Localization of Factor IXa and Factor VIIIa Interactive Sites*

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The contribution of the catalytic and noncatalytic domains of factor IXa to the interaction with its cofactor, factor VIIIa, was evaluated. Two proteolytic fragments of factor IXa, lacking some or all of the serine protease domain, failed to mimic the ability of factor IXa to enhance the reconstitution of factor VIIIa from isolated A1/A3-C1-C2 dimer and A2 subunit. Both fragments, however, inhibited this factor IXa-dependent activity. Selective heat denaturation of the serine protease domain eliminated its effect on factor VIIIa reconstitution. Modification of factor IXa with dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR-IXa) stabilized this domain, and heat-treated DEGR-IXa retained its ability to enhance factor VIIIa reconstitution. These results indicate the importance of the serine protease domain as well as structures residing in the factor IXa light chain (γ-carboxyglutamic acid and/or epidermal growth factor domains) for cofactor stabilizing activity. In the presence of phospholipid, the A1/A3-C1-C2 dimer produced a saturable increase in the fluorescence anisotropy of fluorescein-Phe-Phe-Arg chloromethyl ketone-modified factor IXa (Fl-FFR-IXa). This effect was inhibited by a factor IXa fragment comprised of the γ-carboxyglutamic acid and epidermal growth factor domains. The difference in FI-FRR-IXa anisotropy in the presence of A1/A3-C1-C2 dimer (Δr = 0.043) compared with factor VIIIa (Δr = 0.069) represented the contribution of the A2 subunit. A peptide corresponding to factor VIII A2 domain residues 558-565 decreased the factor VIIIa dependent-anisotropy of FI-FRR-IXa to a value similar to that observed with the A1/A3-C1-C2 dimer. These results support a model of multiple interactive sites in the association of the enzyme- cofactor complex and localize sites for the A1/A3-C1-C2 dimer and the A2 subunit to the factor IXa light chain and serine protease domain, respectively.

Factors VIII and IX are essential plasma glycoproteins, which when absent or defective, result in hemophilia A and hemophilia B, respectively. Factor VIII is synthesized as a 300-kDa protein (1, 2) with domain structure A1, A2-B, A3-C1-C2 (3) that is subsequently processed and circulates in plasma as a series of Me2+ heterodimers (4–6). Factor VIII is converted to its active form, factor VIIIa, upon proteolytic cleavage by thrombin (7). Factor VIIIa is a heterotrimer composed of the A1, A2, and A3-C1-C2 subunits. The A1 and A3-C1-C2 subunits retain the Me2+ ion linkage and can be isolated as a stable dimer (8, 9). The A2 subunit is weakly associated with the dimer primarily through electrostatic forces (10) and at physiologic pH readily dissociates resulting in the loss of factor VIIIa activity (11–13). Under the appropriate conditions, factor VIIIa activity can be reconstituted from the isolated A2 subunit and the A1/A3-C1-C2 dimer (10, 12, 14).

Factor IX circulates in blood as a single chain zymogen. It is composed of an NH2-terminus Gla domain which is rich in γ-carboxyglutamic acid, followed by two EGF-like domains, a 35-residue activation peptide and the serine protease domain (see Ref. 15 for review). In the intrinsic blood coagulation pathway, factor IX is activated to factor IXa by factor XIa and Ca2+ (15). Upon activation, the 35-residue activation peptide is released to yield factor IXa light chain, comprised of the Gla and two EGF domains, linked via a disulfide bond to the heavy chain which contains the serine protease domain (16).

Factor VIIIa functions as a cofactor for the serine protease factor IXa, which in the presence of Ca2+ and a membrane surface, converts factor X to factor Xa. Factor VIIIa increases the kcat for this reaction by several orders of magnitude (17). Association of factor VIIIa with factor IXa in the presence of phospholipid stabilizes cofactor activity (18). While reconstitution of heterotrimer factor VIIIa from the isolated A2 subunit and A1/A3-C1-C2 dimer is enhanced severalfold in the presence of factor IXa and phospholipid (14), prolonged incubation of factor VIIIa with factor IXa results in a loss of factor VIIIa activity due to proteolytic cleavage within the A1 domain (19). However, stable enhancement of factor VIIIa reconstitution can be achieved in the presence of active site-inhibited factor IXa (14).

Sites of interaction between factor IXa and its cofactor are not well defined. In addition to its role in binding phospholipid (20) and endothelial cell (21) surfaces, the isolated factor IX Gla domain has also been shown to inhibit the factor VIIIa-dependent conversion of factor X to Xa by factor IXa (22). Furthermore, a monoclonal antibody specific for an epitope within the

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2 The abbreviations used are: Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; DEGR-CK, 2,6-dansyl glutamyl-L-glycyl-L-arginyl-chloromethyl ketone; DEGR-IXa, factor IXa modified with DEGR-CK; Fl-FRR-IXa, factor IXa modified with fluorescein D-phenylalanyl-D-phenylalanyl-L-arginyl-L-arginyl chloromethyl ketone; PPACK, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone; FPR-IXa, factor IXa modified with PPACK; PS, phosphatidylserine; PC, phosphatidylycholine; PE, phosphatidylethanolamine.
heavy chain of factor IXa was shown to interfere with the binding of factor VIIIa to factor IXa suggesting the serine protease domain also interacts with factor VIIIa (23). Similarly, little information is known about sites within factor VIII(a) involved in its interaction with factor IXa. Recently it has been shown that the light chain of factor VIII (likely within the A3 domain) contains a high affinity binding site for factor IXa (24). In addition, peptides corresponding to the sequence surrounding the activated protein C cleavage site in the A2 subunit of factor VIIIa (Arg562) (8), inhibit the factor IXa-dependent enhancement of factor VIIIa reconstitution (25). Thus, interaction of factor IXa and factor VIIIa appears to involve multiple sites on each protein.

In this study we demonstrate that the serine protease domain of factor IXa is essential for stabilizing the structurally labile, factor VIIIa heterotrimer and likely contributes to this association by interaction with the A2 subunit. Additional results obtained with proteolytic fragments of factor IX indicate that the Gla and/or EGF domains contain an interactive site for the A1/A3-C1-C2 dimer factor VIIIa. These results support a model for the factor VIIIa-factor IXa interaction consistent with proposed surface proximal and surface distal regions of the molecules.

MATERIALS AND METHODS

Reagents—The reagents DEGR-CK and PPACK (Calbiochem), phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (Sigma) were purchased from the indicated vendors. Phospholipid vesicles (PSPCPE) composed of 20% phosphatidylserine, 40% phosphati
dylycholine, and 40% phosphatidylethanolamine were prepared using octyl glucoside as described previously (26). The synthetic peptide SVDQRGNQ which corresponds to factor VIII residues 558–565 was a generous gift from Debra Pittman of the Genetics Institute. Human factor VIIIa was obtained following CM-Sepharose chromatography of thrombin-activated human factor VIII (27). Purification of factor VIIIa subunits from recombinant factor VIII was as described previously (28). Human factor IXa and human α-thrombin were purchased from Enzyme Research Laboratories.

Preparation of Factor IX Fragments—Factor IXa was isolated from fresh-frozen human plasma by affinity chromatography on a column of immobilized anti-factor IX monoclonal antibody (anti-FIX-Sepharose) according to Tharakan et al. (29) using the same affinity matrix. The 45-kDa fragment consisting of the Gla and two EGF domains plus the amino-terminal half of the serine protease domain was prepared by digestion of factor IX with thrombin followed by affinity chromatography on the anti-FIX-Sepharose. The 25-kDa fragment containing the Gla and two EGF domains was prepared by digestion of factor IX with chymotrypsin followed by affinity chromatography on the same column. The conditions for digestion and purification were as described previously (30). Both fragments were subjected to 10 cycles of NH2-terminal sequence analysis on a Hewlett Packard model G10005 sequencer. The sequences were exactly the same as determined previously (30).

Factor IXa was modified with active site specific reagent DEGR-CK as described elsewhere (31). Fluorescin-Phe-Phe-Arg-factor IXa (Fi-FFR-IXa) was purchased from Molecular Innovations, Wayne MI.

Assays—Factor VIIIa activity was measured by a one stage clotting assay using substrate plasma that has been chemically depleted of factor VIII activity (32). Reconstitution of factor VIIIa activity from the isolated A1/A3-C1-C2 dimer and A2 subunit was performed at room temperature in buffer A (20 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM CaCl2, 0.01% Tween 20) with 0.5 mmgl bovine serum albumin and 0.1 mmgl phospholipid in the form of dispersions. Purification measurements of the thermal unfolding of factor IXa and DEGR-IXa were performed with a protein concentration of 0.1 mg/ml by monitoring the ratio of intrinsic fluorescence intensity at 350 nm to that at 320 nm with excitation at 280 nm in an SLM 8000-C fluorometer. The temperature was controlled with a circulating water bath programmed to raise the temperature at ~1 °C/min. Changes in the ratio parameter provide a sensitive method for detecting melting transitions and determination of their midpoints (Tm).

Fluorescence Anisotropy—Fluorescence anisotropy measurements were made using a SPEX Fluorolog 212 spectrometer operated in the L format. The excitation wavelength was 495 nm (5-nm band pass) and the emission wavelength was 520 nm (14-nm band pass). Reactions were carried out at room temperature in buffer A with 0.1 mg/ml phospholipid in the form of PSPCPE in a quartz micro cell (200 μl, final volume). Anisotropy measurements were made by manually rotating the polarizers and monitoring fluorescence for 10 s at each position. Three determinations were made at each position and the average value obtained. Prior to addition of Fl-FFR-IXa, blank readings for the buffer containing phospholipid were made and subtracted from subsequent determinations. Following addition of Fl-FFR-IXa, baseline readings for the binding of porcine factor VIIa to fluorescently modified factor IXa (33) using the following equations:

\[
\begin{align*}
    r = f_0 + f_s(n_{Eo} - f_0) \\
    f_s = \frac{B}{n_E} \\
    B = (n_E + k_d + C_o)^2 - 4n_Ek_d \\
    \frac{n_Ek_d}{2}
\end{align*}
\]

where \( r \) is the anisotropy of Fl-FFR-IXa in the absence of added A1/A3-C1-C2 dimer, \( r_{sat} \) is the anisotropy of Fl-FFR-IXa in the presence of saturating levels of dimer, \( f_s \) is the fraction of Fl-FFR-IXa binding sites that are occupied by the dimer and is defined as, \( f_s = \frac{B}{n_E} \), where \( B \) is the concentration of bound A1/A3-C1-C2 dimer and \( C_o \) is the total concentration of Fl-FFR-IXa.

For experiments in which Fl-FFR-IXa or the 25-kDa factor IX fragment were added to displace Fl-FFR-IXa, reactions initially contained Fl-FFR-IXa (20 μM) in buffer A with 0.1 mg/ml PSPCPE. The change in Fl-FFR-IXa anisotropy upon addition of A1/A3-C1-C2 (50 μM) was determined. Subsequent anisotropy measurements were made following successive additions of either FPR-IXa or the 25-kDa factor IX fragment.

RESULTS

Role of the Serine Protease Domain in Factor IXa-dependent Enhancement of Factor VIIIa Reconstitution—The serine protease domain of factor IX is structurally labile and melts at a temperature of ~42–55 °C depending on pH with an irreversible loss of esterolytic activity (30). Alternatively, the Gla and EGF modules of factor IX melt at much higher temperatures (30). Modification of the active site of several serine proteases with peptidyl chloromethyl ketone inhibitors stabilizes the serine protease domain thus increasing their melting temperatures to variable extents (34). Fluorescence detected thermal denaturation indicated that factor IXa melts with a midpoint (Tm) near 58 °C, while factor IXa that has been modified with the active site inhibitor dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR-IXa) melts with a midpoint (Tm) of 65 °C (Fig. 1). Incubation of factor IXa at 60 °C for 10 min followed by fluorescence detected thermal denaturation revealed the complete elimination of the characteristic transition curve indicating that unfolding was irreversible. However, identical treatment of DEGR-IXa resulted in a transition curve similar to the original, although the magnitude was somewhat decreased, indicating that at least 40% of the molecules retained their native structures (data not shown).

To examine the role the serine protease domain of factor IXa serves in its interaction with factor VIIIa, we took advantage of the sensitivity of this domain to elevated temperature and monitored the capacity of factor IXa to enhance reconstitution of factor VIIIa from isolated subunits. Factor IXa or DEGR-IXa was incubated for 5 min at 60 °C prior to reaction with factor VIIIa subunits. This treatment destroyed greater than 98% of the proteolytic activity of factor IXa as determined by its in...
ability to convert factor X to factor Xa in the presence of factor VIIIa and phospholipid (data not shown). Reconstitution of factor VIIIa activity from the isolated A1/A3-C1-C2 dimer and the A2 subunit was carried out in the presence of the various factor IXa forms (Fig. 2). Untreated, native factor IXa resulted in a transient enhancement of factor VIIIa reconstitution that decayed over the time course of the reaction, consistent with our previously reported observation (14). The transient nature of this reaction reflects the subsequent inactivation of factor VIIIa following factor IXa-catalyzed cleavage within the A1 subunit (19, 35). Modification of the factor IXa active site by DEGR-CK yields catalytically inactive enzyme that still retains its ability to enhance factor VIIIa reconstitution (14) (Fig. 2, diamonds). Furthermore, the enhancement activity observed with DEGR-IXa is stable and occurs to a greater extent since the protease activity is nonfunctional. However, heat-treated factor IXa was unable to enhance the reconstitution of factor VIIIa activity while heat-treated-DEGR-IXa, whose serine protease domain was stabilized by the active site modification, retains its ability to enhance factor VIIIa reconstitution. These results suggest that the serine protease domain is necessary for the factor IXa-dependent enhancement of this factor VIIIa intersubunit interaction.

Effect of Factor IX Fragments on Factor IXa-dependent Enhancement of Factor VIIIa Reconstitution—Fragments of factor IX were prepared following limited proteolysis and used to further investigate the structural requirements for the interaction of factor IXa with factor VIIIa. A 45-kDa fragment consisting of the Gla and two EGF domains plus the NH2-terminal portion of the serine protease domain and a 25-kDa fragment containing only the Gla and two EGF domains were prepared as described under "Materials and Methods." Neither the 45-kDa fragment nor the 25-kDa factor IX fragment was able to enhance the reconstitution of factor VIIIa activity when present at micromolar concentrations (data not shown). This result was consistent with the above data, suggesting the importance of a structurally intact serine protease domain for this activity.

To determine whether the Gla and/or EGF domains participate in the interaction between factor IXa and factor VIIIa, competition experiments were performed in which the ability of the 25-kDa factor IX fragment to inhibit the DEGR-IXa-dependent enhancement of factor VIIIa reconstitution was examined. As shown in Fig. 3, factor VIIIa reconstitution from 10 nM subunits was enhanced approximately 8-fold in the presence of 10 nM DEGR-IXa. However, the 25-kDa fragment was able to inhibit this DEGR-IXa-dependent effect with 50% inhibition occurring at ~4 μM. The 45-kDa fragment, which contained the Gla and EGF domains as well as the amino-terminal portion of the serine protease domain, also inhibited the DEGR-IXa-dependent enhancement of reconstitution with 50% inhibition seen at ~3 μM (data not shown). This effect did not result from titration of the phospholipid vesicles by the high levels of fragment in the reaction since increasing the phospholipid concentration 10-fold failed to reverse the inhibition (data not shown). This result suggests that the Gla and/or EGF domains of factor IX interact with one or more factor VIIIa subunits, and this interaction, like the interaction with the serine protease domain, is necessary for the factor IXa-dependent stabilization of factor VIIIa.

Interaction of A1/A3-C1-C2 Dimer with Factor IXa—Fluorescence anisotropy was used to examine the interaction of the A1/A3-C1-C2 dimer with fluorescently modified factor IXa (FIFR-IXa) in the presence of PSPCPE vesicles. Titration with A1/A3-C1-C2 dimer produced a saturable increase in the fluorescence anisotropy of FIFR-IXa in the presence of PSPCPE (Fig. 4), suggesting that the dimer was capable of binding factor IXa and altering its active site. The data were fit to a model that has been used previously to describe the binding of porcine factor VIIIa to fluorescently labeled factor IXa (33). This model assumes reversible binding to n identical noninteracting sites on FIFR-IXa. Nonlinear least squares fit of the data to this equation yielded an apparent Kd ~11 nM and a stoichiometry of ~5 A1/A3-C1-C2 molecules/FIFR-IXa. The greater than unity stoichiometry has been observed previously using purified factor VIIIa preparations (27) and may reflect a high mole fraction of inactive dimer molecules, possibly resulting from chromatographic procedures.

The ability of a nonfluorescent, active site modified factor IXa molecule, FPR-IXa to inhibit the binding of FIFR-IXa to the A1/A3-C1-C2 dimer was examined. The fluorescence anisotropy detected thermal denaturation of factor IXa and DEGR-IXa in 20 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM CaCl2, and 0.01% Tween-20 was determined as described under "Materials and Methods."
The fluorescence anisotropy of Fl-FFR-FIXa alone was 0.205.

The interaction of factor IXa with its cofactor, factor VIIIa, was investigated by evaluating the contribution of both the catalytic (serine protease) and noncatalytic (Gla and two EGF) domains of the molecule. In the present study, we demonstrate that a structurally intact serine protease domain is required for interaction with factor VIIIa, as judged by the factor IXa-dependent enhancement of factor VIIIa reconstitution. Two proteolytic fragments of factor IX in which all or part of the serine protease domain are deleted, failed to enhance factor VIIIa reconstitution. Furthermore, selective thermal denaturation of the serine protease domain abolished the ability of the molecule to enhance factor VIIIa reconstitution. Modification of the factor IXa active site with DEGR-CK stabilized this domain,

**DISCUSSION**

To determine the role of the Gla and/or EGF domains in the interaction of factor IXa and the A1/A3-C1-C2 dimer, the 25-kDa factor IX fragment was used as an inhibitor of the dimer-dependent increase in anisotropy. Successive additions of the factor IX fragment produced a decrease in the fluorescence anisotropy in a reaction containing 20 nM Fl-FFR-FIXa and 50 nM A1/A3-C1-C2 in the presence of 0.1 mg/ml of phospholipid (0.1 mg/ml PSPCPE) vesicles. A 50% inhibition of the anisotropy increase occurred at \( \sim 1 \mu M \). This fragment was observed to be about 10-fold less potent of the serine protease domain abolished the ability of the molecule to enhance factor VIIIa reconstitution. The interaction of factor IXa with its cofactor, factor VIIIa, was investigated by evaluating the contribution of both the catalytic (serine protease) and noncatalytic (Gla and two EGF) domains of the molecule. In the present study, we demonstrate that a structurally intact serine protease domain is required for interaction with factor VIIIa, as judged by the factor IXa-dependent enhancement of factor VIIIa reconstitution. Two proteolytic fragments of factor IX in which all or part of the serine protease domain are deleted, failed to enhance factor VIIIa reconstitution.
The above results indicate the importance of the serine protease domain for the cofactor stabilizing activity and are consistent with previous studies that proposed a factor VIIIa-interactive site on the factor IXa heavy chain. Earlier studies described a monoclonal antibody that binds an epitope within factor IXa heavy chain (serine protease domain) residues 181 and 310 (36) and interferes with subsequent binding of factor VIIIa (23). However, the exact mechanism of this inhibition is unknown since creation of recombinant factor IX molecules, in which surface residues in this region were individually replaced with factor X residues from the homologous position, eliminated or reduced affinity for the antibody yet retained normal clotting activity (37).

A role for the noncatalytic domains of factor IXa in the interaction with its cofactor was indicated by the capacity of the 25-kDa factor IX fragment to inhibit the DEGR-IXa-dependent enhancement of factor VIIIa reconstitution. Localization of factor VIIIa interactive sites within these domains remains controversial. Lin et al. (38) reported that recombinant factor IX in which the two EGF-like domains have been replaced by the corresponding domains of factor XA possessed only 4% normal biological activity, while recombinant factor IX, in which only the first EGF domain was replaced with the corresponding region from factor XA, retained normal activity. This suggests that the first EGF domain is not likely to be involved in the interaction with factor VIIIa. In contrast, mutational analysis has indicated that Tyr69, located within the first EGF domain, is essential for the factor VIIIa-dependent conversion of factor XA by factor IXa (39). Evidence that the Gla domain may be required for interaction with factor VIIIa is based on the observation that a fragment of bovine factor IX consisting of the isolated Gla domain weakly inhibited (Ki = 10 μM) the factor VIIIa-dependent conversion of factor X to XA as well as inhibit the factor IXa-dependent conversion of factor X to XA (40). However, the conformation around the active site of factor IXa is altered by the cofactor (42). The A3-C1-C2 dimer of factor VIIIa produces a greater increase in the anisotropy of Fl-FFR-IXa than the A1/A3-C1-C2 dimer, thus suggesting a role for the A2 subunit in modulating the factor IXa active site. Recently, Duffy et al. (33) showed that the fluorescence anisotropy of factor IXa is increased in the presence of factor VIII and that a further incremental increase is observed upon thrombin-catalyzed activation. This incremental increase likely reflects contribution from cleavage of both the light chain (Arg12499) and the heavy chain (Arg172) of factor VIII. A hybrid factor VIII molecule possessing a native heavy chain plus thrombin-cleaved light chain increased the anisotropy of Fl-FFR-IXa to a value that was intermediate to that of factor VIII and factor VIIIa (40). Furthermore, addition of thrombin to fully activate the hybrid was required to observe the maximal anisotropy effect. Recently, we reported that residues 558–565 of the A2 subunit contained a factor IXa interactive site based upon the observation that a peptide corresponding to this region was able to inhibit the factor VIIIa-dependent conversion of factor X to XA as well as inhibit the factor IXa-dependent enhancement of factor VIIIa reconstitution (25). We now show that this peptide can block the interaction of the A2 subunit with factor IXa based upon its capacity to eliminate the A2-dependent contribution to the anisotropy increase of Fl-FFR-IXa in the presence of factor VIIIa. The ability of A2 subunit to alter the conformation around the active site is compatible with the localization of this interaction to the serine protease domain of factor IXa.

Based upon the above results we suggest the following model for the relative orientation of factors VIIIa and IXa and surface in the intrinsic tenase complex. Factor IXa is an elongated molecule with its light chain (Gla domain) involved in binding the phospholipid surface, whereas the heavy chain contains the serine protease domain (see Ref. 41 for review). Recently, Mucucumarana et al. (42) determined that the active site of factor IXa is located high above the membrane surface (>70 Å), and this distance is unaffected by the presence of factor VIIIa. However, the conformation around the active site of factor IXa is altered by the cofactor (42). The A3-C1-C2 subunit of factor VIIIa contains the binding site for phospholipid (C2 domain) (43) as well as the high affinity site for factor IXa (A3 domain) (24). Since this factor VIIIa subunit is also surface proximal, it is reasonable to speculate an interaction between the A3 domain and the light chain of factor IXa. This inference is supported by the inhibition of the A1/A3-C1-C2 dimer-dependent interaction of factors IXa with the free factor VIII light chain (Kd = 14 nM) (24) and human factor VIIIa (Kd = 23 nM) (27) suggests little if any contribution of factor VIII heavy chain-derived subunits (A1 and A2) to the binding energy. However, factor VIII light chain produces a fractional increase in the fluorescence anisotropy of Fl-FFR-IXa compared with the A1/A3-C1-C2 dimer, suggesting that the A1 subunit may contribute to the change in conformation near the active site. FPR-IXa eliminated the dimer-dependent anisotropy increment by displacing Fl-FFR-IXa from the complex demonstrating a direct physical interaction of the Gla and/or EGF domains with the A1/A3-C1-C2 subunit. The observed 10-fold reduction in inhibitor potency of the fragment compared with the intact FPR-IXa molecule could result from partial denaturation of the fragment during isolation and/or indicate that additional sites of dimer interaction are located within the serine protease domain.

**Table I**

| Sample | Anisotropy |
|--------|------------|
| Fl-FFR-IXa | 0.195 ± 0.005 (12) |
| Fl-FFR-IXa + FVIIIa | 0.199 ± 0.012 (6) |
| Fl-FFR-IXa + FVIIIa + Fl-FFR-IXa | 0.264 ± 0.003 (5) |
| Fl-FFR-IXa + FVIIIa + Fl-FFR-IXa | 0.246 ± 0.004 (6) |
| Fl-FFR-IXa + A1/A3-C1-C2 | 0.238 ± 0.009 (5) |
| Fl-FFR-IXa + A1/A3-C1-C2 + FVIIIa | 0.230 ± 0.005 (5) |

* a Reactions were performed as described under "Materials and Methods" and contained Fl-FFR-IXa (20 nM), and FVIIIa (80 nM), A1/A3-C1-C2 (80 nM), and FVIII558–565 (300 μM) where indicated.

Values represent the mean ± S.D. Number of separate determinations are indicated in parentheses. Comparison of the factor VIIIa-dependent anisotropy of Fl-FFR-IXa in the absence (0.264 ± 0.003) and presence of peptide (0.246 ± 0.006) using the student t-test yields a p = 0.0006. The factor VIIIa-dependent anisotropy of Fl-FFR-IXa in the presence of the peptide (0.246 ± 0.006) is not statistically different, p = 0.14, from the anisotropy value of A1/A3-C1-C2 dimer in the presence of peptide (0.238 ± 0.005).

The change in conformation near the active site. Fl-FFR-IXa elicits the dimer-dependent anisotropy increment by displacing Fl-FFR-IXa from the complex demonstrating a direct physical interaction of the Gla and/or EGF domains with the A1/A3-C1-C2 subunit. The observed 10-fold reduction in inhibitor potency of the fragment compared with the intact FPR-IXa molecule could result from partial denaturation of the fragment during isolation and/or indicate that additional sites of dimer interaction are located within the serine protease domain.
anisotropy increase of Fl-FFR-IXa by the 25-kDa fragment. The factor VIIIa A1 subunit is linked by divalent metal ion(s) to the A3-C1-C2 subunit and is likely oriented above the surface since its presence is required for the anisotropy effect and it contains a site (Arg<sup>366</sup>) (19) proteolyzed by factor IXa. We predict that the factor VIIIa A2 subunit is also localized above the surface since it contains a primary cleavage site for activated protein C (Arg<sup>362</sup>) (8), an homologous enzyme to factor IXa (see Ref. 41 for review) whose active site is likely similarly positioned. Additional support for the surface distal positioning of A2 subunit is inferred from its direct effect on the magnitude of factor VIIIa-dependent anisotropy of Fl-FFR-IXa when the contribution of A2 is eliminated. Thus, we propose that factor IXa contains at least two interactive sites for factor VIIIa and include: (i) a surface-proximal site possessing a high affinity interaction and involving the light chain of factor IXa and the factor VIII A3-C1-C2 subunit and is likely oriented above the surface and (ii) a surface-distal site(s) involving participation of the serine protease domain of factor IXa and the A2 (plus possibly A1) subunit(s) of factor VIIIa.

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