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The Gla Domain of Factor IXa Binds to Factor VIIIa in the Tenase Complex*

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During blood coagulation factor IXa binds to factor VIIIa on phospholipid membranes to form an enzymatic complex, the tenase complex. To test whether there is a protein-protein contact site between the γ-carboxyglutamic acid (Gla) domain of factor IXa and factor VIIIa, we demonstrated that an antibody to the Gla domain of factor IXa inhibited factor VIIIa-dependent factor IXa activity, suggesting an interaction of the factor IXa Gla domain with factor VIIIa. To study this interaction, we synthesized three analogs of the factor IXa Gla domain (FIX1–47, with Phe-9, Phe-25, or Val-46 replaced, respectively, with benzoylphenylalanine (BPA), a photoactivatable cross-linking reagent. These factor IX Gla domain analogs maintain native tertiary structure, as demonstrated by calcium-induced fluorescence quenching and phospholipid binding studies. In the absence of phospholipid membranes, FIX1–47 was able to inhibit factor IXa activity. This inhibition is dependent on the presence of factor VIIIa, suggesting a contact site between the factor IXa Gla domain and factor VIIIa. To demonstrate a direct interaction we did cross-linking experiments with FIX1–47BPA, FIX1–47BPA, and FIX1–46BPA. Covalent cross-linking to factor VIIIa was observed primarily with FIX1–47BPA and to a much lesser degree with FIX1–46BPA. Immunoprecipitation experiments with an antibody to the C2 domain of factor VIIIa indicate that the factor IXa Gla domain cross-links to the A3-C1-C2 domain of factor VIIIa. These results suggest that the factor IXa Gla domain contacts factor VIIIa in the tenase complex through a contact site that includes phenylalanine 25 and perhaps valine 46.

Factor IX is composed of a single polypeptide comprising an N-terminal γ-carboxyglutamic acid (Gla)-rich domain, amino acid acid stack domain, and C-terminal serine protease domain. The Gla domain contains 12 γ-carboxyglutamic acid residues and a posttranslationally modified glutamic acid residue. γ-Carboxyla-
as the first epidermal growth factor domain of factor IXa and the A3 domain of factor VIIIa (22). Furthermore, a naturally occurring mutation of the Gla domain of factor IX leads to loss of factor VIIIa-mediated tenase activity (23). In this report, we use a synthetic analog of the factor IX Gla domain, FIX\textsubscript{1–47} (Fig. 1), which we modify by replacing a specific hydrophobic amino acid with the photoactivatable amino acid, benzoylphenylalanine. With these Gla domain peptides, we provide direct evidence using a cross-linking strategy for the binding of the factor IX Gla domain to the light chain of factor VIIIa.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Factor IXa and factor X were purchased from Hematologic Technologies Inc. Recombinant human factor VIII formulated in sucrose (Kogenate FS) was received as a gift from Bayer Canada and activated to factor VIIIa as described previously (24). Bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), and d ansylated phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids. Chromogenic substrate S-2765 was purchased from Chromogenix. Anti-FIX antibodies were purchased from American Diagnostica. Antibodies to the Glu domain of factor IXa were prepared as previously described (6). An antibody to γ-carboxyglutamic acid was a gift from Dr. Johan Stenflo (25). The FIX\textsubscript{1–47}BPA analogues were synthesized as previously described (4).

**Enzymatic Assays**—The activation of factor X by factor IXa was measured with a two-step amidolytic substrate assay. For inhibition experiments with the FIX\textsubscript{1–47}BPA peptides or with anti-FIX CaCl\textsubscript{2}-specific antibodies, factor IXa (20 nM) was incubated with 500 nM factor X, 4 mM factor VIIIa, and the specified concentrations of the FIX\textsubscript{1–47} peptides or anti-FIX CaCl\textsubscript{2}-specific antibodies in 150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM CaCl\textsubscript{2}, and 0.1% bovine serum albumin for 60 min at 25 °C. The reaction was then stopped with 150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, and sonicated in a bath sonicator until the solution cleared. The suspension of phospholipid vesicles was centrifuged, first at 160,000 \( \times \) g for 30 min and then at 250,000 \( \times \) g for 90 min in a Beckman L8–80M ultracentrifuge using a Ti-70.1 rotor. The supernatant contained the small unilamellar phospholipid vesicles. Phospholipid concentrations were determined by phosphorus analysis (27).

**Cross-linking Experiments**—Covalent cross-linking of BPA-labeled FIX\textsubscript{1–47} peptides to factor VIIIa was performed through photoactivation of β-benzoyl-phenylalanine substituted for specific hydrophobic residues in FIX\textsubscript{1–47}. Each of the three peptides (FIX\textsubscript{1–47}BPA, FIX\textsubscript{1–47}25BPA, or FIX\textsubscript{1–47}46BPA), at a concentration of 1 \( \mu \)M, was mixed with factor VIIIa (100 nM) in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5 mM CaCl\textsubscript{2}. The samples were irradiated with a B-100 AP ultraviolet lamp (UVP) for 20 min at a distance of 5.5 cm. Non-covalent Ca\textsuperscript{2+}-mediated protein-protein binding was reversed by the addition of SDS and EDTA and heating at 37 °C for 5 min. The samples were run on a 7.5% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. The membrane was probed with a primary mouse monoclonal antibody to γ-carboxyglutamic acid, followed by the addition of a secondary antibody, sheep anti-mouse IgG horseradish peroxidase-linked antibody. The blots were developed with an ECL reagent (Amersham Biosciences, UK) according to the manufacturer’s protocol.

**Immunoprecipitation**—Immunoprecipitation experiments were carried out with ESH8, a monoclonal antibody to the C2 domain of factor VIIIa. After irradiation and photo-induced cross-linking, the samples were immunoprecipitated with ESH8, subjected to SDS-PAGE, and analyzed by Western blotting as described for the cross-linking experiments.

**RESULTS**

The tenase complex, assembled on phospholipid membranes, is composed of the enzyme, factor IXa, and the cofactor, factor VIIIa. To test the hypothesis that the factor IXa Glu domain might bind to factor VIIIa, inhibition of factor IXa-catalyzed activation of factor X was performed in the presence or absence of factor VIIIa using an antibody to the calcium-stabilized...
conformation of factor IXa (anti-FIX-Ca(II)-specific antibodies). The epitope recognized by this antibody is targeted against residues 1–11 within the Gla domain of factor IXa. Because anti-FIX-Ca(II)-specific antibodies inhibit factor IX binding to phospholipid membranes (6), phospholipid membranes were omitted from this assay system. In the absence of phospholipid membranes, factor IXa can catalyze the conversion of factor X to factor Xa albeit at a significantly reduced rate. Anti-FIX-Ca(II)-specific antibodies inhibited the generation of factor Xa by factor IXa, if factor VIIIa was present (Fig. 2A). In the absence of factor VIIIa, there was no inhibition of the conversion of factor X to factor Xa by factor IXa in the presence of the indicated concentrations of an antibody to the calcium-bound form of the factor IXa Gla domain. B, factor IXa (20 nM) and factor X (500 nM) were incubated without factor VIIIa (4 nM) ( ) for 60 min at room temperature in 5 mM CaCl2 in the presence of the indicated concentrations of an antibody to the calcium-bound form of the factor IXa Gla domain. B, factor IXa (20 nM) and factor X (500 nM) were incubated without factor VIIIa ( ) for 120 min at room temperature in 5 mM CaCl2 in the presence of the indicated concentrations of an antibody to the calcium-bound form of the factor IXa Gla domain.

A peptide containing the Gla and aromatic amino acid stack domains of factor IXa, FIX1-47, was also used to study factor IXa Gla domain-factor VIIIa interactions. FIX1-47 has been previously demonstrated to be a good model for studying the biochemical properties of the factor IXa Gla domain (28). To demonstrate direct binding between the Gla domain of factor IXa and factor VIIIa, analogs of FIX1-47 containing benzoyl-phenylalanine (BPA) were synthesized. BPA forms covalent bonds with adjacent structures within a 3 Å radius when irradiated at 350 nm (30). It has been previously used to identify the residues that constitute a phospholipid binding site in factor IXa (4). Three analogs were chosen based upon the positioning of the BPA in the Gla domain, and each had different amino acids replaced with BPA (Fig. 1). Phe-9 and Val-46 were replaced, respectively, in two peptides (FIX1-479BPA and FIX1-4746BPA) and have been previously characterized (4). A third peptide was prepared with Phe-25 replaced by BPA. All three peptides bind calcium similarly to unsubstituted FIX1-47 (28), suggesting that the BPA substitution does not disrupt tertiary structure (Fig. 3A). Fifty percent fluorescence quenching occurred at 210, 100, and 317 μM CaCl2 for FIX1-47 (2.4 μM), and FIX1-4746BPA (1.5 μM), and FIX1-479BPA (1.5 μM) for phospholipid membranes (PS:PC: dansylated PE = 25:65:10) in Tris-buffered saline/5 mM CaCl2, measured by fluorescence energy transfer. The dissociation constant, KD, was 1.2 μM, and the binding was reversible with EDTA. Kd and the line of best fit were calculated using non-linear regression employing SigmaPlot 8.0 for Windows (SPSS). Calculations were based on a bimolecular model as previously described (29).

Fig. 2. An antibody to the Gla domain of factor IXa inhibits factor VIIIa-mediated activation of factor X by factor IXa. A, factor IXa (20 nM) and factor X (500 nM) were incubated with factor VIIIa (4 nM) ( ) for 60 min at room temperature in 5 mM CaCl2 in the presence of the indicated concentrations of an antibody to the calcium-bound form of the factor IXa Gla domain. B, factor IXa (20 nM) and factor X (500 nM) were incubated without factor VIIIa ( ) for 120 min at room temperature in 5 mM CaCl2 in the presence of the indicated concentrations of an antibody to the calcium-bound form of the factor IXa Gla domain.

Fig. 3. Properties of the FIX1-47 peptides containing BPA. A, intrinsic fluorescence perturbations upon calcium binding. Changes in intrinsic fluorescence in FIX1-479BPA ( ), FIX1-4725BPA ( ), or FIX1-4746BPA ( ) upon binding to calcium was monitored at 340 nm. Varying concentrations of calcium chloride were added, as indicated, to 1.5 ml of 20 μM peptide in 20 mM Tris-HCl, pH 7.4, at 25 °C. Fluorescence were measured by irradiation at an excitation wavelength of 280 nm (5-nm bandpass) and monitored at an emission wavelength of 340 nm (5-nm bandpass). B, binding of FIX1-4725BPA to phospholipid vesicles. The binding of FIX1-4725BPA to phospholipid vesicles (PS:PC: dansylated PE = 25:65:10) in Tris-buffered saline/5 mM CaCl2 was measured by fluorescence energy transfer. The dissociation constant, KD, was 1.2 μM, and the binding is reversible with EDTA. KD and the line of best fit were calculated using non-linear regression employing SigmaPlot 8.0 for Windows (SPSS). Calculations were based on a bimolecular model as previously described (29).
The Factor IXa Gla Domain Binds to Factor VIIIa

FIG. 4. Inhibition of the catalytic function of the factor VIIIa-factor IXa complex but not factor IXa alone by FIX1-47,25BPA. A, factor IXa (20 nM) and factor X (500 nM) were incubated with factor VIIIa (4 nM) for 60 min at room temperature in 5 mM CaCl₂ and the indicated concentrations of FIX1-47,25BPA. The line of best fit was calculated with a non-linear least-square fit regression curve using SigmaPlot 8.0 for Windows (SPSS). B, factor IXa (20 nM) and factor X (500 nM) were incubated without factor VIIIa for 120 min at room temperature in 5 mM CaCl₂ and the indicated concentrations of FIX1-47,25BPA. Shown is a representative experiment of four performed in duplicate.

The photoactivatable peptides, FIX1-47,9BPA, FIX1-47,25BPA, or FIX1-47,46BPA, were added to factor VIIIa (100 nM) in the presence of CaCl₂ (5 mM) and irradiated for 20 min at 350 nm. Non-covalent interactions dissociated upon treatment with SDS and EDTA. The sample was run on an SDS-PAGE gel and developed with an anti-γ-carboxyglutamic acid antibody. For each reaction, equal quantities of material were loaded on each lane. A3/C1/C2, A2, and A1 refer to the expected migration positions of the factor VIIIa component peptides.

coupled to factor VIIIa migrated on SDS gels in a position just above that of the light chain of factor VIIIa, suggesting a covalent complex between FIX1-47,25BPA and the light chain of factor VIIIa. To substantiate this result, we immunoprecipitated the cross-linked material with ESH8, monoclonal antibody to the C2 domain of factor VIIIa. The immunoprecipitate was run on an SDS-PAGE and Western blotted with an anti-Gla antibody. This result, shown in Fig. 6, substantiates the conclusion that the region on factor VIIIa to which FIX1-47,25BPA binds is the light chain (A3-C1-C2 domain).

To establish the specificity of the cross-linking of FIX1-47,25BPA to the light chain of factor VIIIa, full-length factor IXa was employed at different concentrations to inhibit cross-linking. As shown in Fig. 7, the addition of increasing amounts of factor IXa led to complete inhibition of the cross-linking of FIX1-47,25BPA to factor VIIIa.

DISCUSSION

In the present work, we demonstrate that the Gla domain of factor IXa interacts with the light chain of factor VIIIa. A
schematic model of this complex, assembled on phospholipid membranes, is presented in Fig. 8. Two contact sites on factor IXa have previously been implicated in this interaction. Using fluorescence anisotropy, Fay et al. demonstrated a contact site between the A2 domain of factor VIIIa and the serine protease domain of factor IXa. This interaction was characterized by an affinity of ~300 nM (31–33). Another contact site of higher affinity (~15 nM) has been identified between the A3 domain of factor VIIIa and the first epidermal growth factor domain of factor IXa (34). In the current study, we show that the Gla domain of factor IXa and light chain of factor VIIIa, both phospholipid-binding structures, also bind to each other.

Because factor VIIa is homologous with factor IXa, the contact sites through which tissue factor activates factor VIIa provide useful comparisons to the contact sites through which factor VIIIa activates factor IXa. On-off rates using both fluorescence spectroscopy and surface plasmon resonance demonstrate three interactive sites between the enzyme, factor VIIa, and its cofactor, tissue factor. A site of interaction between the Gla domain of factor VIIa and tissue factor is one site of interaction analogous to that identified in the present work. This site is considered to be of lower affinity in comparison to the other sites of interaction. The similar properties that we describe for the factor VIIa-factor IXa Gla domain complex are consistent with that report (35). Fluorescent labeling of the involved residues in tissue factor that contact factor VIIa confirm that these sites become protected from solvent upon binding factor VIIa (36).

Two independent lines of evidence suggest that the contact between the light chain of factor VIIIa and the factor IXa Gla domain is physiologically important. First, a naturally occurring mutation in the Gla domain of factor IXa leading to hemorrhagia B affect function only in the presence of factor VIIIa. This propisitus, with a substitution of glycine 12 by arginine, has a severe bleeding phenotype (23). Second, factor IXa-factor VIIIa interactions can be inhibited by a peptide comprising the Gla domain of factor IXa, FIX₁₋₄₇, in the presence of phospholipid bilayer.²

² M. Shenone, B. Furie, and B. C. Furie, unpublished data.

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Our data suggest an interaction between the C-terminal half of the Gla domain of factor IXa and the light chain of factor VIIIa. In the folded Gla domain Phe-25 is located at the distal end from the omega loop and phospholipid binding site and not too distant from residue 46. The side chains of Phe-25 and Val-46 project from the backbone of the Gla domain at an angle greater than 90 degrees from one another. Nevertheless, it is possible that the side chains of both residues interact with the light chain of factor VIIIa, although from the cross-linking data Phe-25 may interact more effectively than Val-46 (5). The failure of FIX₁₋₄₇ to cross-link to factor VIIIa suggests that the face of the omega loop, including Phe-9, does not contact factor VIIIa; in the presence of membranes it is buried in the phospholipid bilayer.

In this study, we identify a region of the factor IXa Gla domain containing Phe-25 and Val-46 as a contact site with factor VIIIa. These contact sites are localized to the C-terminal portion of the Gla domain, which is similar to two homologous serine proteases, factor VIIa and protein C. The crystal structure of the factor VIIa-tissue factor complex demonstrates a direct interaction between the C-terminal region of the Gla domain of factor VIIa and tissue factor (37). In the case of the
protein C-protein S complex, replacement of the Gla domain of protein C with that of prothrombin renders protein C activity independent of protein S, its cofactor. This protein S dependence localizes to residues 23–46 of the Gla domain of protein C (38).

In conclusion, our studies demonstrate a specific interaction between the Gla domain of factor IXa and the light chain of factor VIIIa. Given that both the Gla domain of factor IXa and the C2 domain of factor VIIIa are membrane-binding structures, we envision these regions bound to membranes and to each other. Whether the occupancy of this contact site promotes allosteric activation of the factor VIIIa-factor IXa complex on phospholipid membranes remains to be determined.

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