Factor IXa:Factor VIIIa Interaction

HELIX 330–338 OF FACTOR IXa INTERACTS WITH RESIDUES 558–565 AND SPATIALLY ADJACENT REGIONS OF THE A2 SUBUNIT OF FACTOR VIIIa

S. Paul Bajaj‡§, Amy E. Schmidt‡, Akash Mathur‡, K. Padmanabhan‡, Degang Zhong‡, Maria Mastri¶, and Philip J. Fay‖

From the ‡Department of Medicine, Saint Louis University School of Medicine, St. Louis, Missouri 63104, the §Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, and the ¶Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.


Published, JBC Papers in Press, February 14, 2001, DOI 10.1074/jbc.M011680200

The physiologic activator of factor X consists of a complex of factor IXa, factor VIIIa, Ca2+ and a suitable phospholipid surface. In one study, helix 330 (162 in chymotrypsin) of the protease domain of factor IXa was implicated in binding to factor VIIIa. In another study, residues 558–565 of the A2 subunit of factor VIIIa were implicated in binding to factor IXa. We now provide data, which indicate that the helix 330 of factor IXa interacts with the 558–565 region of the A2 subunit. Thus, the ability of the isolated A2 subunit was severely impaired in potentiating factor X activation by EGR-IXa helixVII and by a helix replacement mutant (IXa helixVII in which helix 330–338 is replaced by that of factor VII) but it was normal for an epidermal growth factor 1 replacement mutant (IXa PCEGF1 in which epidermal growth factor 1 domain is replaced by that of protein C). Further, affinity of each 5-dimethylaminonaphthalene-1-sulfonfonyl (dansyl)-Glu-Gly-Arg-IXa (dEGR-IXa) with the A2 subunit was determined from its ability to inhibit wild-type IXa in the tenase assay and from the changes in dansyl fluorescence emission signal upon its binding to the A2 subunit. Apparent Kd(A2) values are: dEGR-IXa WT or dEGR-IXa PCEGF1 ~100 nM, dEGR-IXa R338Q ~1.8 μM, and dEGR-IXa R333Q >10 μM. In additional experiments, we measured the affinities of these factor IXa molecules for a peptide comprising residues 558–565 of the A2 subunit. Apparent Kd(peptide) values are: dEGR-IXa WT or dEGR-IXa PCEGF1 ~4 μM, and dEGR-IXa R338Q ~62 μM. Thus as compared with the wild-type or PCEGF1 mutant, the affinity of the R333Q mutant for the A2 subunit or the A2 558–565 peptide is similarly reduced. These data support a conclusion that the helix 330 of factor IXa interacts with the A2 558–565 sequence. This information was used to model the interface between the IXa protease domain and the A2 subunit, which is also provided herein.

Physiologic blood clotting begins by exposure of blood to tissue factor (TF) at an injury site and formation of the complex between TF and plasma factor VIIa. The TF-VIIa complex formed activates both factors IX and X (1, 2). Factor IXa thus generated forms a stoichiometric complex with factor VIIIa and also activates factor X in the presence of Ca2+ and a suitable phospholipid (PL) surface (1, 2). The role of factor VIIIa in this complex is to increase the kcat by several orders of magnitude while PL primarily reduces the Km for the substrate factor X.

Human factor IX circulates in blood as a single chain protein of 415 amino acids. Upon activation by factor Xa/Ca2+ or TF-VIIa/Ca2+, two peptide bonds in factor IX are cleaved with resultant formation of a serine protease, factor IXa, and release of an activation peptide (3). Factor IXa is composed of a light chain consisting of residues 1–145 and a heavy chain consisting of an amino-terminal γ-carboxyglutamatic acid (Gla)-rich domain (residues 1–40), a short hydrophobic segment (residues 41–46), and two epidermal growth factor (EGF)-like domains (EGF1 residues 47–84 and EGF2 residues 85–127), whereas the heavy chain contains the serine protease domain, which features the catalytic triad residues, namely His211 (c57),2 Asp209 (c102), and Ser365 (c195) (5). The Gla domain contains several high and low affinity Ca2+-binding sites, whereas EGF1 and protease domain each contain a high affinity Ca2+ site (7). For proper binding of factor IXa to PL and factor VIIIa, all of the Ca2+ sites in factor IXa must be filled (7, 8).

Factor VIII is synthesized as a single chain molecule containing several domains (A1-A2-B-A3-C1-C2) (9), with a molecular mass of ~300 kDa (10, 11). The A domains are homologous to the ceruloplasmin domains and to the A domains of factor Va (12), whereas the C domains are homologous to the galactose lipid binding domain and to the regions within neutaminidase (13). Factor VIII circulates as a divalent metal ion-dependent, noncovalent heterodimer resulting from proteolytic cleavage at the B/A3 junction that generates a heavy chain (A1-A2-B) and a light chain (A3-C1-C2). This procotector factor is cleaved by thrombin at Arg372-Ser373, Arg420-Ser421, and Arg1689-Ser1690. This paper is available online at http://www.jbc.org

1 The work was supported by National Institutes of Health Grant HL36365 and American Heart Association Grant 9950228N (both to S. P. B.) and by National Institutes of Health Grants HL03616 and HL38199 (to P. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 For comparison, the factor IX amino acid numbering system is used. The numbers with a prefix c (e.g. c57) in parentheses refer to the chymotrypsin equivalents for the protease domain of factor IXa (6).
to yield factor VIIa, a heterotrimer composed of A1, A2, and A3-C1-C2 subunits (14, 15). The A1 and A3-C1-C2 subunits remain associated with a divalent metal ion-dependent linkage, whereas the A2 subunit is weakly associated with the A1 and A3-C1-C2 dimer (16, 17). Although intact factor VIIIa is required for maximal enhancement of factor IXa activity, recent results demonstrate that the isolated A2 subunit stimulates factor IXa activity by ~100-fold (18).

Ca$^{2+}$-dependent assembly of factor IXa and factor VIIIa on a suitable PL surface is essential for hemostasis since defects or deficiency in the proteins result in severe bleeding diatheses, namely hemophilia A (factor VIII deficiency) or hemophilia B (factor IX deficiency) (Ref. 12; see the hemophilia A mutation database and the Haemophilia B database of point mutations (factor IX deficiency) (19). Suitable PL surface is essential for hemostasis since defects or deficiency in the proteins result in severe bleeding diatheses, namely hemophilia A (factor VIII deficiency) or hemophilia B (factor IX deficiency) (Ref. 12; see the hemophilia A mutation database and the Haemophilia B database of point mutations (factor IX deficiency) (19).

In this assembly, the Ca$^{2+}$-loaded form of the Glu domain of factor IXa binds to PL (21), whereas EGF1-3/EGF2 subunit residues 558–565 (Ser-Val-Asp-Gln-Arg-Gly-Asn-Gln) was observed and short additions and deletions, both available via the World Wide Web. In this assembly, the Ca$^{2+}$-loaded form of the Glu domain of factor IXa binds to PL (21), whereas EGF1-3/EGF2 subunit residues 558–565 (Ser-Val-Asp-Gln-Arg-Gly-Asn-Gln) was observed and short additions and deletions, both available via the World Wide Web.

Calculated point mutations (factor IX deficiency) (Ref. 12; see the hemophilia A mutation database and the Haemophilia B database of point mutations (factor IX deficiency) (Ref. 12; see the hemophilia A mutation database and the Haemophilia B database of point mutations (factor IX deficiency) (Ref. 12; see the hemophilia A mutation database and the Haemophilia B database of point mutations (factor IX deficiency).

To test the impact of the 558–565 region on the factor IXa:A2 subunit complex. EC50 is the functional constant, and the rates of formation of factor Xa were determined at increasing concentrations of the A2 subunit. Reaction mixtures contained 5 nM factor IXa, 250 nM factor X, 25 μM PL, and various concentrations of the A2 subunit in TBS/BSA, pH 7.5 containing 5 mM CaCl2. Reactions were carried out at 37 °C for 5–20 min and stopped by adding 1 μl of 500 mM EDTA. The amount of factor Xa generated was measured by hydrolysis of S-2222 as described previously (25, 34). The Kd and Kd values were obtained using the program GraFit from Erithacus Software.

The IC50 (concentration of inhibitor required for 50% inhibition) was determined by fitting the data to IC50 four-parameter logistic equation (Equation 1) by non-linear regression analysis using the program GraFit from Erithacus Software.

\[ V = \frac{V_{\text{max}}L}{EC_{50} + L} \]  

\[ y = \frac{a}{1 + (IC_{50})^b} + \text{background} \]

\[ y \] is the rate of formation of factor Xa at a given concentration of the A2 subunit, denoted by \( L \), and \( V_{\text{max}} \) is the rate of factor Xa formation by the factor IXa:A2 subunit complex. \( IC_{50} \) is the functional constant, defined as the concentration of free A2 subunit yielding 50% of the \( V_{\text{max}} \). The background rate of factor Xa generation was obtained by carrying out the reaction in the absence of the A2 subunit. This represented less than 1% of the \( V_{\text{max}} \) and was subtracted before data analysis. To obtain \( IC_{50} \) values as a function of substrate concentration, a series of experiments were performed in which factor X was varied from 50 nM to 1 μM.

**Determination of the Apparent Kd(A2) of Binding of dEGR-IXa Proteins with the A2 Subunit—**The \( EC_{50} \) (functional \( K_d \)) of binding of each factor IXa protein with the A2 subunit was measured essentially as described previously for its interaction with the intact factor VIIIa (25, 34). For these experiments, concentrations of factor IXa and factor X were kept constant, and the rates of formation of factor Xa were determined at increasing concentrations of the A2 subunit. Reaction mixtures contained 5 nM factor IXa, 250 nM factor X, 25 μM PL, and various concentrations of the A2 subunit in TBS/BSA, pH 7.5 containing 5 mM CaCl2. Reactions were carried out at 37 °C for 5–20 min and stopped by adding 1 μl of 500 mM EDTA. The amount of factor Xa generated was determined by S-2222 hydrolysis as described previously (25, 34). The \( IC_{50} \) was obtained by fitting the data to a single-site ligand binding equation (Equation 1) by non-linear regression analysis using the program GraFit from Erithacus Software.

\[ V = \frac{V_{\text{max}}L}{EC_{50} + L} \]  

\[ y = \frac{a}{1 + (IC_{50})^b} + \text{background} \]
factor Xa formation in the absence of dEGR-IXa, and $s$ is the slope factor. Each point was weighted equally, and the data were fitted to Equation 2 using the nonlinear regression analysis program GraFit from Erithacus Software. The background value represented $5\%$ of the maximum rate of factor Xa formation in the absence of dEGR-IXa.

To obtain apparent $K_d$ values for the interaction of dEGR-IXa proteins with A2, we used the following equation as described by Cheng and Prusoff (36) and further elaborated by Craig (37).

$$K_{d(app)} = \frac{IC_{50}}{1 + (\Delta [EC_{50}])} \quad (Eq. 3)$$

$K_{d(app)}$ is the concentration of factor IXa$_{WT}$, and $EC_{50}$ is the concentration of factor IXa$_{WT}$ that gives a $50\%$ maximum response in the absence of the competitor at a specified concentration of factor X used in the experiment.

**Fluorescence Quenching of the Dansyl Moiety in dEGR-IXa by the A2 Subunit**—Effect of the A2 subunit on the emission intensity of the dansyl moiety in each dEGR-IXa protein was determined using the SLM AB2 spectrophotometer. Each reaction mixture contained 220 nM dEGR-IXa in 20 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM CaCl$_2$, 0.01% Tween, 200 nM/µL BSA, and 100 µM PL vesicles. The excitation wavelength was 340 nm (slit width, 8 nm) and the emission wavelength was 540 nm (slit width, 8 nm). First, blank values (in triplicate) were obtained for the buffer containing PL. dEGR-IXa was then added, and the emission intensity in the absence of the A2 subunit was recorded. Each reaction mixture was subsequently titrated with the A2 subunit, and the emission readings (in triplicate) were obtained at each point. The fluorescence emission intensity at each point was corrected for increases in the reaction volume prior to analysis of the data. The volume of added A2 subunit did not exceed $10\%$ of the total volume. Data are presented as $F/F_0$, where $F_0$ is the emission intensity in the absence of A2 subunit and $F$ is the intensity at a given A2 subunit concentration.

**Determination of the Apparent $K_{d(peptide)}$ of binding of each factor IXa to the A2 558–565 peptide**—The apparent $K_{d(peptide)}$ for binding of each factor IXa to the A2 558–565 peptide was determined by its ability to inhibit the respective IXa:A2 subunit interaction, as measured by reduction in the rate of factor X activation in the tenase system. The reaction mixtures for both IXa$_{WT}$ and IXa$_{NP}$ contained 100 nM factor IXa, 30 nM A2 subunit, 250 nM factor X, and 25 µM PL in TBS/BSA, pH 7.5, with 5 mM CaCl$_2$. The reaction mixture for IXa$_{RE339Q}$ contained 300 nM factor IXa instead of 100 nM used for IXa$_{WT}$ or IXa$_{NP}$; concentrations of other components were the same. The amount of factor Xa generated was determined by hydrolysis of S-2222. The $IC_{50}$ values were obtained using Equation 2. Here, $y$ is the rate of activation of factor X by various factor IXa proteins. The kinetic constants for the interaction of factor IXa with A2 subunit and PL normally. Further, in the absence of factor VIIIa, activation of factor X by these IXa mutants is not impaired.

**RESULTS AND DISCUSSION**

**Activation of Factor X by Various Factor IXa Proteins in the Presence of Only Ca$^{2+}$ and PL**—The kinetic constants for the activation of factor X were obtained by various factor IXa proteins in the presence of Ca$^{2+}$ and several concentration of PL. This analysis was performed to establish whether or not the factor IXa proteins under investigation bind to Ca$^{2+}$ and PL normally and possess a functional active site. The kinetic constants obtained under these conditions in the absence of factor VIIIa are listed in Table I. All mutants activated factor X normally, and the specificity constant (kcat/Km) for each mutant at different PL concentrations did not differ appreciably from that observed for IXa$_{WT}$ or IXa$_{NP}$. The increase in kcat values at higher concentrations of PL for WT or for a given mutant may reflect binding of factor IXa and factor X to different PL vesicles (42, 43). Further, our $K_m$ and kcat values are in close agreement with the earlier published data (18, 43). Consistent with earlier observations (43), we also observed a slight increase in kcat for each factor IXa protein at higher concentrations of PL. Cumulatively, our data presented in Table I indicate that the factor IXa mutants under investigation interact with Ca$^{2+}$ and PL normally. Further, in the absence of factor VIIIa, activation of factor X by these IXa mutants is not impaired.

<table>
<thead>
<tr>
<th>Protein</th>
<th>PL</th>
<th>K0</th>
<th>Km</th>
<th>Kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>IXa$_{WT}$</td>
<td>10</td>
<td>0.10</td>
<td>0.012</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.16</td>
<td>0.022</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.21</td>
<td>0.032</td>
<td>0.156</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.63</td>
<td>0.062</td>
<td>0.098</td>
</tr>
<tr>
<td>IXa$_{NP}$</td>
<td>10</td>
<td>0.09</td>
<td>0.010</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.12</td>
<td>0.012</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.24</td>
<td>0.024</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.57</td>
<td>0.038</td>
<td>0.066</td>
</tr>
<tr>
<td>IXa$_{NP}$</td>
<td>10</td>
<td>0.13</td>
<td>0.010</td>
<td>0.076</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.23</td>
<td>0.018</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.26</td>
<td>0.028</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.54</td>
<td>0.038</td>
<td>0.070</td>
</tr>
<tr>
<td>IXa$_{RE339Q}$</td>
<td>10</td>
<td>0.11</td>
<td>0.012</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.18</td>
<td>0.018</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.23</td>
<td>0.030</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.61</td>
<td>0.040</td>
<td>0.066</td>
</tr>
<tr>
<td>IXa$_{R333Q}$</td>
<td>10</td>
<td>0.12</td>
<td>0.014</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.20</td>
<td>0.020</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.25</td>
<td>0.028</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.55</td>
<td>0.042</td>
<td>0.076</td>
</tr>
</tbody>
</table>

**FIG. 1.** Effect of the isolated A2 subunit of factor VIIIa on the rate of activation of factor X by various factor IXa proteins. Rate of formation of factor Xa by each factor IXa protein was measured as described under “Experimental Procedures.” The reaction mixtures contained 5 nM factor IXa, 250 nM factor X, and various concentrations of A2 subunit. The buffer used was TBS/BSA, pH 7.5 containing 25 µM PL and 5 mM CaCl$_2$. The proteins used are: IXa$_{WT}$ (closed circles), IXa$_{NP}$ (open circles), IXa$_{RE339Q}$ (closed triangles), and IXa$_{R333Q}$ (open triangles). The data were fitted to a single-site binding equation (Equation 1).
of factor X, the concentration of factor IXa was held constant at 100 nM IXaWT, 30 nM A2 subunit, 250 nM factor X, 25 μM PL and 5 mM CaCl2. Factor Xa concentration was measured by S-2222 hydrolysis.

The presence of the A2 subunit in the reaction mixtures enhanced the factor X-activating activity of IXa by a factor X (23). More importantly, our data with the IXa VIIhelix mutant indicate that the EGF1 domain of factor IXa does not interact with the A2 subunit of factor VIIIa. In further experiments, we measured the EC50 values for interaction of various factor IXa mutants. These data are presented in Fig. 1. At each concentration of factor X, the concentration of factor IXa was held constant at 5 nM and the rate of factor Xa generation was determined in the presence of increasing concentrations of the A2 subunit. The EC50 values ranged from 200 to 280 nM at lower concentrations of factor X (<150 nM) to ~200 nM at higher concentrations of factor X (>1 μM) for both IXaWT and IXaPCEGF1. Our functional Kd (EC50) values ranging from 200 to 280 nM for the interaction of IXaWT (or IXaPCEGF1) and the A2 subunit employing different factor X concentrations are consistent with the EC50 values obtained earlier using similar conditions for IXa and the A2 subunit (18). From these observations, we conclude that factor X does not appreciably influence the functional Kd of IXa:A2 subunit interaction. This is in contrast to the results obtained using factor VIIIa where factor X reduces the functional Kd of IXa:VIIIa interaction by ~10-fold (34). These results support previous observations that the A1 subunit of factor VIIIa interacts with factor X (23). More importantly, our data with the IXaPCEGF1 mutant indicate that the EGF1 domain of factor IXa does not interact with the A2 subunit of factor VIIIa.
The steady state inhibition of IXa WT:A2 subunit interaction by A2 Subunit with dEGR-IXa Proteins—
with IXaWT and IXaPCEGF1, and similarly modulates the emissions. Consistent with the data presented in Figs. 1 and 3, with the values obtained from steady state inhibition experiments, the fluorescence quenching measurements is in agreement as the absolute temperature (298 K), and $T$ is the gas constant (1.987  10^{-3} \text{ kcal mol}^{-1} \text{ deg}^{-1}$), $K_d$ is the dissociation constant.

The change in Δ$G^{a}$ values between the mutant and WT is given in parentheses.

ND, not determined.

**Table II**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Apparent $K_{d(A2)}$ (μM)</th>
<th>Δ$G^{aA2}$ (kcal mol$^{-1}$)</th>
<th>Apparent $K_{d(peptide)}$ (μM)</th>
<th>Δ$G^{peptide}$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IXaWT</td>
<td>100 ± 11</td>
<td>9.54</td>
<td>4 ± 1</td>
<td>7.36</td>
</tr>
<tr>
<td>IXaPCEGF1</td>
<td>114 ± 15 (1'$)</td>
<td>8.47 (0.07)'</td>
<td>4 ± 1 (1)'</td>
<td>7.36 (0.00)'</td>
</tr>
<tr>
<td>IXaR333Q</td>
<td>1850 ± 82 (18)</td>
<td>7.82 (1.72)</td>
<td>62 ± 9 (15)</td>
<td>5.74 (1.62)</td>
</tr>
<tr>
<td>IXaVIIhelix</td>
<td>&gt;10$^4$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a The -fold change in apparent $K_d$ values (mutant/WT) is given in parentheses.

b Gibbs free energy values were calculated using the equation, Δ$G = -RT\ln K_d$, where $R$ is the gas constant (1.987  10^{-3} \text{ kcal mol}^{-1} \text{ deg}^{-1}$), $T$ is the absolute temperature (298 K), and $K_d$ is the dissociation constant.

c The change in Δ$G^{a}$ values for the interaction of various factor IXa proteins with the A2 subunit and the A2 558–565 peptide

Determination of Apparent $K_{d(A2)}$ Values for the Interaction of A2 Subunit with dEGR-IXa Proteins—Here, we investigated the steady state inhibition of IXaWT:A2 subunit interaction by different dEGR-IXa proteins. These data are presented in Fig. 3. The IC_{50} values were obtained using Equation 2, and the respective apparent $K_{d(A2)}$ values were obtained using Equation 3. eGFP-IXaWT and eGFP-IXaPCEGF1 interacted with the A2 subunit with similar $K_{d(A2)}$ values, whereas dEGR-IXaR333Q interacted with the A2 subunit with a $K_{d(A2)}$ value of 1.8 μM and dEGR-IXaVIIhelix failed to compete with factor IXaWT up to 12 μM concentration. The apparent $K_{d(A2)}$ values obtained from the inhibition data (Fig. 3) and IC_{50} values (200–280 nM) obtained from the potentiation of factor X activation data (Figs. 1 and 2) for the factor IXaWT and IXaPCEGF1 are in close agreement with each other. Of significance is the observation that the mutations in the helix 330 (c162) of the protease domain of factor IXa severely impairs its interaction with the A2 subunit.

Effects of the A2 Subunit on the Fluorescence Emission of eGFP-IXa Proteins—Since dansyl emission is quite sensitive to its environment, we examined the changes in dansyl emission intensity (excitation wavelength, 340 nm; emission wavelength, 540 nm) of eGFP-IXa proteins in the presence of increasing concentrations of the A2 subunit. Reaction mixtures contained 220 nm each of factor IXa, 100 μM PL, and various concentrations of the isolated A2 subunit. The results are presented in Fig. 4. For IXaWT or IXaPCEGF1, a dose-dependent decrease in the fluorescence emission of the dansyl probe was observed. However, little if any change in the emission intensity was observed when the A2 subunit was titrated into the reaction mixtures containing factor IXaR333Q or IXaVIIhelix. A nonlinear least squares fitting of the data for IXaWT and IXaPCEGF1 to a bimolecular association model yielded a plateau value of 0.59 ± 0.05 for $F/F_0$ and an apparent $K_{d(A2)}$ value of 82 ± 18 nM for each protein. Further, the $K_{d(A2)}$ for IXaR333Q or IXaVIIhelix could not be calculated. These results suggest that the isolated A2 subunit interacts equivalently with IXaWT and IXaPCEGF1, and similarly modulates the emission of the active site-labeled dansyl probe. The apparent $K_{d(A2)}$ value of 82 nM for factor IXaWT or IXaPCEGF1 obtained using the fluorescence quenching measurements is in agreement with the values obtained from steady state inhibition experiments. Consistent with the data presented in Figs. 1 and 3, these fluorescence results suggest that the factor IXaR333Q and IXaVIIhelix mutations are severely impaired in their interactions with the A2 subunit.

Determination of Apparent $K_{d}$ Peptide Values for Binding of Factor IXa Proteins to the A2 558–565 Peptide—The data presented thus far strongly indicate that the A2 subunit interacts with residues of the helix 330 (c162) of factor IXa. Previous studies also suggest that residues 558–565 of the A2 subunit are involved in binding to factor IXa (18). However, it is not known whether the 558–565 peptide region of the A2 subunit represents the site of direct interaction with the helix 330 of factor IXa. We investigated this possibility by measuring the affinity of the A2 558–565 peptide for IXaWT, IXaPCEGF1, and IXaR333Q. These data are presented in Fig. 5. The A2 558–565 peptide inhibits the interaction of IXaWT and IXaPCEGF1 with similar IC_{50} values of 18 nM. However, the A2 558–565 peptide inhibited the IXaR333Q:A2 subunit interaction with an IC_{50} value of 70 μM, which is 9-fold higher than the value obtained for IXaWT or IXaPCEGF1 (Fig. 5). We next used the Cheng and Prusoff relationship (36, 37) to calculate apparent $K_{d}$ (peptide) values for each factor IXa protein. These apparent $K_{d}$ (peptide) values along with the changes in Gibbs free energy are listed in Table II. The peptide bound to IXaWT and IXaPCEGF1 with an apparent $K_{d}$ of 4 μM and to IXaR333Q with an apparent $K_{d}$ of 62 μM. Thus, the affinity of the A2 558–565 peptide for IXaWT and IXaPCEGF1 is similar, whereas it is reduced 15-fold for the IXaR333Q.

Notably, comparison of the data presented in Figs. 3 and 5 reveal that the change in apparent $K_{d(A2)}$ or $K_{d(peptide)}$ for IXaR333Q is similar as compared with the apparent $K_{d(A2)}$ or $K_{d(peptide)}$ obtained for IXaWT (or IXaPCEGF1). Further, the difference in Δ$G^{a}$ for the interaction of A2 subunit with IXaWT (or IXaPCEGF1) and IXaR333Q is 1.72 kcal mol$^{-1}$ (Table II). This difference in Δ$G^{a}$ is essentially the same as that (1.62 kcal mol$^{-1}$) obtained for the interaction of A2 peptide with IXaWT (or IXaPCEGF1) and IXaR333Q. If the A2 558–565 peptide bound to a different region than the helix 330 of factor IXa, then one would expect it to bind to IXaR333Q with the same affinity as that for IXaWT. Since this is not the case, our data support a conclusion that the helix 330 (c162) in factor IXa is most likely in direct contact with the 558–565 region of the A2 subunit.

Modeling of the Interface between the Protease Domain of Factor IXa and the A2 Subunit of Factor VIIIa—Based upon the preceding information, we modeled the interface between the protease domain of factor IXa (Ref. 5, Protein Data Bank code 1RFN) and the A2 subunit (see “Experimental Procedures”) by bringing together the helix 330 of factor IXa and the 310 helical turn in residues 558–565 of the A2 subunit and maximizing the interaction among the charged residues. Emphasis was also given for interactions involving hydrogen bonds and hydrophobic contacts. An important guiding principle in the construction of this interface model was that the Gla domain of factor IXa and the C2 domain of factor VIIIa must be oriented such that each may contact the PL surface. To achieve

The present IC_{50} value (18 nM) for the A2 peptide inhibition of the IXaWT-A2 subunit interaction is 5-fold lower than the IC_{50} value (40 μM) obtained from the inhibition studies of the A2 subunit enhancement of IXaNP activity (18). This difference in IC_{50} values is most likely due to the different concentrations (30 nM in present study versus 240 nM in previous study) of the A2 subunit used in the two studies.
this, the A2 structure (along with the A1 and A3 subunits) was rotated and translated as a rigid body. The principle approach used was that described earlier by Tulinsky and co-workers (44) in building the prothrombin model from the structures of prothrombin fragment 1 and the fragment 2-thrombin complex. Minor adjustments in the side chains of both proteins were also made. All residues in the interface of both proteins were checked for distances to ensure no improper contacts (45). The interface model that resulted from this approach is shown in Fig. 6A. In this display, the Gla domain of factor IXa and the C2 domain of factor VIIIa are projecting away from the viewer.

In addition to the A2 558–565 region and the factor IXa 330–338 region, other spatially nearby regions that may play important roles in the interaction of A2 subunit with the protease domain were also noted. The details of the composite interface region are shown in Fig. 6B. It appears that electrostatic forces might play a significant role in the interaction between the A2 subunit and the protease domain, and an electrostatic potential for the interface calculated using the program GRASP (46) is shown in Fig. 6C. Further, in addition to the electrostatic interactions outlined in Fig. 6, hydrophobic and polar uncharged interactions between Thr^{443} (c175) and Tyr^{445} (c177) of factor IXa and His^{444} of the A2 subunit were observed. Moreover, a hydrogen bond between Asn^{258} (c93) of factor IXa and Ser^{709} of the A2 subunit could also be formed. Importantly, a significant hydrophobic patch involving Ile^{566} and Met^{567} in the A2 subunit and Ile^{298} (c129B), Tyr^{295} (c128), Phe^{299} (c133), Phe^{302} (c133), Phe^{378} (c208), and Phe^{98} (EGF2 domain) in factor IXa was noted. Thus, it appears that the hydrogen bonds as well as the hydrophobic and electrostatic interactions all play important roles in the proposed interface between factor IXa and the A2 subunit. In this context, an apparent K_d(A2) of ~100 nM observed for this interaction reflects the net change in free energy involved in making and breaking such bonds.

A factor IXa-interactive site comprising residues 484–509 in the A2 subunit that was identified using a monoclonal antibody (47) does not appear to contact the protease domain in our interface model. However, it should be noted that, in a previous study (48), Lollar et al. concluded that this same monoclonal antibody does not interfere with the IXa:VIIIa interaction. The

Fig. 6. Interface model between the factor IXa protease domain and the A2 subunit of factor VIIIa. The coordinates for the human factor IXa structure are from the Brookhaven Protein Data Bank (code 1RFN) and the coordinates for the A1, A2, and A3 subunits (12) of factor VIIIa are based upon homology models built using ceruloplasmin coordinates (Protein Data Bank code 1KCW). A, schematic representation of the interface model. Ribbon structure for each protein is depicted. The IXa protease domain is in light blue, and the EGF2 domain is in red. The A1 subunit is in yellow, the A2 subunit is in magenta with residues 484–509 in white, and the A3 subunit is in cyan with the COOH terminus in red. The Gla and the EGF1 domains of factor IXa and the C1 and C2 domains of factor VIIIa are not shown. The interface residues of factor IXa protease domain and of the A2 subunit are shown as CPK space-filling models. The molecules are oriented such that the Gla domain of factor IXa and the C2 domain of factor VIIIa are projecting away from the viewer. The Gla domain in factor IXa and the C2 domain of factor VIIIa bind to the PL surface.

B, detailed interface between factor IXa protease domain and the modeled A2 subunit. Only the charged residues that participate in the binding interactions are depicted. The hydrophobic residues that participate in this interaction are discussed in the text. The orientation of the molecules is the same as in A. Chymotrypsin numbering system for the factor IXa protease domain is used. Corresponding factor IX numbering system are 338 (c170), 332 (c164), 333 (c165), 346 (c178), 403 (c233), 293 (c126), and 410 (c240). Factor IXa residues are labeled light blue, and A2 subunit residues are labeled magenta. C, electrostatic potential between the factor IXa protease domain and the A2 subunit interface as determined using the program GRASP (46). Blue represents positive, red represents negative, and white represents neutral residues.
reason(s) for the differing results obtained in the two studies (47, 48) is not fully understood. Further, in the proposed interface model shown in Fig. 6A, the 484–509 region in the A2 subunit is not in close proximity to the 558–565 interface region and shows no apparent contacts with factor IXa. However, we cannot exclude the possibility of a change in the conformation of the A2 subunit upon binding the protease domain, which may juxtapose (and subsequently involve) this region. Alternatively, the monoclonal antibody may prevent the association of the A2 subunit with factor IXa through steric interference.

Analysis of Hemophilia Data Bases—Of significance is the observation that numerous mutations in the helix 330 (c162) of factor IXa cause hemophilia B (see Ref. 25 and the Haemophilia B data base of point mutations and short additions and deletion), whereas several mutations in or near factor VIII residues 558–565 result in hemophilia A (Ref. 12; see hemophilia data base, World Wide Web). Further, Arg321 (c165) in our interface model (Fig. 6) interacts with Glu440 residue of the A2 subunit, and mutations in the Arg321 (c165) that eliminate the charge (Arg → Gln or Leu) cause severe hemophilia B (see Haemophilia B data base of point mutations and short additions and deletion, available via the World Wide Web). Further, Asn346 (c178) of factor IXa interacts with both Lys470 and Glu445 of the A2 subunit, and a mutation of Asn346 (c178) to Asp causes hemophilia B (see Haemophilia B data base). Similarly, Arg303 (c233) in our model interacts with Glu633 of the A2 subunit and mutations in Arg303 (c233) to Trp or Gln cause hemophilia B (see Haemophilia B data base). Moreover, Arg438 (c170) of factor IXa interacts with Asp560 of the A2 subunit, and mutations in both of these residues result in hemophilia (HAM- SERTS data base and Haemophilia B data base, both available via the World Wide Web). In addition, Arg562 contained within the A2 558–565 peptide region is cleaved by activated protein C (17), and factor IXa selectively protects this site from cleavage (49). In support of this observation, Arg562 of the A2 subunit along with Glu661 interacts with Asp332 (c164) in our interface model and change of Asp332 (c164) to Tyr results in hemophilia B (see Haemophilia B data base).

Mutations in the hydrophobic patch of the interface model are also known to cause bleeding diathesis. Thus, mutation of Phe287 (c208) to Val or Leu in factor IXa causes hemophilia B (see Haemophilia B data base, available via the World Wide Web), and change of Ile566 to Thr or Arg in the A2 subunit causes hemophilia A (Ref. 12; see hemophilia data base, World Wide Web). The mutation of Ile566 to Arg is expected to disrupt the hydrophobic interaction. However, Thr substitution yields Asn-X-Thr consensus sequence that leads to a new N-glycosylation site at Asn664, which could disrupt the Factor IXa:A2 subunit interaction. Moreover, change of Phe302 (c133) to Ala has been shown to impair the interaction of factor IXa with factor VIIIa (19). Mutations of Phe302 (c133) to Ala and Phe287 (c208) to Val or Leu are expected to diminish the hydrophobic interactions involving Ile566 and Met567 of the A2 subunit. The change of Ile566 to Arg in the A2 subunit would have similar consequences.

Concluding Remarks—Previous studies have indicated that the helix 330 (c162) of the protease domain (25) and 558–565 region of the A2 subunit (18) represent important determinants for the interaction of factor IXa and factor VIIIa, respectively. However, it was not known whether these two regions interact with each other in the IXa:VIIIa complex. The present study provides evidence that these two regions may form an interface and interact with each other involving hydrophobic as well as electrostatic forces (Fig. 6). Modeling of the interface suggests that other spatially nearby regions may also participate in the interaction of factor IXa with factor VIIIa. Several mutations in the proposed interface cause hemophilia A or B and are known to impair IXa:VIIIa interaction. Thus, our interface model is compatible with the existing biochemical as well as with the two-dimensional electron crystallography data of Stoylova et al. (20). However, the three-dimensional co Crystal structure of the factor IXa protease domain and A2 subunit will be required to fully establish this view. In light of these considerations, we emphasize that our interface model is an interim model subject to refinement as new biochemical and experimentally determined structural data become available.

Acknowledgments—We thank Tomasz Heyduk and Jim Kiefer for useful discussions. We also thank Lisa Regan and James Brown of the Laboratory for Molecular Medicine and Debra Pittman of the Genetics Institute for providing the recombinant factor VIII, and Jennifer Chandler for excellent technical assistance.

REFERENCES

S. Paul Bajaj, Amy E. Schmidt, Akash Mathur, K. Padmanabhan, Degang Zhong, Maria Mastri and Philip J. Fay

doi: 10.1074/jbc.M011680200 originally published online February 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011680200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 22 of which can be accessed free at http://www.jbc.org/content/276/19/16302.full.html#ref-list-1