The Factor IX γ-Carboxyglutamatic Acid (Gla) Domain Is Involved in Interactions between Factor IX and Factor Xla

Aysar Aktimur‡, Melanie A. Gabriels, David Gailani‡, and John R. Toomey§

From the ‡Departments of Pathology and Medicine, Vanderbilt University, Nashville, Tennessee 37232 and the §Cardiovascular and Urogenital Diseases Center of Excellence, GlaxoSmithKline, King of Prussia, Pennsylvania 19406

Received for publication, December 13, 2002
Published, JBC Papers in Press, December 20, 2002, DOI 10.1074/jbc.M212748200

During hemostasis, factor IX is activated to factor IXa by factor VIIa and factor Xla. The glutamic acid-rich γ-carboxyglutamatic acid (Gla) domain of factor IX is involved in phospholipid binding and is required for activation by factor VIIa. In contrast, activation by factor Xla is not phospholipid-dependent, raising questions about the importance of the Gla for this reaction. We examined binding of factors IX and IXaβ to factor Xla by surface plasm reversal. Plasma factors IX and IXaβ bind to factor Xla with Kᵦ values of 120 ± 11 nM and 110 ± 8 nM, respectively. Recombinant factor IX bound to factor Xla with a Kᵦ of 107 nM, whereas factor IX with a factor VIIa Gla domain (rFIX/VII-Gla) and factor IX expressed in the presence of warfarin (rFIX-desGla) did not bind. An anti-factor IX Gla monoclonal antibody was a potent inhibitor of factor IX binding to factor Xla (Kᵦ 34 nM) and activation by factor Xla (Kᵦ 33 nM). In activated partial thromboplastin time clotting assays, the specific activities of plasma and recombinant factor IX were comparable (200 and 150 units/mg), whereas rFIX/VII-Gla activity was low (<2 units/mg). In contrast, recombinant factor IXaβ and activated rFIX/VIIa-Gla had similar activities (80 and 60% of plasma factor IXaβ), indicating that both proteases activate factor X and that the poor activity of zymogen rFIX/VII-Gla was caused by a specific defect in activation by factor Xla. The data demonstrate that factor Xla binds with comparable affinity to factors IX and IXaβ and that the interactions are dependent on the factor IX Gla domain.

Coagulation factor IX (FIX, 1) EC 3.4.21.22 is the zymogen precursor of a plasma serine protease, factor IXαβ (FIXαβ), which is required for formation and maintenance of a fibrin clot at a site of blood vessel injury (1–3). The importance of this protein to normal hemostasis is demonstrated by the severe bleeding abnormality (hemophilia B) associated with its deficiency state (4, 5). FIX is a member of a family of proteases, including the hemostasis-related proteins prothrombin, factor VII, factor X, and protein C (the "vitamin-K dependent proteases"), which require specific post-translational modifications for normal activity (3, 6). At the N terminus of the mature forms of these proteins is a region rich in glutamic acid called the γ-carboxyglutamatic acid or "Gla" domain. Glutamic acid residues in the Gla domain are modified by the addition of a carboxyl group to the γ-carbons in a reaction catalyzed by the vitamin K-dependent enzyme γ-glutamyl carboxylase (6, 7). Proper γ-carboxylation is required for Gla domain binding to calcium and phospholipid, two properties that are indispensable for proper protease activity during coagulation (8). In vivo, Gla domain-dependent protease-substrate interactions take place on the phospholipid membranes of damaged cells and activated platelets. Binding to phospholipid accelerates the conversion of substrate to product by decreasing the Kᵦ for the reactions several orders of magnitude (8–10). The use of coumarin compounds as therapeutic anticoagulants is based on their ability to interfere with vitamin K metabolism, causing incomplete γ-carboxylation of the Gla domains of prothrombin and factors VII, IX, and X (8). In addition to phospholipid, protease-substrate interactions involving vitamin K-dependent proteins require a protein cofactor, which further improves catalytic efficiency by increasing the kᵦ for the reactions (9).

FIX is a 56-kDa polypeptide comprised of C-terminal trypsinlike catalytic (heavy chain) domain and an N-terminal noncatalytic (light chain) region separated by an 11-kDa activation peptide (1, 11). Two proteolytic cleavages are required to liberate the activation peptide from the remainder of the molecule to produce FIXαβ (11, 12). Activation may occur by two distinct mechanisms mediated by the plasma serine proteases factor VIIa (EC 3.4.21.21) and factor Xla (EC 3.4.21.27) (2, 3, 13, 14). Activation of FIX by factor VIIa is a typical coagulation protease-substrate interaction requiring calcium, phospholipid, and a protein cofactor (the membrane protein tissue factor) (13, 15). In this reaction the Gla domains of both FIX and factor VIIa form critical interactions with the phospholipid surface (16–20). The importance of the FIX Gla domain to FIX activation by factor Xla is less certain because the reaction appears to involve a mechanism distinctly different from typical vitamin K-dependent protease-substrate interactions. Although calcium is required (1, 21, 22), phospholipid has little influence on the process (1, 11, 23). Indeed, factor Xla lacks a Gla domain, suggesting that it may interact poorly with phospholipid (3, 24, 25). Furthermore, a protein cofactor has not been identified for FIX activation by factor Xla, at least when the reaction occurs in liquid plasma. These observations raise the possibility that the FIX Gla domain is not required for activation by factor Xla. This evidence not withstanding, there are some data to support a role for the Gla domain in FIX activation by factor Xla. A Gla domain mutation at amino acid 4 associated with hemo-
Philippe B interferes with factor XIa-mediated activation of FIX (26), as does failure to remove the FIX propeptide from the N terminus (27). Monoclonal antibodies directed against FIX-Gla block activation of FIX by factor XIs (28, 29). However, the antibodies interfere with other activities such as FIX activation by factor VIIa/tissue factor and factor X activation by FIXaβ. Therefore, nonspecific steric interference cannot be ruled out as the cause of the inhibition. In this report we describe studies on FIX binding to factor XIa. The work demonstrates that both FIX and FIXβ bind to factor XIa but not zymogen factor XI and that the FIX Gla domain plays a critical role in the interactions.

**EXPERIMENTAL PROCEDURES**

**Plasma and Recombinant FIX Proteins—**Plasma-derived FIX, FIXαα, and FIXββ were purchased from Enzyme Research Laboratories (South Bend, IN). The cDNAs for human wild type FIX and chimera FIX/VII-Gla were gifts from Dr. Darrell Stafford, University of North Carolina, Chapel Hill (30). FIX/VII-Gla contains the factor VII signal peptide, propeptide, Gla domain, and aromatic stack region (factor VII amino acids 50–148) and the FIX epidermal growth factor-like (EGF) 1 and 2 domains, activation peptide, and catalytic domain (factor IX amino acids 50–415). cDNAs were ligated into the mammalian expression vector pJ2C10 (which contains a constitutively expressed neomycin phosphotransferase gene) (31). 50 million HEK293 cells (a human kidney fibroblast line, ATCC CRL 1573) were cotransfected with 40 μg of expression construct and 2 μg of plasmid pRSVneo, which contains a gene conferring resistance to neomycin. Transfection was by electroporation (Electrocell Manipulator 600 BTX, San Diego). Transfected cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum containing the neomycin analog G418 at 500 μg/ml. G418-resistant clones were transfected to 96-well culture plates, and supernatants were tested for protein expression by enzyme-linked immunosorbent assay using goat anti-human FIX antibodies (Affinity Biologicals, Hamilton, ON). Expressing clones were expanded in 175-cm² culture flasks, and the medium was changed by a serum-free method (Medium (Mediatech, Herdon, VA) supplemented with 10 μg/ml vitamin K₃ (phytadione, Abbot). The medium was exchanged every 48–72 h. Conditioned medium was supplemented with benzamidine to a final concentration of 5 mM and stored at −20 °C pending purification. Recombinant proteins are designated by the prefix “r” to distinguish them from plasma-derived proteins (no prefix). To generate rFIX that is incompletely γ-carboxylated (rFIX-desγ), transfected HEK293 cells expressing rFIX were grown in medium supplemented with 5 μg/ml sodium warfarin (Sigma) instead of vitamin K.

**Purification of Recombinant Proteins—**Proteins were purified from conditioned medium by monoclonal antibody affinity chromatography. Antibodies were affinity purified to 5 ml of AffiGel Blue (Bio-Rad) at a concentration of 10 mg/ml of gel. For rFIX, the antibody used was humanized murine monoclonal IgG SB 249417 (GlaxoSmithKline, King of Prussia, PA), a calcium-dependent antibody that recognizes the properly γ-carboxylated FIX Gla domain (32). One to two liters of conditioned medium were run across the column, followed by washing with 25 ml Tris-HCl, pH 7.5, 100 mM NaCl (TBS) containing 2.5 mM CaCl₂. Elution was with TBS containing 25 mM EDTA. For rFIX-desγ and rFIX/VII-Gla, a monoclonal murine IgG against the FIX catalytic domain (kindly provided by Dr. George Broze, Washington University, St. Louis, MO) was used. Washing was with TBS containing 2.5 mM CaCl₂ and elution was with TBS containing 2.5 mM CaCl₂ and 2.0 mM sodium thiocyanate. Protein containing fractions from elutions were pooled and concentrated in an Amicon concentration, dialyzed against TBS, and stored at −80 °C. Protein concentration was determined by dye binding assay (Bio-Rad).

**Recombinant Factor Xla-Alaββ—**Preparation of recombinant factor XI has been described previously (31). Briefly, HEK293 cells were transfected with an expression construct consisting of the human factor XI cDNA in pJC10 to generate factor Xla-Alaββ. Recombinant factor XI was purified from conditioned medium by affinity chromatography using monoclonal IgG 1G5.12 (31). This process was used to generate a recombinant variant of factor XI in which the active site serine residue at amino acid position 557 was changed to alanine (factor XI-Ala557) by site-directed mutagenesis. Factor XI-Ala557 was converted to the “active” form (factor XIa-Alaββ) by diluting to 300 μg/ml in TBS containing 5 μg/ml human factor XIIa (Enzyme Research Laboratory) and incubating at 37 °C. Conversion of the 80-kDa zymogen to the 45-kDa heavy and 35-kDa light chains of the “activated” species was followed by reducing SDS-PAGE.
Factor IX-Gla Domain Binds to Factor XIa

results

reaction buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2.5 mM CaCl₂, and 0.05% CHAPS) was incubated at 37 °C for 30 min. After incubation, FIXαβ generation was determined by adding the reaction mixture to an equal volume of a detection mixture comprising reaction buffer and 66% ethylene glycol containing 2 mM FIXα p-nitroanilide-conjugated peptide substrate S299 (American Diagnostica, Greenwich, CT). After incubating for 10 min at 37 °C, the release of p-nitroanilide as a measure of FIXα activity was determined at a wavelength of 405 nm using a spectrophotometric microplate reader. In the absence of FIX in the assay, no p-nitroanilide signal was detected. Data were fit to a single site competition model, and an inhibition constant (Kᵢ) was calculated by nonlinear regression (GraphPad Prism).

Time Courses of FIX Activation followed by Western Immunoblot and SDS-PAGE—Plasma-derived or recombinant FIX proteins were diluted to 100 nM in TBS containing 2.5 mM CaCl₂, and the solution was warmed to 37 °C in a water bath. Reactions were started by the addition of plasma-derived factor XIa to a final concentration of 1 nM. At time points between 0 and 60 min, 30-μl volumes were removed and mixed with 10 μl of 4× nonreducing SDS-sample buffer (500 mM Tris-Cl, pH 6.8, 40% glycerol, 10% SDS). Samples were size fractionated on 12% polyacrylamide gels followed by transfer to nitrocellulose membranes. Blots were developed with goat anti-human FIX polyclonal IgG (Affinity Biologicals) using an ECL chemiluminescence Western blotting detection kit (Amersham Biosciences). The intensities of bands on autoradiographs for Western blots of recombinant protein activation by factor XIa were measured using a Bio-Rad Imaging XI (Decimal model GS-670). Measurements for bands representing the zymogen (56 kDa band) and active protease (45 kDa band) were determined for each lane. For each lane, the value for the active protease was divided by the sum of the signals for the zymogen and active protease for each time point to determine the percent zymogen converted to protease.

Cleavage of FIX by Russell’s viper venom protease (RVVP) was assessed as follows. Plasma-derived or recombinant FIX proteins were diluted to 1.0 μM in TBS containing 2.5 mM CaCl₂, and the solution was warmed to 37 °C in a water bath. Reactions were started by the addition of RVVP (Enzyme Research Laboratory) to a final concentration of 15 nM. At time points between 0 and 120 min, 30-μl volumes were removed and mixed with 10 μl of 4× reducing SDS-sample buffer (500 mM Tris-Cl, pH 6.8, 40% glycerol, 10% SDS, 10% β-mercaptoethanol). Samples were size fractionated on 12% polyacrylamide gels, and gels were stained with GelCode Blue stain reagent (Pierce).

RESULTS

Binding of FIX and FIXαβ to Factor XIa Studied with SPR—Initially, binding of plasma-derived FIX in solution to immobilized plasma-derived factor XIa was studied. FIX concentrations between 4 nM and 2 μM were tested using 2-min association and 90-s dissociation times. FIX rapidly associates with, and dissociates from, factor XIa (Fig. 1A) in a process that is dependent on calcium (data not shown). Dissociation appears to be nearly complete, indicating that little nonspecific irreversible binding to components of the flow cell is occurring. A plot of plasma FIX bound to factor XIa as a function of FIX concentration is shown in Fig. 1B (open circles). The plot was derived from data in Fig. 1A corrected for nonspecific binding as determined by the simultaneous infusion of the identical concentrations of FIX across the reference flow cell containing immobilized plasma kallikrein. Certain characteristics of the FIX-factor XIa interaction (the rapid dissociation rate in particular) preclude a kinetic fit of the data. We used equilibrium binding analysis to determine the binding constant, where binding at steady state is plotted against the concentration of FIX, and Kᵢ is determined in the traditional manner as the concentration of analyte (FIX) occupying 50% of available binding sites. Using this method, a Kᵢ for the FIX-factor XIa binding interaction of 120 ± 11 nM was obtained. This result is in reasonably good agreement with published values of Kᵢ for activation of FIX by factor XIa (160–180 nM) determined by chromogenic substrate assay (31, 35). Interestingly, FIXαβ also bound to plasma factor XIa with a Kᵢ of 110 ± 8 nM (Fig. 1B, open squares). Apparently liberation of the activation peptide during FIX activation does not alter the affinity of the protein for factor XIa. Neither FIX nor FIXαβ bound to zymogen factor XI (Fig. 1B, closed circles and closed squares), indicating that factor XI undergoes conformational changes upon conversion to factor XIa which exposes the FIX binding site.

A significant concern when using SPR to study the binding of a substrate to its enzyme is, of course, that the substrate may be converted to product by the enzyme on the surface of the flow cell. In the case under consideration, this could confound interpretation of results for binding of FIX to factor XIa. To address this issue, we prepared a recombinant version of factor XIa, factor XIa-Alaα557, in which the active site serine of the catalytic domain was replaced with alanine. Wild type factor XIa expressed in HEK293 cells has been shown to have activity similar to that of plasma-derived factor XIa in plasma and purified protein based assays (31, 36). As expected, factor XIa-Alaα557 lacks activity in plasma clotting assays and does not cleave the factor XIa chromogenic substrate S-2366 (data not shown). The binding of FIX and FIXαβ to factor XIa-Alaα557 was studied by SPR in the same manner as binding to plasma-derived factor XIa (Fig. 1C, open circles and squares). The Kᵢ values for binding of FIX and FIXαβ to factor XIa-Alaα557 (152 ± 41 and 129 ± 27 nM, respectively) are comparable with those obtained with plasma-derived factor XIa. Again, FIX and FIXαβ do not bind to uncleaved “zymogen” factor XIa-Alaα557 (Fig. 1C, closed circles and squares). The results indicate that conversion of FIX to FIXαβ by immobilized factor XIa, if it...
occurring, does not influence the results of the binding assays appreciably. The studies demonstrate that both zymogen and activated FIX bind to factor XIa with similar affinity and that a catalytically functional factor XIa molecule is not required for binding.

Recombinant FIX Proteins and Activity in Plasma Clotting Assays—To determine the importance of the FIX Gla domain in binding to, and activation by, factor XIa, recombinant versions of FIX with altered Gla domains were prepared (Fig. 2A). Recombinant proteins were expressed in the human fibroblast cell line HEK293 because this line has been shown to γ-carboxylate properly a high percentage of expressed recombinant vitamin K-dependent protein (37). In a standard aPTT assay, plasma-derived FIX demonstrated a specific activity of 200 units/mg (1 unit equaling the FIX activity in 1 ml of normal plasma). rFIX expressed in the presence of vitamin K had a specific activity of 75% (150 units/mg) of that of plasma FIX in the aPTT assay. FIX expressed in the presence of the vitamin K antagonist warfarin (rFIX-desγ) demonstrated significantly reduced activity (<1% normal activity or <2 units/mg of protein) when tested under the same conditions. As shown in Fig. 2B, the presence of warfarin in the cell culture results in a protein that is not recognized by a monoclonal antibody (SB 249417) that requires calcium and a properly γ-carboxylated FIX Gla domain for protein recognition. This demonstrates that adding warfarin to the cell culture system effectively interferes with γ-carboxylation of the Gla domain. rFIX in which the Gla domain has been replaced by the corresponding domain from factor VII (rFIX/VII-Gla; Fig. 2A) was expressed in the presence of vitamin K. This chimeric protein also demonstrated poor activity in the aPTT assay (<1% normal activity or <2 units/mg of protein).

The aPTT assay requires FIX to be activated by factor XIa and the FIXαβ subsequently generated to activate factor X. Poor activity in this assay could, therefore, reflect a defect in one or both of these steps. To define further the abnormalities in rFIX-desγ and rFIX/VII-Gla, we attempted to activate recombinant proteins by incubating them with a high concentration (100 nM) of factor XIa before testing them in clotting assays (modified PTT assay). This step removes the requirement for activation by factor XIa from the clotting process. rFIX-desγ could not be activated to FIXαβ by prolonged incubation with high concentrations of factor XIa. rFIX/VII-Gla was completely converted to the active form; however, it was noted that activation was considerably slower than with wild type rFIX (see below) or plasma FIX. In the modified PTT assay, rFIXαβ demonstrated ~80% of the activity of plasma-derived FIXαβ, consistent with results from the conventional aPTT assay. Interestingly, rFIXαβ/VII-Gla also demonstrated significant activity in this assay (~60% of the activity of plasma FIXαβ). This suggests that the factor VII Gla domain is a reasonably good substitute for the FIX Gla domain when FIXαβ is incorporated into the factor Xase complex in plasma with zymogen factor X, factor VIIIa, calcium, and phospholipid. Furthermore, the finding strongly indicates that the poor performance of rFIX/VII-Gla in the aPTT assay is caused by a specific defect in activation by factor XIa rather than a global abnormality of protein structure which affects multiple FIX functions.

Recombinant FIX Protein Binding to Factor XIa Studied with SPR—Recombinant proteins were tested by SPR for their ability to bind to factor XIa (Fig. 2C). rFIX binds to plasma factor Xa with a Kd of 107 nM, a value similar to those obtained for plasma-derived FIX and FIXαβ (Fig. 1, B and C). In contrast, neither rFIX-desγ nor rFIX/VII-Gla demonstrated binding above background (Fig. 2C). These data demonstrate that proper γ-carboxylation of the Gla domain is required for FIX binding to factor XIa and that the factor VII Gla domain is not an adequate substitute in this interaction.

The humanized murine monoclonal antibody SB 249417 recognizes the FIX Gla domain in a calcium-dependent manner (32) and is sensitive to conformational changes to the domain which accompany incomplete γ-carboxylation (Fig. 2B). Furthermore, the antibody does not recognize rFIX/VII-Gla in Western blot assays or enzyme-linked immunosorbent assays (data not shown), indicating that binding involves epitopes specific to the FIX Gla domain. The effect of SB 249417 on FIX binding to factor XIa was examined using SPR. As shown in Fig. 3A, the antibody is a potent inhibitor of binding, with a Kd of 34 nM (EC50 320 nM).

**FIG. 2. Recombinant FIX proteins.** A, nonreducing SDS-PAGE (12% gel) of recombinant proteins (~200 ng) purified by monoclonal antibody affinity chromatography. Staining is with GelCode Blue. The positions of molecular mass standards are shown on the left of the figure. It is not clear why rFIX/VII-Gla appears as two bands; however, this was a consistent finding in several protein preparations. B, Western blots of rFIX expressed in HEK293 cells grown in the presence of 10 μg/ml vitamin K (VK) or 3 μM warfarin (W). The blot on the left was developed with goat polyclonal anti-human factor IX IgG and the blot on the right with humanized murine monoclonal antibody SB 249417, which recognizes the properly γ-carboxylated FIX Gla domain. C, binding of rFIX, rFIX/VII-Gla, or rFIX-desγ to plasma factor XIa studied with SPR. rFIX concentrations between 4 nM and 1 μM were tested. ○, rFIX; ●, rFIX-desγ; and △, rFIX/VII-Gla.
inhibitor of FIX activation by factor Xla, with a \( K_i \) of 33 nM (Fig. 3B). The chromogenic substrate assay used to follow this reaction is relatively insensitive to FIXa and, therefore, may not detect low levels of activation. For this reason, conversion of rFIX molecules to rFIXa by a low concentration of factor Xla (1 nM) was examined by western immunoblot (Fig. 4). As can be seen in Fig. 4A, zymogen wild type rFIX undergoes nearly complete conversion to rFIXa within 1 h under the conditions of the assay (Fig. 4A). No activation of rFIX-desγ was observed (Fig. 4B). rFIX/VII-Gla appears to be slowly converted to FIXaβ, with a small increase in the activated form detectable at late time points (Fig. 4C). This finding is consistent with the earlier observation that prolonged incubation of rFIX/VII-Gla with high concentrations of factor XI will eventually result in complete conversion to the active protease. This indicates that the activation cleavage sites on rFIX/VII-Gla are accessible to factor Xla. Progress curves for activation of the recombinant FIX proteins were constructed using densitometry measurements from Western blot autoradiographs (Fig. 4D). The initial slopes of the progress curves (first 5 min for rFIX, 60 min for rFIX-desγ and rFIX/VII-Gla) were 4.1 nM/min for activation of rFIX, 0.2 nM/min for activation of rFIX/VII-Gla, and 0.0 nM/min for rFIX-desγ. Thus, the initial rate of activation of rFIX was ~20-fold greater than for rFIX/VII-Gla.

The venom of Dabois russelli (Russell’s viper) contains a protease, RVVP, which cleaves FIX between Arg50 and Val193 at the C terminus of the activation peptide to produce an active FIX intermediate called FIXαα (38). As with the activation of FIX by factor Xla, this reaction is enhanced by calcium but not by phospholipid (39). rFIX proteins were incubated in the presence of purified RVVP and calcium (Fig. 5). rFIX and rFIX/VII-Gla are converted to FIXαα in a similar manner, consistent with this reaction not having a specific requirement for the FIX Gla domain. Furthermore, this experiment demonstrates that the conformation of rFIX/VII-Gla is sufficiently like FIX that it interacts properly with RVVP. In contrast, rFIX-desγ is not cleaved by RVVP, indicating that poor γ-carboxylation induces significant enough conformational changes in the protein to interfere with activation. Significant structural alteration induced by poor γ-carboxylation could explain our inability to activate rFIX-desγ with high concentrations of factor Xla (100 nM), even with prolonged periods of incubation (>12 h).

**DISCUSSION**

Activation of FIX, a key step in the formation and maintenance of a fibrin clot, is controlled by at least two distinct mechanisms. Initiation of fibrin formation involves binding of factor VIIa in plasma to the membrane protein tissue factor at a site of blood vessel injury (2, 3). Factor VIIa/TF activates factor X and FIX in reactions that require calcium and a phospholipid surface (13, 40, 41). Activated factor X (factor Xa) then converts prothrombin to thrombin, which initiates fibrin formation. It is postulated that factor VIIa/TF is inhibited relatively early in the coagulation process by the TF pathway inhibitor (2, 42). FIXβ and its cofactor, factor VIIIa, would be required for generation of factor Xa to sustain coagulation after inhibition of factor VIIa/TF. In this model, bleeding in hemophilia (congenital deficiency of factor VIII or FIX) is caused by poorly sustained factor X activation rather than a failure to initiate fibrin formation (2, 43). Given the severity of the bleeding disorders associated with complete deficiencies of FIX and factor VII, it appears that FIX activation by factor VIIa is important for most hemostatic challenges. In contrast, factor XI deficiency is associated with bleeding that typically requires a more severe hemostatic insult (trauma or surgery) (44). FIX activation via factor Xla, therefore, is most likely required in certain situations to supplement FIXαα produced through factor VIIa/TF.

Factor XI has an unusual structure for a coagulation protease. The protein is a disulfide bond linked dimer of two iden-
The importance of the dimeric structure of factor XIa to FIX involves interactions remote from the activation cleavage sites. It supports the notion that binding of FIX and factor XIa activation peptide is activated by factor XIa (48). Furthermore, it suggests that the C-terminal half of A2 is also involved in FIX binding (47). Zymogen factor XI, unlike factor XIa, does not cleave small chromogenic substrates. This indicates that conversion of zymogen to active protease involves a conformational change that gives small molecules, as well as portions of FIX, access to the catalytic active site. The SPR studies demonstrate that conversion to factor XIa is also required for binding of FIX and FIXαβ to the protease. Binding sites for FIX are probably masked in the zymogen and become available for binding upon activation. Catalytic activity is not required for the binding interaction because factor XIa lacking an active site serine residue binds normally to FIX and FIXαβ. It is interesting that both FIX and FIXαβ bind to factor XIa. This indicates that the activation peptide is not required for the binding interaction, which is consistent with a report showing that relocation of activation peptide is activated by factor XIa (48). Furthermore, it supports the notion that binding of FIX and factor XIa involves interactions remote from the activation cleavage sites. The importance of the dimeric structure of factor XIa to FIX binding and activation is not entirely clear. In solution, a monomorphic variant of factor XIa activates FIX with kinetic parameters that are similar to those for activation by wild type factor XIa (31). However, recent work suggests that the physiologic environment for this reaction may be the surface of activated platelets (46, 49, 50). In this environment, the dimeric structure of factor XI is necessary for normal FIX activation, possibly because one polypeptide of the dimer is required for binding to the platelet, whereas the other interacts with FIX (46, 49, 50).

The structural elements on FIX required for binding to factor XIa have not been clearly defined. The noncatalytic portion of the molecule contains (from N terminus to C terminus) the Gla domain, a short aromatic stack region, two EGF-like domains, and the activation peptide (3). Some Gla domain mutations causing hemophilia B are associated with poor factor Xla mediated activation, as is the failure to remove the propeptide from the N terminus of the molecule (26, 27). Similarly, single amino acid substitutions in the EGF-1 (factor IX New London) (51) and EGF-2 (52) domains associated with cross-reactive material-positive hemophilia B are activated poorly by factor XIa. However, all of these mutations interfere with other activities of FIX in addition to activation by factor XIa. A rFIX protein containing the factor VII EGF1 domain is activated normally by factor XIa (53, 54), whereas a study employing alanine scanning mutagenesis on portions of the EGF2 domain identified only a single amino acid (position 89) that appeared to be necessary for normal activation by factor XIa (55).

Using SPR, plasma coagulation, antibody inhibition, and purified protein activation assays we have demonstrated that the FIX Gla domain either contains all or a portion of the factor XIa binding site or is required for the proper conformation of the binding site. The results with chimera rFIX/VII-Gla are particularly intriguing. In its active form, this molecule appears to activate factor X reasonably well in a plasma clotting assay and is activated by the snake venom protease RVVP in a manner similar to wild type FIX. This indicates that rFIX/VII-Gla is structurally and catalytically similar to FIX and strongly suggests that the failure of the protein to demonstrate activity in a standard aPTT assay is because of a specific defect in factor XIa-mediated activation. SPR confirmed that this protein has a profound defect in binding to factor XIa. Activation of factor X by FIXαβ is a phospholipid-dependent reaction involving the Gla domains of both proteins (8). It would appear that the Gla domain of factor VII is similar enough to that of FIX to substitute for it in this reaction. In contrast, factor VII-Gla cannot substitute for FIX-Gla during FIX activation by factor XIa, a reaction not influenced by phospholipid. Liebman and co-workers (28) demonstrated that a Fab fragment of a monoclonal antibody that interacts with the factor IX Gla domain phospholipid-binding epitope inhibited activation of factor IX by factor XIa. This group postulated that factor XIa may have a surface domain with features similar to those of phospholipid vesicles which may mediate the interaction with factor IX. However, phospholipid, although not enhancing FIX activation by factor XIa, does not inhibit the reaction either (23). This suggests that the FIX Gla domain may contain one or more epitopes for a protein-protein interaction with factor XIa and that the epitopes are distinct from the phospholipid-binding elements on the domain. This is not to say that FIX binding to phospholipid is not relevant to activation of the protein by factor XIa. As mentioned above, a physiologic site for the reaction may well be the surface of activated platelets. Although factor XIa binds to platelets largely through a protein-protein interaction involve-
Factor IXa-Gla Domain Binds to Factor Xa

It is likely that FIX would bind to the platelet surface, at least in part, through a phospholipid-protein interaction involving the Gla domain. Work is under way to identify specific residues in the Gla domain involved in the interaction between FIX and factor Xa, to determine whether they are distinct from those required for phospholipid binding.

The notion that a Gla domain may be involved in a binding interaction not involving phospholipid has precedent. Amino acids 3-11 in the FIX Gla domain are critical for high affinity binding of the protein to aortic endothelial cells (57). It appears that this interaction is caused by binding of FIX to collagen IV and not phospholipid in the cell membrane (58). Using scanning force microscopy, Wolberg and colleagues (59) demonstrated that FIX binds specifically to collagen IV in the later molecular collagens domain. Regan and co-workers (60) have shown that the protein C Gla domain is involved in a protein-protein interaction with the protein C receptor on endothelial cells. A recombinant prothrombin molecule containing the protein C Gla domain bound specifically to cells expressing the protein C receptor. Along similar lines, Lockett and Mast (61) presented data suggesting that an interaction between the C terminus of TF pathway inhibitor and the factor Xa Gla domain is required for proper TF pathway inhibitor mediated-inhibition of factor Xa. Thus, Gla domain involvement in protein-protein interactions may be common.

Acknowledgments—We thank Mao-Fu Sun for technical expertise and Jean McClure for graphics work and preparation of the manuscript.

REFERENCES

1. Osterud, B., Bouma, B., and Griffin, G. (1978) J. Biol. Chem. 253, 5846-5851
2. Broze, G., Girard, T., and Novotny, W. (1990) Biochemistry 29, 7539-7546
3. Davie, E., Fujikawa, K., and Kissel, W. (1991) Biochemistry 30, 10363-10370
4. Pollak, E., and High, K. (2001) in The Molecular Basis of Blood Diseases (Stamatoyannopoulos, G., Majerus, P., Perlmutter, R., and Varmus, H., eds) 4th Ed., pp. 89-101, W. B. Saunders Co., Philadelphia
5. Suttle, J. (1985) Annu. Rev. Biochem. 54, 459-477
6. Wu, S.-M., Chen, W.-F., Drazner, D., and Stafford, D. (1991) Science 254, 1634-1636
7. Mann, K., Nesheim, M., Church, W., Haley, P., and Krishnaswamy, S. (1990) Blood 76, 1-16
8. Mann, K., Jenni, R., and Krishnaswamy, S. (1988) Annu. Rev. Biochemistry 57, 915-956
9. West, J. and Dahlback, B. (2001) in The Molecular Basis of Blood Diseases (Stamatoyannopoulos, G., Majerus, P., Perlmutter, R., and Varmus, H., eds) 3rd Ed., pp. 680-697, W. B. Saunders Co., Philadelphia
10. Sutcliffe, J. (1985) Annu. Rev. Biochem. 54, 459-477
11. Wu, S.-M., Cheung, W.-F., Drazner, D., and Stafford, D. (1991) Science 254, 1634-1636
12. Mann, K., Kissel, W., and Davie, E. (1991) Biochemistry 30, 10363-10370
13. Pollak, E., and High, K. (2001) in The Molecular Basis of Blood Diseases (Stamatoyannopoulos, G., Majerus, P., Perlmutter, R., and Varmus, H., eds) 4th Ed., pp. 89-101, W. B. Saunders Co., Philadelphia
14. Suttle, J. (1985) Annu. Rev. Biochem. 54, 459-477
15. Mann, K., Kissel, W., and Davie, E. (1991) Biochemistry 30, 10363-10370
16. Pollak, E., and High, K. (2001) in The Molecular Basis of Blood Diseases (Stamatoyannopoulos, G., Majerus, P., Perlmutter, R., and Varmus, H., eds) 4th Ed., pp. 89-101, W. B. Saunders Co., Philadelphia
17. Freedman, S. J., Blostein, M. D., Baleja, J. D., Jacobs, M., Furie, B. C., and Furie, B. (1996) J. Biol. Chem. 271, 16227-16236
18. Stoylova, S., Gray, E., Barrowcliffe, T. W., Kennell-Cook, G., and Holzenbusch, A. (1998) Biochem. Biophys. Acta 1383, 175-178
19. Zhang, E., St. Charles, R., and Tulinsky, A. (1999) J. Mol. Biol. 285, 2089-2104
20. Morriss, J. (2001) in Hemostasis and Thrombosis: Basic Principles and Clinical Practice (Colman, R., Hirsh, J., Marder, V., Clowes, A., and George, J., eds) 4th Ed., pp. 89-101, Lippincott, Williams and Wilkins, Philadelphia
21. Bajaj, S. (1982) J. Biol. Chem. 257, 4127-4133
22. Walsh, P., Bradford, H., Sinha, D., Piperno, J., and Tuszyński, G. (1984) J. Clin. Invest. 73, 1329-1332
23. Mannhalter, C., Schiffman, S., and Deutsch, E. (1984) Br. J. Haematol. 56, 261-271
24. Fujikawa, K., Chung, D., Hendrickson, L., and Davie, E. (1986) Biochemistry 25, 2417-2424
25. McMullen, B., Fujikawa, K., and Davie, E. (1991) Biochemistry 30, 2056-2060
26. de la Salle, C., Charmandier, J. L., Ravunat, C., Ohlmann, P., Hartmann, M. L., Schuller, S., Bischoff, R., Ebel, C., Roeschlin, D., and Balland, A. (1993) Nouv. Rev. Fr. Hematol. 35, 473-480
27. Wojciech, E., Van Den Berg, M., Polet, S. R., and Bertina, R. M. (1997) J. Biochem. 73, 629-632
28. Liebman, H. A., Furie, B. C., and Furie, B. (1987) J. Biol. Chem. 262, 7605-7612
29. Reina, C., Himber, J., Burcklen, I., Moran, P., Peck, M., Suggett, S., Devaux, B., and Kirchhofer, D. (1999) Thromb. Haemostasis 82, 1118-1125
30. Toomey, J., Smith, K., and Stafford, D. (1991) J. Biol. Chem. 266, 19198-19202
31. Sun, Y., and Gailani, D. (1996) J. Biol. Chem. 271, 29023-29028
32. Toomey, J., Blackburn, M. N., Storer, B. L., Valocic, R. E., Koster, P. F., and Feuerstein, G. Z. (2000) Thromb. Res. 106, 73-79
33. Bell, W., and Alton, H. (1954) Nature 174, 880-881
34. McMullen, B., Fujikawa, K., and Davie, E. (1991) Biochemistry 30, 2056-2060
35. Sun, M.-F., Zhan, M., and Gailani, D. (1999) J. Biol. Chem. 274, 36373-36378
36. Zhan, M., Abel-Razek, T., Sun, M., and Gailani, D. (1998) J. Biol. Chem. 273, 31153-31159
37. Yan, S. C., Ranazzo, P., Chao, Y. B., Walls, D. J., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) Biochemistry 29, 1528-1538
38. Zhan, M., Abel-Razek, T., Sun, M., and Gailani, D. (1998) J. Biol. Chem. 273, 31153-31159
39. Regan, L., Mollica, J., Rezaie, A., and Esmon, C. T. (1997) J. Clin. Invest. 99, 2627-2638
40. Lockett, J. C., and Mast, A. E. (2002) Biochemistry 41, 4898-4907

Downloaded from http://www.jbc.org/ by guest on July 23, 2018
The Factor IX γ-Carboxyglutamic Acid (Gla) Domain Is Involved in Interactions between Factor IX and Factor XIa

Aysar Aktimur, Melanie A. Gabriel, David Gailani and John R. Toomey

J. Biol. Chem. 2003, 278:7981-7987.
doi: 10.1074/jbc.M212748200 originally published online December 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M212748200

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 57 references, 29 of which can be accessed free at http://www.jbc.org/content/278/10/7981.full.html#ref-list-1