type Factor IXa than for DEGR chimeric Factor IXa. These data indicate that DEGR chimeric Factor IXa has a higher affinity for Factor VIIIa than does wild-type Factor IXa.

Animal studies. The animal studies were conducted (*a*) to confirm that increased clotting activity is not due to contamination of the zymogen with activated Factor IX since Factor IXa is rapidly cleared in vivo; (*b*) to determine if Factor $IX_{VIIEGF1}$ functions well in vivo; and (*c*) to investigate the potential of this chimeric Factor IX for future therapeutic use. Equal amounts of chimeric and wild-type Factor IX were infused into a hemophilia B dog as described in Methods (0.08 mg/kg). The dog exhibited no redness or swelling at the site of injection, and showed no evidence of thrombotic complications with either infusion. Results from this experiment demonstrated that the clearance of Factor IX antigen and activity were similar for both chimeric and wild-type molecules (Fig. 7). The whole blood clotting time was shortened from longer than 40 min to a normal value of < 10 min with each infusion, suggesting that both the chimeric and wild-type proteins function well in vivo. As can be seen in Fig. 7 *A*, the amount of chimeric Factor IX recovered was less than the wild-type Factor IX as measured by an ELISA based on a monoclonal capture antibody that recognized the heavy chain of Factor IX and a rabbit anti–human Factor IX–detecting antibody. In control experiments, the ELISA reading for chimeric Factor IX was ~10% less than for wild-type Factor IX (as expected). The recovered antigen level of the chimeric Factor IX, however, was 23–65% lower than the wild-type at the same time points. This data suggests that factors other than differences in the ELISA account for the lower recovery. Even given a lower recovery, the chimeric Factor IX had up to fourfold higher activity than did wild-type Factor IX with an average increase in activity that was 2–3 times higher than wild-type Factor IX (Fig. 7 *B*).

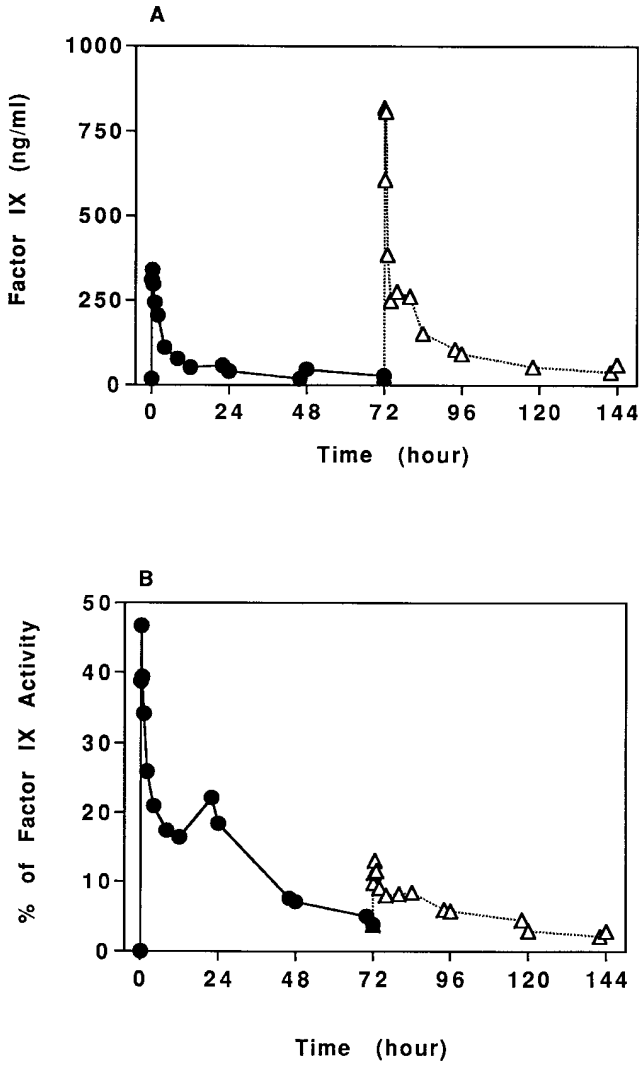

Figure 7. Factor IX activity and antigen levels from a hemophilia B dog. Factor IX antigen levels in the dogs (*A*) were determined by ELISA. Factor IX activity levels in canine plasma (*B*) were determined by clotting assays, and were compared to a standard curve as described in Methods. *Open triangles* show the data for normal Factor IX. *Closed circles* show the data for Factor $IX_{VIIEGF1}$.

Discussion

Our initial observation showed that Factor $IX_{VIIEGF1}$ had at least twofold higher clotting activity than normal Factor IX. Two obvious explanations could account for this observation: (*a*) a discrepancy in the amount of protein used in the assay, or (*b*) chimeric Factor IX contaminated with small amounts of Factor IXa. Careful protein measurements were repeated in more than ten separate experiments using absorbance at 280 nm ($\epsilon 1\% = 13.3$) as well as the Bradford method to confirm the protein concentration. In addition, both chimeric and normal Factor IX were fully activated by the addition of Factor XIa and calcium. Even after full activation, the chimera still retained about twofold higher clotting activity than did the wild-type protein, indicating that the higher activity was not due to contamination with activated Factor IX. In experiments not shown, the active site of both proteins was titrated using antithrombin III, which showed that the same number of active sites were present in both protein preparations.

Other possible mechanisms to explain increased activity in the chimeric molecule include: (*a*) increased activation rate by Factor XIa; (*b*) increased activation rate by Factor VIIa-tissue factor (although in theory there is no tissue factor present in a

one-stage clotting assay); (*c*) enhanced activation of Factor X in the absence of Factor VIIIa; (*d*) increased catalytic activity toward Factor X; or (*e*) increased affinity for Factor VIIIa. As shown in results, both Factor $IX_{VIIEGF1}$ and normal Factor IX were activated similarly by Factor XIa and Factor VIIa tissue factor (Figs. 1 and 2). These experiments show that neither activation pathway contributes to the higher activity of the chimeric protein. The possibility that replacing the EGF1 domain of Factor IX with that of Factor VII would alter the function of the chimeric protein so as to bypass the requirement for Factor VIIIa was excluded since both wild-type and chimeric Factor IXa had the same activity in a system containing Factor IXa, phospholipid, and calcium, but in the absence of Factor VIIIa.

To exclude the possibility that chimeric Factor IXa had increased catalytic activity toward Factor X in the presence of Factor VIIIa, we showed that the K_m and k_{cat} of both activated proteins toward Factor X were essentially similar (Fig. 5 and Table I). These data indicate that the turnover of Factor X reflecting increased catalytic activity does not explain the increased activity of the chimeric molecule. On the other hand, our data shows that the K_d of the activated chimera for Factor VIIIa is 10-fold lower than that for wild-type Factor IX while the V_{max} for Factor Xa generation remains the same. The lower K_d of the chimera indicates that Factor $IX_{VIIEGF1}$ has a higher affinity for Factor VIIIa than for the normal protein. This conclusion is supported by the experiments using DEGR-CK–inhibited Factor IXa. The DEGR chimeric Factor IXa is five to sixfold more potent than DEGR wild-type Factor IXa, as measured by inhibition of intrinsic Xase activity.

Many investigators have tried to pinpoint the functional role of each domain in Factor IX. Some have suggested that the EGF1 domain of Factor IX is involved in binding to Factor VIIIa (25). Others have suggested that the same domain is required for Factor VIIa-tissue factor activation of Factor IX, and for Factor IXa interaction with Factor X (11). Zhong et al. (11) reported that when the first EGF domain of Factor IX was substituted with the same domain of protein C, that, in contrast to the results with Factor $IX_{VIIEGF1}$, activity was markedly reduced. One possible reason for the low activity of the protein C chimera is the poor sequence homology between the EGF1 of Factor IX and protein C as shown in Fig. 8. As can be seen, the EGF1 domain of protein C has nine more residues and one more disulfide linkage than that of Factor IX. Replacing the EGF1 domain of Factor IX with that of protein C might simply interrupt the optimal conformation of the chimera and result in a loss of function. A chimeric Factor IX with the EGF1 domain replaced by that of Factor X was reported to maintain a near normal clotting activity (8). Fig. 8 shows that the EGF1 domains of Factors IX, VII, and X share the same number of residues, and exhibit a high sequence homology. Thus, the overall structure of the chimera factor IX_{XEGF1} may be preserved by the domain exchange so that its activity is also preserved. A recent report for the crystal structure of porcine Factor IXa suggests several residues in the EGF1 domain (Ile-66, Tyr-69, and Trp-72) may be involved in contact with Factor VIIIa (26). The related residues are highly conserved in both Factor X and Factor VII (Leu-66, Tyr-69, and Trp-72 in Factor X, and Leu-65, Tyr-68, and Phe-71 in Factor VII). Therefore, replacing the EGF1 domain of Factor IX with that of Factor VII or Factor X would not necessarily be expected to lower the clotting activity.

Changing the EGF1 domain of Factor IX to Factor VII altered 12 residues between Cys51 and Cys82. The position of these residues was mapped onto a crystal structure of porcine Factor IX using the coordinates deposited in the Brookhaven data bank by Dr. Brandstetter and colleges (26). EGF1 residues in the porcine structure that differed from human were mutated to the appropriate residue using the program Insight II (Molecular Simulations Incorporated, San Diego, CA). EGF1 residues were also mutated to the appropriate residue in Factor VII. Comparing these two models revealed that all of the residues that were changed were significantly surface-exposed. A number of the changes were very conservative (Asn54 to Ser, Ile66 to Leu, Lys80 to Arg), and seem unlikely to account for the increased activity of the chimeric protein. One significant change, however, is in the sequence Pro74-Phe75 in Factor IX that is replaced with Leu-Pro in Factor $IX_{VIIEGF1}$. In the model of human Factor IX EGF1, Phe 75 inserts into EGF2 between the parallel sheets that include residues 94-96 and 121-123. This modeled insertion of Phe75 into the EGF2 domain (which is only a model since in the porcine crystal structure the corresponding residue is a valine) may have two effects on the overall structure. One effect is that Phe75 creates a pocket in the EGF2 domain that may anchor EGF2 in a particular orientation relative to EGF1. The other effect is that Phe75 might distort the backbone structure of Arg94. Arg94 in the porcine crystal structure forms a salt bridge with Asp78 of the EGF1 domain, and may serve to orient EGF1 relative to EGF2. It may be that replacing the EGF1 of Factor IX with that of Factor VII in which Phe75 is replaced by Pro removes a constraint on the relative orientation of EGF1 and EGF2, and confers flexibility on the chimeric molecule such that it can achieve a conformation that allows it to interact effectively with Factor VIIIa.

A 10-fold lower K_d allows the same amount of enzyme–cofactor to be complexed for the chimeric Factor IXa–factor VIIIa at a lower concentration than normal Factor IXa–Factor VIIIa. Since the V_{max} for both Factor IXa–Factor VIIIa com-

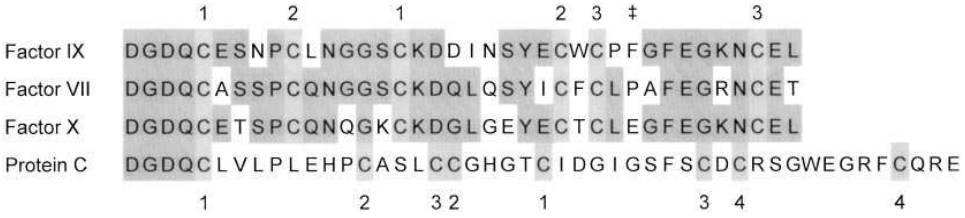

Figure 8. Sequences of the EGF1 domain of human Factor IX, VII, X, and protein C. These sequences are arranged so that the first cysteine residues are aligned. Cysteine residues are shown in *boxes with light shading.* Identical residues are shown in *boxes with darker shading.* Factor IX and X sequences start at residue 47, while Factor VII and protein C sequences start at residue 46. The numbers associated with cysteine residues show the disulfide bonding pattern. Residue 75 in Factor IX is indicated by ‡ and is changed from Phe to Pro in Factor VII. As discussed in the text, changing this residue may be significant for orientation of EGF1 relative to EGF2.

plexes to activate Factor X are the same, once the cofactor, Factor VIIIa, was saturated, the Factor Xa generation rate becomes the same. This suggests that Factor $IX_{VIIEGF1}$ would function significantly better at a lower concentration. Therefore, we performed the animal studies at a dose (0.08 mg/kg) lower than that commonly used (0.16 mg/kg [27]). Surprisingly, after infusion into a hemophilia B dog, the measured antigen level of the chimeric Factor IX was lower than that of normal Factor IX at each related time point. This suggests that the in vivo compartmentalization of the two proteins may not be the same. Since the higher activity of Factor $IX_{VIIEGF1}$ is caused by a higher affinity for Factor VIIIa, the practical increase in activity would depend on the available amount of the activated proteins (Factor IXa and Factor VIIIa). In our animal studies, the assayed activity for Factor $IX_{VIIEGF1}$ was 200–400% greater than that of normal Factor IX at each related time point. If one takes the lower antigen level of the chimera into account and normalizes it, the specific activity for the chimera would be $> 300\%$ of its counterpart.

It has been shown that chimeric plasma proteins may function better than their parent molecules (28–30). The reasons for the increased activity can include reduced plasma clearance (28, 29) or enhanced structural stability of the whole molecule (30). Our studies provide for another mechanism since we show that the increased activity of Factor $IX_{VIIEGF1}$ is caused by an enhanced interaction with cofactor. This better interaction gives Factor $IX_{VIIEGF1}$ increased activity in in vitro assays as well as in vivo in a hemophilia B dog model. Results from these experiments show that the disappearance of Factor IX antigen and activity are similar for the chimeric and normal molecules in a dog, suggesting they have comparable biological half-lives. In addition, no thrombotic complications were observed, although this is admittedly a short-term test. This suggests that the Factor $IX_{VIIEGF1}$ is not only a promising therapeutic agent for conventional intravenous therapy of hemophilia B, but also a potential promising construct for gene replacement therapy of hemophilia B.

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