The light chain of activated factor IX comprises multiple interactions between both epidermal growth factor-like domains that contribute to enzymatic activity and binding of factor IXa to its cofactor factor VIIIa. To investigate the association between factor IXa-specific properties and surface-exposed structure elements, chimeras were constructed in which the interconnection between the modules Leu^{84}-Thr^{87} and the factor IX-specific loop Asn^{89}-Lys^{91} were exchanged for corresponding regions of factor X and factor VII. In absence of factor VIIIa, all chimeras displayed normal enzymatic activity. In the presence of factor VIIIa, replacement of loop Asn^{89}-Lys^{91} resulted in a minor reduction in factor IXa activity. However, chimeras with substitutions or insertions in the spacer between the epidermal growth factor-like domains showed a major defect in response to factor VIIIa. Of these chimeras, some displayed a normal response to isolated factor VIII A2 domain as a cofactor in factor X activation. Surprisingly, chimeras containing elongated inter-domain spacers from factor X or VII displayed reduced response to both complete factor VIIIa and the isolated A2 domain. Moreover, these chimeras still displayed effective association with immobilized A2 domain as assessed by surface plasmon resonance. We conclude that both sequence and length of the junction Leu^{84}-Thr^{87} between both epidermal growth factor-like domains contribute to the enhancement of factor IXa enzymatic activity that occurs upon assembly with factor VIIIa.

Factor IX (FIX) is a vitamin K-dependent serine protease precursor that participates in the process of blood coagulation (1, 2). Factor IX is converted into an active serine protease upon cleavage by either factor Xla (FXIa) or the factor VIIa (FVIIa)-tissue factor complex (3, 4). Proteolysis occurs at restricted cleavage sites and results in the formation of the two-chain enzyme factor IXa (FIXa). FIXa is composed of a light chain that contains an N-terminal Gl domain followed by a short hydrophobic region and two epidermal growth factor (EGF)-like domains (5). The heavy chain comprises the protease domain with the catalytic center. FIXa shares its typical domain structure with related serine proteases of the blood coagulation cascade, including FVIIa, factor Xa (FXa), and activated protein C (6, 7). All of these enzymes contain two EGF-like domains that display considerable sequence homology with the EGF-like modules in factor IXa (8), indicating that these domains are similar in structure and function. Indeed, FIXa, FXa, and FVIIa are similar in that calcium binding to the first EGF-like module is essential for conserving the linkage between its N-terminal half and the N-terminal Gl domain in these proteins (for review, see Ref. 9). However, with respect to the relative orientations of the two EGF-like modules, the three-dimensional crystal structures reveal that this is different in each of these proteases. The connection between these modules is flexible within the structures of FVIIa and FXa (6, 10–13), whereas in FIXa the relative orientation of both domains is much more restricted because of a variety of interdomain contacts (14). These include a salt bridge between residues Glu^{78} in the first EGF-like domain and Arg^{84} in the second EGF-like domain (15) and hydrophobic interaction between the regions Phe^{76}-Phe^{77} and Lys^{106}-Val^{110} at the interface between both domains (16). The salt bridge is crucial for interaction of FIXa with its cofactor, factor VIIIa (FVIIa) (15), whereas the hydrophobic contact contributes to FIXa enzymatic activity (16).

In the present study, we focused on other FIXa-specific elements that are exposed at the interface between both EGF-like domains, including the sequence Leu^{84}-Thr^{87} that connects both domains and the loop Asn^{89}-Lys^{91} at the N-terminal part of the second EGF-like domain. To this end, FIX chimeras were constructed in which these regions were exchanged for the corresponding residues of FX and FVII. The spacer sequence between both EGF-like modules in FIXa, FXa, and FVIIa varies not only in sequence but also in length (4, 6 and 8 residues, respectively). To evaluate whether the length of the connecting segment or the particular amino acid residues therein are important for FIXa function, two additional FIX/FX chimeras were constructed with partial replacements. In one chimera, the FIX residues were replaced by the corresponding number of FX residues, whereas in the second variant the two extra residues of FX were inserted immediately after the FIX spacer.
Activated FIX chimeras were characterized with particular reference to enzymatic activity and response to FVIIia. Our results demonstrate that in particular the junction Leu84-Thr87 between the two EGF-like modules and to some extent also the loop Asn89-Lys91 contribute to respond to the cofactor FVIIia. For chimeras comprising the complete spacer sequence of FX or FVII, impaired sensitivity to intact FVIIia was accompanied by a reduced stimulation by the isolated FVIII A2 domain. Our present data support a model in which the various structural elements that comprise the interface between the EGF-like domains serve a variety of factor IXa functions, including the elements that comprise the interface between the EGF-like domains. See also Figs 85–89/FXII, FIX 84–87/FVII, FIX 89–93/FX, and FIX 89–93/FVII chimeras (see also Fig. 1). Chimeric FIX DNA fragments were obtained by the overlap extension polymerase chain reaction mutagenesis method (18). Sequence analysis was performed to verify the sequence of the constructs. Transfection of FIX-transfected plasmids to Madin-Darby canine kidney cells was performed as described previously (15). Cells expressing appropriate proportions of FIX were maintained cell factories Dulbecco’s modified Eagle’s medium supplemented with 2.5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mg/ml amphotericin B, 0.8 μg/ml dextrose, and 5 μg/ml vitamin K. FIX-containing medium was filtered to remove cell debris and concentrated ~10-fold employing a hollow fiber cartridge (Hemoflow F5, Fresenius, Bad Homburg, Germany). Benzamidine was added to a final concentration of 10 mM, and concentrates were stored at −20 °C. FIX was purified from medium by immuno-affinity chromatography using monoclonal antibody CLB-FIX 14 according to an established procedure (15, 16, 20, 21). Activated FIX was prepared by incubation of 1.4 μM FIX with 7 mM FIXa in 100 mM NaCl, 5 mM CaCl2, and 50 mM Tris (pH 7.4) for 1 h at 37 °C. Activation was stopped by the addition of EDTA (10 mM) and benzamidine (10 mM). Activated FIX was loaded on Q-Sepharose FF and washed with 150 mM NaCl, 5 mM benzamidine, 50 mM Tris (pH 7.4) to remove FIXa. FIXa was eluted from the column by the addition of 500 mM NaCl, 5 mM benzamidine, and 50 mM Tris (pH 7.4). FIXa preparations were dialyzed against 100 mM NaCl, 5% glycerol, 50 mM Tris (pH 7.4), and subsequently against the same buffer containing 50% glycerol. Final FIXa preparations were stored at −20 °C. In agreement with previous studies (20), electrophoretic analysis demonstrated that purified recombinant FIX and FIXa preparations displayed the same mobility as their plasma-derived counterparts, indicating that no propeptide-containing unprocessed FIX species were present. As previously documented, the expression system employed produces FIX with normal Ca2+-dependent properties (20). In accordance with this notion, Gly analysis (22) revealed that recombinant FIX and FIXa preparations used in this study contained 9–12 mol of Glu/mol of protein, with an average Glu content (mean ± S.D.) of 11.1 ± 1.2 mol/mole of FIXa.

Proteins—Human FVIII was purified as described previously (23). Stable FVIIIA preparations were prepared by thrombin activation of CM-Sepharose chromatographed essentially as described elsewhere (16). FVIII A2 domain was isolated from FVIIIa using S-Sepharose chromatography as described (24). Plasma-derived FX and FIX were purified as outlined previously (25). Mononuclear antibody CLB-FIX 14 has been described previously (23) and was purified from culture medium employing protein A-Sepharose as recommended by the manufacturer. Ovalbumin was obtained from NBS Biologicals (Huntingdon, UK). Purified antithrombin and human serum albumin were obtained from the Division of Products of CLB (Amsterdam).

**Protein Concentrations**—Protein was quantified by the method of Bradford (26) using human albumin as a standard. FVIIia activity was assayed by a spectrophotometric method employing bovine coagulation factor X as substrate and a specific colorimetric reagent (Chromogenix, FVIII, Chromogenix, Mölndal, Sweden). Pooled plasma, which was calibrated against the World Health Organization standard 91-666, was used as a standard. The amount present in 1 ml of human plasma (1 unit/ml) was assumed to correspond to 0.4 nM. FVIIIA concentrations were determined using a 1-stage clotting assay (16). The concentration of FIX was determined and described previously (20) assuming that 1 unit FIX corresponds to 0.14 μM FX. Concentrations of wt-FIXa and FIXa chimeras were measured by active site titration using antithrombin as described (19). Active site titration revealed that more than 90% of the protein in the FIX preparations represented active enzymes.

**Amidolytic Activity—**Hydrolysis of CH3SO2-LGR-pNA was essentially performed as described previously (19). Briefly, 50 μl of a solution of CH3SO2-LGR-pNA (0–8 mM) was added to 50 μl of a solution containing 200 nM FIXa and 5 mM Ca2+ in a microtiter plate (Costar, flat bottom type). Initial rates of substrate hydrolysis were measured by monitoring the absorbance at 405 nm in time. Absorbance values were converted into molar concentrations using a molar extinction coefficient of 9.65 × 104 M−1 cm−1 for p-nitroaniline (pNA) and a path length of 0.0167 cm. See also Fig. 1.

**Surface Plasmon Resonance Studies—**The interaction between FIXa and the isolated FVIII A2 domain was assessed by surface plasmon resonance employing a BIACore T20000 000 biosensor system (Biacore AB, Uppsala, Sweden). Purified FIXa A2 domain was immobilized on a CM5 chip using the amine coupling kit as prescribed by the manufacturer at a density of 35.7 fmol/mm2. FIX or FIXa variants were applied in varying concentrations at a flow rate of 20 μl/min in a buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM CaCl2, and 0.005% (v/v) ovalbumin, 0.05 μM Tris (pH 7.4) at 37 °C. Studies performed in the absence of FVIII, various concentrations of FIX (0–500 nM), and a fixed concentration of FIXa (15 nM) were used. In the presence of FVIII (0.4 nM), a fixed concentration of the substrate FX (100 nM) and various concentrations of the enzyme FIXa (0–3 nM) were used. FIXa was then preactivated for 5 min by thrombin (5 nM) before addition. The effect of A2 domain was assessed using fixed concentrations of FIXa (10 nM) and FX (200 nM) and various concentrations of A2 domain (0–100 nM). In experiments in which FXa formation was studied in the absence of FVIII, FIXa (1.7–4 μM) was added to a buffer containing 20 mM Hepes (pH 7.4), 1 mM NaCl, 10 mM EDTA, and 0.005% (v/v) Tween 20. Data were analyzed using BIAevolution software 3.1.

**RESULTS**

**Construction of Recombinant FIXa Variants—**Recombinant FIX/FVII and FIX/FIXa chimeras were constructed in which the sequence connecting the EGF-like domains (i.e., residues Leu84–Thr87) and the loop Asn89–Lys91 in the EGF2 domain were exchanged for the corresponding sequence of both human FVII and FX (Fig. 1). With regard to the spacer between the EGF-like domains, it should be noted that in FVII as well as in FX, the sequence and length differs from that in FIX. Consequently, functional effects of the replacements could be attributed to either the variation in length or to the difference in sequence. To distinguish between these possibilities, two additional constructs were made, one FIX chimera in which the sequence Leu84–Asp85Val86Thr87 was exchanged for the sequence Leu-Phe-Thr-Arg (FIX85–87/FX85–87/Pr) and one chimera in which the “extra” FX residues, Lys and Leu, were inserted immediately after the FIX sequence Asp85Val86Thr87 (FIX85–87/FX85–87/Pr), Recombinant FIX variants were expressed and purified by immunoaffinity chromatography (see “Experimental Procedures”). All FIX mutants were activated by FIXa
under conditions similar to those for recombinant wt-FIXa. All FIX chimeras could completely be converted into the active form, FIXa. The final preparations of activated normal and chimeric FIX were more than 90% active as determined by active site titration using antithrombin.

Amidolytic Activity of Recombinant FIXa Chimeras—To explore whether substitutions in the FIXa light chain affect activity toward a small synthetic peptide substrate, hydrolysis of various concentrations of synthetic substrate CH₃SO₃⁻LGR-pNA was monitored in the presence of FIXa chimeras and Ca²⁺ ions. All chimeras displayed similar rates of substrate hydrolysis compared with that of wt-FIXa, as demonstrated by the calculated catalytic efficiency (k_{cat}/K_m) (Table I). These results indicate that replacement of regions Leu^84-Thr^87 and Asn^92-Lys^91 in the light chain of FIX for corresponding sequences of FVII and FX does not have major effects on FIXa amidolytic activity.

Enzymatic Activity of FIXa Chimeras toward FX—To investigate whether proteolytic activity of FIXa chimeras toward FX was affected, FX activation by FIXa was assessed in the presence of phospholipids and Ca²⁺ ions but in the absence of FVIIIa. wt-FIXa and FIXa chimeras appeared to be similar in their ability to activate FX. Data were fitted into the Michaelis-Menten equation to calculate the apparent catalytic efficiency (k_{cat}/K_m) (Table I). These results suggest that replacement of FIX sequences by the corresponding sequences of FVII or FX in regions 84–87 or 91–92/FX were more than 90% active as determined by active site titration using antithrombin.

Effect of Isolated FVIII-A2 Domain on FX Activation by FIXa Variants—Fay and Koshub (29) demonstrate that FIXa activity can be enhanced by the isolated A2 domain of FVIII (29). Because FIXa chimeras described in the present study display an impaired FVIIIa-mediated stimulation, the effect of isolated A2 domain on FIXa activity was determined. As shown in Fig. 4, the activity of wt-FIXa was increased by the A2 domain in a dose-dependent manner. It should be mentioned that A2 domain-mediated stimulation is 65-fold lower compared with intact FVIIIa (Table II). Stimulation by A2 domain has been proposed to involve the FIXa protease domain residues 333–339 and, in particular, residue Arg^333 (21, 30). As expected, control experiments showed that the variant FIXα-R333Q was associated with a total lack of response to the A2 domain (Table II). Surprisingly, the FIX chimera FIXα_85–87/FX^{A2}_{TRK} also displayed a sharply reduced response to the isolated A2 domain and, as such, behaved similar to the FIXα-R333Q variant (Table II, Fig 4). The activity of both chimeras FIXα_85–87/FX^{A2}_{TRK} and FIXα_85–87/FX^{A2}_{KL} was enhanced by the A2 domain at least as efficiently as observed for wt-FIXa, whereas the stimulation of the FIXα_84–87/FVII was decreased to the level activation of
Hydrolysis of various concentrations of CH$_2$SO$_3$-Leu-Gly-Arg-pNA (0–8 mM) by FIXa (100 nM) and activation of FX (0–500 nM) by FIXa (15 nM) in the absence of FVIIIa were assayed as described under “Experimental Procedures.” Catalytic efficiency ($k_{cat}$/K$_m$) for hydrolysis of CH$_2$SO$_3$-Leu-Gly-Arg-pNA was calculated as described (21). Data for FX activation were fitted into the Michaelis-Menten equation in order to obtain apparent $k_{cat}$ and $K_m$ values. The apparent catalytic efficiency ($k_{cat}$/K$_m$) was inferred from $k_{cat}$ and $K_m$ values. Mean values (± S.D.) of 3–4 experiments are presented.

### Table I

| Amidolytic activity | FX activation in the absence of FVIIIa |
|---------------------|----------------------------------------|
| $k_{cat}$/K$_m$ $\times 10^{-2}$ | $K_{m,app}$ |

| Mutant | $k_{cat}$/K$_m$ $\times 10^{-2}$ | $K_{m,app}$ $\mu$M | $k_{cat}$/K$_m$ $\times 10^{-2}$ |
|--------|---------------------------------|----------------|----------------|
| wt-FIXa| 1.7 ± 0.2                       | 0.17 ± 0.03   | 1.8 ± 0.3      |
| FIXa$_{85-97}$/FX$^{\text{Thr}}$ | 2.0 ± 0.1                       | 0.12 ± 0.01   | 3.8 ± 0.9      |
| FIXa$_{85-97}$/FX$^{\text{KL}}$ | 1.8 ± 0.1                       | 0.18 ± 0.02   | 3.8 ± 0.1      |
| FIXa$_{85-97}$/FX$^{\text{ThrKLI}}$ | 1.9 ± 0.1                       | 0.16 ± 0.01   | 3.3 ± 0.1      |
| FIXa$_{84-91}$/FX$^{\text{II}}$ | 1.5 ± 0.1                       | 0.14 ± 0.01   | 2.8 ± 0.1      |
| FIXa$_{89-91}$/FX$^{\text{II}}$ | 1.9 ± 0.1                       | 0.16 ± 0.02   | 3.0 ± 0.1      |
| FIX$_{89-91}$/FX$^{\text{II}}$ | 2.1 ± 0.1                       | 0.14 ± 0.02   | 4.1 ± 0.2      |

**DISCUSSION**

In the present study we have explored the role of two structure elements that are exposed at the interface between the two EGF-like domains, the small loop Asn$_{89}$-Lys$_{91}$ at the N-terminal end of the EGF2 domain and the connection between the two EGF-like domains, residues Leu$_{84}$-Thr$_{87}$. Both elements are FIX-specific and are completely different in the related coagulation factors FVII and FX (Fig. 1). The rationale for directing our studies to FIX-specific elements at the junction between the two EGF-like domains was 2-fold. First, the crystal structures reveal that FIX is distinct from FX and FVII in that it contains a variety of inter-domain contacts. These restrict flexibility between the two EGF-like modules to a minimum (6, 14). Second, we have previously demonstrated that such inter-domain contacts support the interaction of FIXa with its cofactor FVIIIa (15) or contribute to the enzymatic properties of the protease domain in a FVII-independent manner (16). It thus seems reasonable to suppose that those con-
Connection between Factor IX EGF-like Domains

TABLE II

FX activation by FIXa in the absence and presence of FVIIa and FVIII A2-domain

| Experimental Procedures. |
|--------------------------|
| cofactor concentrations were 0.4 nM for FVIIIa and 150 nM for isolated A2 domain. |

| Stimulation factor | mol FXa(min × mol FIXa) | mol FXa(min × mol FIXa) | mol FXa(min × mol FIXa) |
|-------------------|-------------------------|-------------------------|-------------------------|
|                   | FVIIIa                   | + FVIIIa                 | + A2 domain              |
|                   | mol FXa/(min × mol FIXa) | Stimulation factor       | Stimulation factor       |
|                   |                         |                         |                         |
| wt-FIXa           | 0.018                   | 17.3                    | 961                     | 0.23                     | 12.7                     |
| FIXa89–91/FX      | 0.011                   | 6.9                     | 627                     | 0.14                     | 12.7                     |
| FIXa89–91/FVII    | 0.015                   | 6.6                     | 440                     | 0.13                     | 8.7                      |
| FIXa85–87/FX87/FII| 0.014                   | 6.1                     | 436                     | 0.23                     | 16.4                     |
| FIXa85–87/FX87/KL| 0.012                   | 3.3                     | 275                     | 0.18                     | 15.0                     |
| FIXa85–87/FX87/KFL| 0.011                   | 0.7                     | 64                      | 0.038                    | 3.3                      |
| FIXa84–87/FVII    | 0.011                   | 0.2                     | 20                      | 0.015                    | 1.4                      |
| FIXa-R333Q        | 0.021                   | 1.5                     | 80                      | 0.021                    | 1.0                      |
| FIXaE78K          | 0.011                   | 0.7                     | 64                      | 0.016                    | 1.4                      |

FIG. 3. Activation of FX by FIXa chimeras in the presence of FVIIIa but in the absence of phospholipids. FX (1.7 μM) was activated by 0–40 nM wt-FIXa (open circles), FIXa85–87/FX87 (closed circles), FIXa85–87/FX87/KL (open triangles), FIXa85–87/FX87/KFL (closed triangles), and FIXa84–87/FVII (open squares) in the presence of 10 nM FVIIIa in 0.2 mg/ml ovalbumin, 100 mM NaCl, 5 mM CaCl2, and 50 mM Tris (pH 7.4) for 10 min at 37 °C. FXa formation was quantified as described under "Experimental Procedures." Data represent the mean of three independent experiments.

FIG. 4. Stimulation of FIXa-dependent FX activation by isolated FVIII A2 domain. Various concentrations of isolated FVIII A2 domain (0–100 nM) were incubated with 10 nM wt-FIXa (closed circles), FIXa85–87/FX87/KL (open circles), FIXa85–87/FVII (closed triangles), FIXaE78K (open triangles), or FIXa-R333Q (closed squares) in the presence of phospholipids (0.1 mM) for 10 min at 37 °C in 0.2 mg/ml ovalbumin, 100 mM NaCl, 5 mM CaCl2, and 50 mM Tris (pH 7.4). The reaction was initiated by the addition of FX (final concentration 0.2 μM). Subsamples were drawn at various time points, and the amount of FXa generated was quantified as described under "Experimental Procedures." Rates of FX activation were calculated from at least three measurements within the initial 10 min of activation. Data represent the mean of two independent experiments.

In the absence of FVIII, none of the FIX variants that we examined displayed any significant defect in enzymatic activity (Table I). This implies that neither the loop Asn89–Lys91 nor the spacer Leu84–Thr87 supports the enzymatic properties in manner as described for the hydrophobic contact between the regions Phe75–Phe77 and Lys106–Val108 (18). In the presence of FVIII, however, the various FIX chimeras indeed displayed reduced activity, varying from only a minor reduction for chimeras with replacements of loop Asn89–Lys91 (Fig. 2A) to a major defect for chimeras with replacements in the spacer region Leu84–Thr87 (Fig. 2B). The chimera FIXa884–87/FVII, containing the entire spacer sequence from FVII, displayed the most prominent defect. By lacking a response to FVIIIa, this chimera has a phenotype similar to that of FIXa mutants in which the salt bridge between residues Glu78 and Arg94 was disrupted, thus affecting one of the contacts between the two EGF-like modules (14, 15).

Previous studies provide evidence that assembly of the FIXa-FVIIIa complex involves two distinct interactions, one between the FIXa protease domain and the FVIII A2 domain (21, 28, 29) and the other between the FIXa light chain and the FVIII A3 domain (23, 31). In view of the known Kd values for the individual interactions, we previously proposed that FIXa-FVIII complex assembly is driven by the interaction between the respective light chains of FVIII and FIXa on the phospholipid membrane (32). Our present study (Fig. 3) demonstrates that the same mutants that display reduced activity in the presence of the isolated A2 domain are also defective in response to complete FVIIIa under lipid-free conditions. This suggests a predominant role of the A2 domain in complex assembly when lipids are absent. Further, it is striking that FIXa activity toward FX in the presence of the isolated FVIII A2 domain involves the residues Leu84–Thr87 (Fig. 4). The length of this interdomain spacer, but not its sequence, determines the response to FVIIIa in the absence of lipids (Fig. 3). This is in contrast with the data obtained in the presence of phospholipids, which suggest that both spacer length and sequence contribute to response to FVIIIa (Fig. 2B). It is of further interest to compare the cofactor effect of intact FVIIIa with that of the isolated FVIII A2 domain in quantitative terms. In the presence of lipids, the isolated A2 domain is more than 20-fold less effective as a cofactor than complete FVIIIa (Table II). This demonstrates that stimulation of FIXa activity is greatly de-
dependent on interaction with a site beyond the FVIII A2 domain, for instance with the major FIXa binding site in the A3 domain of the FVIII light chain (23).

One question is whether or not FVIIIa directly interacts with the loop Asn93-Lys93 and the residues Leu87-Thr87 at the EGF1-EGF2 domain interface. It is evident that both these structure elements contribute to FVIII-dependent FIX activation in the presence of phospholipids (Fig. 2). In comparison with the domain linker Leu84-Thr87, however, the contribution of loop Asn93-Lys93 is only minor. This opens the possibility that this loop serves another function than FVIII binding. In this regard it is interesting that a FIX chimera with FVII residues in positions 88–99 in the EGF2 domain displays decreased binding to platelets in the presence and absence of FVIII, suggesting a role in assembly of the factor X-activating complex on the platelet membrane (33). The effect of replacements in the domain linker Leu84-Thr87 is particularly prominent when mutation involves the introduction of extra amino acid residues (Fig. 2B). These findings do not exclude an indirect effect of the spacer on assembly with FVIIIa, for instance by maintaining the orientation of the EGF1 domain, and perhaps also the Gla domain, in the FIXa light chain. However, our data are also compatible with a direct role of the spacer in FVIIIa binding, which is apparently lost in the absence of phospholipids (Fig. 3). Recently, cross-linking studies demonstrate a direct interaction between a FIXa fragment of residues 68–94 with a synthetic peptide of the FVIII residues 1804–1818 (34). We previously identified this A3 domain region as a major FIXa binding site in the FVIII light chain (23, 35). It is therefore conceivable that an interaction between the FVIII A3 domain and the FIXa EGF domain interface provides the molecular basis of the lipid-dependent assembly of the enzyme cofactor complex.

One intriguing finding is that the interdomain spacer Leu84-Thr87 in FIXa plays a role in the enhancement of enzymatic activity by the isolated FVIII A2 domain (Fig. 4) without a concomitant role in the association with the A2 domain (Fig. 5). The predominant role of the helix Arg333-Ser339 in A2 domain binding (21, 28, 30) is supported by our observation that the mutant FIXa-R333Q combines a lack of response to the isolated FVIII A2 domain (Fig 4) with a defect in association with the A2 domain (Fig. 5). In contrast, defective rate enhancement is combined with normal A2 domain binding in FIX variants with mutations in the light chain. Variants of this type do not only comprise the chimeras FIXa84–87/FVII and FIXa85–87/FXa85–87/H11011 (open triangles), FIXa-E78K (closed squares), or plasma-derived FIX (open squares) were incubated with FVIII A2 domain coupled to a BIAcore CM5 chip in a BIAcore system as described under “Experimental Procedures.” Association was monitored, and bound FIXa was expressed as the amount of FIXa (in Response Units) associated after 120 s.

![Figure 5](image5.png)

**Figure 5.** Association of FIXa with isolated FVIII A2 domain. Various concentrations (0–100 nM) of wt-FIXa (closed circles), FIXaR333Q (open circles), FIXa85–87/FVII (closed triangles), FIXa-E78K (open triangles), FIXa-R333K (closed squares), or plasma-derived FIX (open squares) were incubated with FVIII A2 domain coupled to a BIAcore CM5 chip in a BIAcore system as described under “Experimental Procedures.” Association was monitored, and bound FIXa was expressed as the amount of FIXa (in Response Units) associated after 120 s.

![Figure 6](image6.png)

**Figure 6.** Molecular sites in FIXa involved in the interaction with FVIII. The representation is based on the crystal structure of porcine FIXa (14). The backbone structure is shown in ribbon format. The space-filling residues represent the FVIII interactive sites in the protease domain comprising residues 300–303 (dark gray), 330–339 (black), and the inter-EGF domain spacer residues 84–87 (light gray). The salt bridge connecting both EGF-like domains (Glu78 and Arg94) and the side chains involved in contact between the EGF2 domain (Asn92 and Phe98) and the protease domain (Tyr295 and Phe299) are indicated in black. In the crystal structure, the distance between Asn92 and Tyr295 is 2.4 Å, whereas Phe98 is located at ~5 Å from Tyr295 in a hydrophobic pocket that also comprises residues Phe299 and Phe302 (14). Residues Glu78 in the EGF1 and Arg94 in the EGF2 domain forming the inter-domain connection by a salt bridge are separated by 2.73 Å.
chain as well as contacts between the light chain and the FIXa protease domain.

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