A Sequential Mechanism for Exosite-mediated Factor IX Activation by Factor Xla*

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Background: Factor Xla proteolytically activates factor IX.

Results: Xla cleaves IX after Arg145, forming IXα, and then after Arg180, forming IXαβ. Both reactions require substrate binding to the Xla A3 domain.

Conclusion: Xla activates IX by an exosite-mediated release-rebind mechanism. Efficiency of the second cleavage is enhanced by changes resulting from the first cleavage.

Significance: The data support a new model for IX activation by Xla.

During blood coagulation, the protease factor Xla (fXla) activates factor IX (fIX). We describe a new mechanism for this process. FIX is cleaved initially after Arg145 to form fIXα, and then after Arg180 to form the protease fIXαβ. FIXα is released from fXla, and must rebind for cleavage after Arg180 to occur. Catalytic efficiency of cleavage after Arg180 is 7-fold greater than for cleavage after Arg145, limiting FIXα accumulation. FIXα contains four apple domains (A1–A4) and a catalytic domain. Exosite(s) on FIXα are required for FIX binding, however, there is lack of consensus on their location(s), with sites on the A2, A3, and catalytic domains described. Replacing the A3 domain with the prekallikrein A3 domain increases $K_a$ for FIX cleavage after Arg145 and Arg180, 25- and 90-fold, respectively, and markedly decreases $k_{cat}$ for cleavage after Arg180. Similar results were obtained with the isolated FIXα catalytic domain, or FIXα in the absence of Ca$^{2+}$. Forms of FIXα lacking the A3 domain exhibit 15-fold lower catalytic efficiency for cleavage after Arg180 than for cleavage after Arg145, resulting in FIXα accumulation. Replacing the A2 domain does not affect FIX activation. The results demonstrate that FIXα activates FIX by an exosite- and Ca$^{2+}$-mediated release-rebind mechanism in which efficiency of the second cleavage is enhanced by conformational changes resulting from the first cleavage. Initial binding of FIXα and FIXαβ requires an exosite on the FIXα A3 domain, but not the A2 or catalytic domain.

Factor IX (fIX) is the zymogen of a protease, factor IXαβ (fIXαβ), which is required for proper formation and maintenance of blood clots at sites of vascular injury (1, 2). Congenital fIX deficiency causes the severe bleeding disorder hemophilia B (1). Human fIX is a 57-kDa protein composed of an N-terminal calcium-binding Gla domain, two epidermal growth factor domains, an activation peptide, and a trypsin-like catalytic domain (1). It shares this structure with coagulation factors VII and X (3). The Gla domains of these proteins facilitate binding to phospholipid membranes.

Conversion of fIX to fIXαβ requires cleavage of the Arg145–Ala146 and Arg180–Val181 peptide bonds to release the activation peptide (2, 4, 5). The physiologic mediators of this process are the serine proteases factor VIIa (fVIIa) and factor XIa (fXla). FIX activation by fVIIa requires Ca$^{2+}$ and phospholipid (6–8). In the presence of the cofactor tissue factor, fVIIa cleaves fIX first after Arg145, forming the inactive intermediate fIXα. FIXα is released from fVIIa and must rebind to the protease to be cleaved after Arg180 to form fIXαβ. As the second cleavage is rate-limiting, fIXα accumulates during fIX activation by fVIIa.

fXla appears to activate fIX by a different process than fVIIa. fXla is a dimer of identical subunits, each containing a trypsin-like catalytic domain and a heavy chain comprised of four apple domains (A1–A4) (9–12). In contrast to fVIIa, fXla lacks a Gla domain. Perhaps as a result, fIX activation by fXla, although Ca$^{2+}$-dependent, does not require phospholipid. During fIX activation by fXla an intermediate does not accumulate (13, 14). This is unrelated to the dimeric structure of the protease, as isolated fXla subunits convert fIX to fIXαβ without intermediate accumulation (14, 15). fXla readily cleaves fIX with an R180A substitution after Arg145, whereas fIX with an R145A substitution undergoes insignificant cleavage after Arg180 (14). This indicates that the structure of fIX facilitates ordered bond cleavage, with the Arg145–Ala146 bond presented first to the fXla active site. Changes in conformation resulting from cleavage after Arg145 may then facilitate cleavage after Arg180. A similar process was reported previously for prothrombin activation (16).

There are conflicting hypotheses regarding the manner in which fXla is able to activate fIX without fIXα accumulation. It has been proposed that fIXα may not be released from fXla prior to conversion to fIXαβ (13, 17). However, at least a portion
of the intermediate must be released, as active site-blocked FXIa competes with FXIa for FXIa binding during FXI activation (14). Active site-blocked FXIαβ is a competitive inhibitor of FXI cleavage by FXIa (18), and FXIα may have similar inhibitory properties. If FXIα is released from FXIa, the lack of FXIα accumulation implies that cleavage after Arg145 is rate-limiting, although this has yet to be demonstrated.

There is compelling evidence that one or more exosites (substrate binding sites distinct from the protease active site) on FXIa are required for normal FXI activation (17–23), but there is a lack of agreement on their locations. It is clear that Ca2+-dependent FXI activation requires the FXIa heavy chain (19, 20). One report indicated that the A2 domain contains a FXI binding site (21), whereas others point to A3 (22, 23). More recent work suggests a distinct FXI binding site may be located on the FXIa catalytic domain (17). Km for FXI activation by the isolated FXIa catalytic domain (a species lacking the heavy chain) was reported to be similar to Km for FXI activation by full-length FXIa, implying the catalytic domain was largely responsible for recognition and specificity of FXI binding. These data are not compatible with those showing that A3 domain substitution results in a marked increase in Km for FXI activation (18, 22, 23).

We conducted a kinetic analysis of FXI and FXIa activation to FXIαβ by FXIa and FXIa variants, to address the conflicting information regarding the mechanism of FXI activation and the locations of substrate binding exosites on FXIa. Binding studies were used to support the kinetic data, which show that FXI activation by FXIα is a largely sequential process in which FXI is converted to FXIa and then to FXIαβ, with a substantial increase in catalytic efficiency of the second cleavage explaining the absence of FXIα accumulation. The A3 domain serves as the major binding site for both FXI and FXIα. The dramatic decrease in catalytic efficiency of forms of FXIαa lacking the functional exosite illustrates the critical role of Ca2+-dependent FXI and FXIα binding to FXIa.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human FXI, FXIαβ, glutaryl-glycyl-arginyl-chloromethyl ketone (EGR-CK), Phe-Pro-Arg-chloromethyl ketone (PFR-CMK), and biotinylated EGR-CK were from Hematologic Technologies (Essex Junction, VT). Factor XIIa (FXIIa) and goat anti-human factor IX IgG (EGR-HP) were from Enzyme Research (South Bend, IN). L-Pyroglutamyl-L-prolyl-L-arginine p-nitroanilide S-(2366) was from Diapharma (West Chester, OH). Soybean trypsin inhibitor-agarose and bovine serum albumin (BSA) were from Sigma. Streptavidin-agarose was from Pierce. Anti-FXI IgG O1A6 (24) and anti-FXI IgG SB249417 (25) have been described.

**Recombinant FXIa**—The system for expressing human wild type FXI (FXI-WT) and FXI variants has been reported (14, 22, 23). HEK293 fibroblasts (ATCC-CRL1573) were transfected with 40 μg of pJVCMV containing a FXI cDNA and 2 μg of pRSVneo encoding a neomycin resistance marker using an Electrocell Manipulator 600, (BTX, San Diego, CA). Cells were initially grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum and 500 μg/ml of G418, then switched to serum-free medium (Cellgro Complete, Mediatech, Herndon, VA). FXI was purified from conditioned media by affinity chromatography using anti-human FXI-IgG 1G5.12 (22, 23).

FXI chimeras in which apple domains are replaced with prekallikrein (PK) apple domains have been described (22, 26). This study used FXI with the PK A2 or A3 domain (FXI/PKA2 or FXI/PKA3) (22), and FXI in which the entire heavy chain is replaced with the PK heavy chain (FXI-CD/PK-HC) (26). In FXI/PKA2, Lys140 was changed to Ser to prevent autoproteolysis.

FXI was converted to FXIa by incubation with FXIIa (100:1 substrate to enzyme molar ratio) at 37 °C for 24 h in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl (TBS). Conversion of the 80-kDazymogen FXI subunits to the 45-kDa heavy chain and 35-kDa catalytic domain of FXIa was confirmed by SDS-PAGE. In studies of FXI activation by FXIa or FXIa variants, removal of FXIa, or inhibition of FXIa, had no effect on fIX or fIXα cleavage.

To prepare isolated FXIa heavy chain (FXIa-HC) and isolated FXIa catalytic domain (FXIa-CD) (18), FXI with Cys362 and Cys482 changed to Ser (fXI-Ser362/Ser482) was activated and passed over a soybean trypsin inhibitor-agarose column. FXIa-CD binds to the column and is eluted with TBS containing 200 mM benzamidine, whereas FXIa-HC flows through the column.

**Preparation of FIXα—FIX (14.3 μM) was incubated with 50 nM FXIa in TBS, pH 7.4, 20 mM EDTA for 12 min at 37 °C. FIXαβ was inhibited by incubating 90 min at 37 °C with biotinylated EGR-CK (10-fold molar excess over fIX). After dialysis against PBS, streptavidin immobilized on agarose resin was added, and incubated for 1 h at room temperature with mixing. Resin was removed by centrifugation, and the supernatant containing FIXα was dialyzed against 50 mM HEPES, pH 7.4, 125 mM NaCl, 1 mg/ml of polyethylene glycol (PEG) 8000.

**Hydrolysis of S-2366 by FXIa—FXIa (6 nm) was incubated with S-2366 (50–2000 μM) in TBS at room temperature. Free p-nitroaniline formation was followed by continuous monitoring of absorbance at 405 nm on a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA). Rates of p-nitroaniline generation (nmol/s) were determined using an extinction coefficient of 9920 OD units (405 nm) per mol per cm of p-nitroaniline. Km and kcat for S-2366 cleavage were determined by nonlinear least squares fitting performed with MicroMath Scientific Software.

**Activation of FIX and FIXα by FXIa**—FIX or FXIα (25 nm to 5 μM) in assay buffer (50 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM CaCl2 (or 25 mM EDTA), 1 mg/ml of PEG 8000) was incubated at room temperature with FXIa (1 to 240 nm active sites, depending on the FXI species and substrate) in tubes coated with PEG 20,000. At various times (0 to 640 min), aliquots were removed into nonreducing SDS-sample buffer, size fractionated on 17% polyacrylamide-SDS gels, and stained with GelCode Blue (Pierce). Gels were imaged on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) using an excitation λ of 685 nm, and emission λ of 720 nm. Conversion of FIX to FIXα and FXIαβ, and FXIα to fIXαβ was assessed by densitometry. To determine the amount of protein per band, standards were run on a separate gel, with one standard also run on the gel with the time course samples. Full progress curves
were constructed from data for disappearance of fIX, and appearance of fIXα and fIXαβ. In some reactions, antibodies to fXla or fIX were included.

Kinetic Analyses—Steady-state kinetic parameters for fIX or fIXα cleavage were obtained by numerical integration fitting of full progress curves of substrate depletion, and intermediate and product formation at substrate concentrations from 25 to 5000 nM; and by analysis of initial rate dependence of substrate depletion as a function of substrate concentration. Rates of cleavage of the Arg145–Ala146 and Arg180–Val181 bonds were analyzed simultaneously with KinTek Explorer Version 2.5 software (27) using the reaction mechanism shown in Equations 1 and 2. IXα* indicates the intermediate bound to fXla in the correct orientation for cleavage after Arg180.

\[
k_{-1} = k_{-1}/k_1 \quad K_m = (k_{-1} + k_2)/k_1 \quad k_{cat1} = k_2 \quad K_i = k_{-2}/k_3
\]

\[
k_{-4}/k_4 \quad k_{cat2} = k_5 \quad K_m = (k_{-4} + k_4)/k_{cat2} = k_5 \quad K_{i2} = k_{-6}/k_6
\]

We reported that the product fIXαβ binds to fXla and is a competitive inhibitor of fIX activation by fXla (18). A similar assumption was made for product inhibition by fIXα. K_d and K_i values were initially constrained to those obtained from surface plasmon resonance studies (below) and full progress curve analysis of fIXαβ formation, and no rapid equilibrium assumptions were imposed on association and dissociation rates. By fitting full progress curves for fIX disappearance, and fIXα and fIXαβ appearance, K_m, k_cat, K_a, and catalytic efficiency (k_cat/K_m) were determined for both cleavages in fIX. The same method was used with fIXα as substrate. As fXla preparations have ~20% fIX contamination, analyses of conversion of fIXα to fIXαβ included the cleavage of the fIX.

Results of numerical analysis were compared with those obtained by conventional Michaelis-Menten analysis of both cleavages. With fIX as substrate, initial velocities (v_0) of cleavage after Arg145 were determined from the initial slopes of progress curves for disappearance of fIX, normalized to 1 nM fXla active sites. Values for v_0 were analyzed with the Michaelis-Menten equation, and values for K_m and k_cat were obtained from direct nonlinear least squares analysis using Scientist Software. With fIXα as substrate, K_m and k_cat for cleavage after Arg180 were determined in a similar manner. Competitive binding of fXla to fIX in the fXla preparation was calculated by a cubic equation, and was taken into account for determining the fXla concentration available for the reaction with fIXα.

Surface Plasmon Resonance—Binding studies were performed on a Biacore T100 flow biosensor (Biacore, Uppsala, Sweden) at 25 °C. FIX, fIXα, or fIXαβ were immobilized on carboxymethyl-dextran flow cells (CM5 sensor chips, GE Healthcare) using amine-coupling chemistry. To prevent cleavage of bound fIX or fXla, fXla active sites were blocked with FPR-CMK. fIXαβ active sites were blocked with EGR-CK. Flow cell surfaces were activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysulfosuccinimide for 5 min (flow rate 10 µl/min), after which the protein (30 µg/ml, pH 4.0) was injected onto the surface. Unreacted sites were blocked for 5 min with 1 M ethanolamine. Analytes (fXla species, 1 to 5000 nM) were perfused through flow cells in HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM CaCl_2, 1 mM benzamidine, 0.005% (v/v) P20) at 10 µl/min for 5 min. After changing to HBS-P buffer without fXla, analyte dissociation was monitored for 10 min. Flow cells were regenerated with HBS-P containing 30 mM EDTA (no CaCl_2). fIXα binding to fIX species was also tested in the absence of Ca^2+ (10 mM EDTA). Data were corrected for nonspecific binding by subtracting signals obtained with analytes infused through a flow cell without coupled protein.

Binding was analyzed with BIAs评价 software (Biacore) using a bivalent binding model for dimers (fXla-WT, fXla/ PKA2, fXla/PKA3) and a 1:1 binding model for monomers (fXla-CD, fXla-CD/PK-HC). K_d values were calculated from the quotient of the derived dissociation (k_d) and association (k_a) rate constants. In addition, a steady state affinity model was used in which K_d was derived from nonlinear regression fitting of the response at equilibrium to fXla concentration.

**FIGURE 1. Purified proteins.** A, nonreducing SDS-polyacrylamide gel stained with GelCode blue of recombinant (1) FXI-CD/PK-HC, (2) FXI-WT, (3) FXI/PKA3, and (4) FXI/PKA2-Ser^482. FIXa-CD/PK-HC is an 80-kDa monomer, whereas other FXI species are 160-kDa dimers. B, purified fXla heavy chain (HC) and catalytic domain (CD) isolated from fXla-Ser^482 and fXla-Ser^482 are compared with fXla-WT (fXla) on nonreducing (left) and reducing (right) SDS-polyacrylamide gels. C, fIX and fIXα on a nonreducing SDS-polyacrylamide gel. Note that fIXα migrates more rapidly than fIX, despite having the same molecular mass. Positions of molecular mass standards are indicated on the left of each panel in kilodaltons.

**RESULTS**

Recombinant fXla—All fXI species migrate at ~160 kDa on nonreducing SDS-PAGE, except for fXI-CD/PK-HC, which is an 80-kDa monomer (Fig. 1A). FXI-Ser^482 and Ser^482 lacks the disulfide bond that links the A4 and catalytic domains after conversion to fXla. Activation of this variant results in an ~90 kDa heavy chain dimer and 35-kDa catalytic domains that dissociate (Fig. 1B). Under reducing conditions the heavy chain dimer dissociates into 45-kDa monomers (Fig. 1B). Chroma-
Factor IX Activation Mechanism

Figure 2. FIX and fIXα cleavage by FIXa-WT. A, nonreducing 17% polyacrylamide SDS gels of 100 nM FIX (top) or fIXα (bottom) in assay buffer with Ca2+ incubated at room temperature with 3 nM (active sites) FIXa-WT. Positions of standards for FIX, FIXa, and fIXα variants are indicated at the right of each panel. B, progress curves of FIX disappearance (□), and FIXα (○), and Fixαβ (▲) generation from panel A (top). Lines represent the least-squares fits to the data. C, initial velocities of cleavage after Arg145 (conversion of fIX to FIXα) by 1 μM FIXa-WT active sites, as a function of FIX concentration. Initial rates were obtained from the slopes of the linear portions of progress curves documenting the disappearance of fIX with time. D, progress curves of fIXα cleavage by FIXa-WT determined from panel A (bottom). E, initial velocities of cleavage after Arg160 (conversion of fIXα to fIXαβ) by 1 μM FIXa-WT as a function of fIXα concentration.

FXIa in which the A3 domain is replaced with the PK A3 domain (FXIa/PK3), and the isolated fIXα catalytic domain (fIXα-CD) were tested for their ability to cleave fIX and fIXα (Fig. 3 and Tables 1 and 2). The cleavage rates of the Arg145-→Ala146 and Arg180→Val181 bonds were markedly reduced compared with FIXa-WT, with pronounced fIXα accumulation. Km values for fIX cleavage after Arg145 by FXIa/PK3 and fIXα-CD were increased ~25-fold compared with FIXa-WT, with 60- and 90-fold decreases in catalytic efficiency, respectively. The catalytic efficiency of cleavage after Arg180 was impaired to an even greater degree, but only kcat/Km could be estimated accurately because saturation was not achieved for the reactions (Fig. 3E). The values for Km in Tables 1 and 2 should, therefore, be considered lower limits for the actual Km. These data indicate that the A3 domain is important for binding of fIX and fIXα to FXIa, and that loss of the A3 binding site has a deleterious effect on both cleavages. fIXα accumulation is the result of an 11–14-fold higher catalytic efficiency for Arg145→Ala146 cleavage compared with Arg180→Val181 cleavage. Progress curve simulations for cleavage of isolated fIXα by FXIa/PK3 and fIXα-CD suggested that Arg180→Val181 is cleaved with similar, low catalytic efficiency when fIX or fIXα is the starting...
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**TABLE 1**

Kinetic parameters for cleavage of fIX and fIXα to fIXαβ by fIXa

| Pro tease | Sub strate | Kcat | Km | Catalytic efficiency | kcat/Km |
|----------|-----------|------|----|----------------------|---------|
| fIXa-WT  | fIX       | 12 ± 1 | 0.10 ± 0.01 | 0.20 ± 0.01 | 60 ± 6 | 0.14 |
| fIXa-WT  | fIXα      | 6.5 ± 0.4 | 4.9 ± 0.4 | 4.9 ± 0.4 | 1.3 ± 0.3 | 5 |
| fIXa-CD  | fIX       | 3.2 ± 0.1 | 4.9 ± 0.2 | 4.9 ± 0.2 | 0.7 ± 0.1 | 3 |
| fIXa-PKA3| fIX       | 4.9 ± 0.1 | 4.9 ± 0.2 | 4.9 ± 0.2 | 1.0 ± 0.1 | 3 |
| fIXa/PKA3| fIX       | 16 ± 1 | 0.03 ± 0.01 | 0.10 ± 0.01 | 160 ± 20 | 0.15 |
| fIXa-CD/PK-HC | fIX | 1.6 ± 0.1 | 2.4 ± 0.2 | 2.4 ± 0.2 | 0.7 ± 0.1 | 5 |

Due to the low affinity between the enzyme and substrate in certain reactions, we were not able to reach saturation for reactions with fIXa-CD, fIXa/PKA3, and fIXa-CD/PK-HC. Thus, values for kcat/Km represents lower estimates for the reaction. kcat/Km is the appropriate parameter for comparing these reactions.

**TABLE 2**

Kinetic parameters for cleavage of fIX and fIXα by fIXa determined from initial velocities

| Pro tease | Sub strate | Bond cleaved | kcat | Km | Catalytic efficiency |
|----------|-----------|--------------|------|----|----------------------|
| fIXa-WT  | fIX       | Arg145       | 10 ± 1 | 0.27 ± 0.07 | 40 ± 10 |
| fIXa-WT  | fIXα      | Arg145       | 7.1 ± 0.3 | 0.09 ± 0.01 | 80 ± 10 |
| fIXa-WT no Ca2+ | fIX | 4.6 ± 0.4 | 1.8 ± 0.3 | 3 ± 1 | 0.8 ± 0.3 |
| fIXa-CD  | fIXα      | Arg145       | 2.4 ± 0.4 | 3 ± 1 | 0.8 ± 0.3 |
| fIXa-CD  | fIXα      | Arg145       | ND | 3.9 ± 0.9 | 1.6 ± 0.4 |
| fIXa-PKA3| fIX       | Arg145       | 6.1 ± 1 | 0.02 ± 0.005 | 2 ± 1 |
| fIXa-PKA3| fIXα      | Arg145       | ND | 6 ± 1 | 0.02 ± 0.005 |
| fIXa-CD/PK-HC | fIX | Arg145       | 5 ± 1 | 3 ± 1 | 0.8 ± 0.3 |
| fIXa-CD/PK-HC | fIXα | Arg145       | ND | 5 ± 1 | 0.8 ± 0.3 |

ND, not determined.

substrate, indicating that the exosite is important for efficient binding of both fIX and fIXα as substrates. Cleavage of fIX or fIXα by fIXa in which the A2 domain was replaced with PK A2 (fIXa/PKA2) was similar to cleavage by fIXa-WT (Table 1) indicating that the A2 domain does not contain a high affinity fIX or fIXα binding site.

The fIXa catalytic domain is attached to the heavy chain by the Cys362–Cys482 disulfide bond (11, 12). The absence of the heavy chain or the substitution of Cys482 with serine may have caused changes to fIXa-CD that reduced its ability to activate fIX, independent of the loss of the A3 exosite. fIXa-CD/PK-HC consists of the fIXa catalytic domain attached to the heavy chain of PK. Like the fXI heavy chain, the PK heavy chain contains four apple domains, and it is connected to the catalytic domain through a single disulfide bond. Activation of fIX and fIXα by fIXa-CD/PK-HC (Tables 1 and 2) was similar to activation by fIXa/PKA3 and fIXa-CD. Taken as a whole, the data indicate that a major exosite for binding of fIX and fIXα is located on the fIXa A3 domain. Loss of this exosite, either in chimeras (fIXa/PKA3 or fIXa-CD/PK-HC) or through absence of the heavy chain (fIXa-CD) results in loss of the high affinity site and a marked increase in Kcat for fIX activation.

The anti-human fXI monoclonal IgG O1A6 binds to the A3 domain (24). In the presence of O1A6, fIX activation by fIXa-WT appears similar to activation by fIXa/PKA3, with decreased rates of cleavage for both bonds and fIXα accumulation (compare Figs. 4, A and B, to 3B). An IgG that binds to the A2 domain (14E11; Ref. 24) had no effect on fIX activation by fIXa (not shown).

Cleavage of fIX and fIXα in the Absence of Calcium Ions—Although most reactions catalyzed by fIXa do not require Ca2+, fIX activation by fIXa is Ca2+-dependent. The fIX Gla domain is involved in the interaction with fXIa (25), and Ca2+ is likely required for proper Gla domain conformation. In the absence of Ca2+, fIX bond cleavage is slow, with accumulation of fIXα (compare Fig. 4, D and E). Kcat values for fIX activation in the absence of Ca2+ are similar to those with fIXa/PKA3 in the presence of Ca2+ (Tables 1 and 2). Absence of Ca2+ had little effect on the overall rate of fIX cleavage by fIXa/PKA3 or fIXa-CD (not shown). The data support the hypothesis that the
Ca\textsuperscript{2+}-dependent interaction between the fXa A3 domain and fIX requires the fIX Gla domain. Consistent with this, fIX activation by fXa in the presence of IgG SB 249417 (25), which binds to the fIX Gla domain, is characterized by significant accumulation of fIXa and slow generation of fXa\beta (Fig. 4C).

**Binding of FXIa to FIX and FIXα**—We studied binding of active site-blocked fXa to immobilized fIX, fIXa, and fXαb using surface plasmon resonance. Zymogen fXI-WT did not bind to any species in the presence of Ca\textsuperscript{2+}, nor did fXla-WT in the presence of 10 mM EDTA (data not shown). In the presence of Ca\textsuperscript{2+}, fXla-WT bound to fIX, fIXa, and fXαb. Binding at equilibrium was plotted as a function of the fXla-WT concentration (Figs. 5, A–C). K\textsubscript{d} for binding to fIX (130 ± 20 nM), fIXa (140 ± 20 nM), and fXαb (60 ± 10 nM) were comparable (Table 3). Isolated fXla heavy chain also bound to these species in a Ca\textsuperscript{2+}-dependent manner (Fig. 5, D and E) with K\textsubscript{d} values of 90 ± 20, 70 ± 10, and 70 ± 10 nM, respectively (Table 3). We were unable to demonstrate binding of fXla-CD, fXla/PKA3, or fXla-CD/PK-HC (Fig. 5F and Table 3) to fIX, fIXa, or fXαb at analyte concentrations up to 5 \mu M. These results indicate that most of the binding energy for the fIX-fXla interaction requires the fXla A3 domain. The Western blots in Fig. 5G show that Fxla-TC inhibits fX conversion to fXαb by fXla, indicating the A3 exosite engages fIX in the absence of the catalytic domain, and accumulation of fIXα in the presence of fXla-TC supports the premise that fIXα is released from fXla.

**DISCUSSION**

The current study was undertaken to establish the mechanism by which fXla converts fIX to fXαb. The data need to be interpreted in light of published work in the field, some of which is in disagreement with the results presented here. It is clear that fIX activation by fVIIa or fXa involves sequential proteolysis of the Arg\textsuperscript{145–146} and Arg\textsuperscript{180–181} peptide bonds (2, 5). The observation that fIX containing an R145A substitution is cleaved poorly by fXla (14) supports the premise that the Arg\textsuperscript{145–146} cleavage site is presented to the fXla active site after binding of fIX, and that conformational changes resulting from cleavage after Arg\textsuperscript{145}, and perhaps repositioning of fIXα on the protease, enhances cleavage after Arg\textsuperscript{180}.

Although fIXα forms during fIX activation by either fVIIa or fXla, it does not accumulate during the latter reaction (13, 14). The fact that fXla is a dimer initially suggested that the two subunits may cleave the two fIX bonds prior to releasing fXαb; however, subsequent work demonstrated that the ability to cleave fIX without fIXα accumulation is intrinsic to each fXla subunit (14, 15). Wolberg *et al.* (13) observed that cleavage rates of Arg\textsuperscript{145–146} and Arg\textsuperscript{180–181} in the intermediates fIXα (fIX cleaved after Arg\textsuperscript{180}) and fIXα (cleaved after Arg\textsuperscript{145}) are similar and comparable with the overall rate for conversion of fIX to fXαb. They suggested that any intermediate formed may not be released prior to conversion to fXαb. However, significant fIXα accumulation was subsequently observed in competitive reactions in which fIX is activated by fXla in the presence of active site-inhibited fXla (14). Some portion of fIXα must be released from fXla, arguing against a mechanism based strictly on the intermediate remaining bound to fXla. There are parallels between fIX activation and prothrombin activation, in this regard. During ordered bond cleavage of prothrombin by fXa, the 150-fold higher catalytic efficiency of the second cleavage relative to the first results in formation of an intermediate (meizothrombin) that does not accumulate, but that can be captured with a tripeptide chloromethylketone (28).

The results reported here support an exosite- and Ca\textsuperscript{2+}-mediated release-rebind mechanism for fIX activation by fXla (summarized in Fig. 6), in which the efficiency of the second cleavage is enhanced by conformational changes resulting from the first cleavage. We propose that binding of fIX to the fXla A3 domain is followed by docking with the active site and cleavage after Arg\textsuperscript{145} to form fIXα. This is followed by a second docking step with the active site after rebinding of fIXα to A3, and cleavage after Arg\textsuperscript{180}. The ratio of the catalytic efficiencies of the two cleavages determines whether fIXα accumulates. If the A3 domain exosite is available, catalytic efficiency for cleavage after Arg\textsuperscript{180} is 7-fold greater than for cleavage after Arg\textsuperscript{145}, and fIXα does not accumulate. In the absence of a functional exo-
site, the rates of both bond cleavages are markedly decreased, but the predominance of the catalytic efficiency of the second cleavage is lost, leading to fIX$^*/H_9251$ accumulation.

The observations that fIX$^*/H_9251$ is captured by active site-inhibited fXIa (14) or fXIa-HC in competition assays, and that a small amount of fIX$^*$ is transiently observed on gels during fIX cleavage by fXIa-WT, support a mechanism involving fIX$^*/H_9251$ release. However, the data do not exclude the possibility that release is partial, with a fraction of fIX$^*/H_9251$ repositioned for cleavage after Arg180, while still bound to fXIa (a transition from fIX$^*/H_9251$/H18528 XIa in Equation 1 directly to fIX$^*/H_9251$* fXIa in Equation 2). Such a mechanism has been termed “processive” in prior studies (13). Using

FIGURE 4. fIX cleavage by fXIa-WT in the presence of monoclonal antibodies, and in the absence of Ca$^{2+}$ ions. Panels A–C, effects of antibodies. Shown are progress curves of fIX disappearance (●), and fIXα (●) and fIXαβ (▲) generation. FIX (100 nM) was activated by 3 nM fXIa-WT (active sites) in the presence of (A) vehicle, (B) 50 nM IgG OA16, or (C) 1000 nM IgG SB 249417. Panels D and E, importance of Ca$^{2+}$. Shown are progress curves of fIX disappearance (●), and fIXα (●) and fIXαβ (▲) generation. FIX (1000 nM) was activated in assay buffer at room temperature by (D) fXIa-WT (5 nM active sites) in the presence of Ca$^{2+}$, or (E) fXIa-WT (40 nM active sites) in the presence of 25 mM EDTA.

FIGURE 5. FXIa binding to fIX, fIX$^*$, and fIXαβ. Surface plasmon resonance was used to assess binding of FXIa perfused over immobilized fIX, fIXα, or fIXαβ in the presence of Ca$^{2+}$ ions at 10 μM/min for 6 min. Dissociation was monitored for 10 min. Panels A–C, FXIa-WT binding. FXIa-WT concentrations tested were: 1, 5, 10, 25, 37.5, 75, 150, 300, and 500 nM. Affinity and kinetic parameters were determined after subtraction of nonspecific binding from the control surface. Nonlinear regression fitting of the steady state equilibrium binding of FXIa-WT to (A) fIX, (B) fIXα, and (C) fIXαβ was performed using a bivalent model. Panels D–F, surface plasmon resonance data for Ca$^{2+}$-dependent binding of FXIa-WT, FXIa-HC, and FXIa-CD binding to fIX. D, shown are curves for FXIa-WT binding to immobilized fIX at analyte concentrations listed above. E, binding curves for FXIa-HC at the same concentrations used for FXIa-WT. F, binding curve for FXIa-CD at a single analyte concentration (5000 nM). Panel G, effect of FXIa-HC on fIX activation by FXIa-WT. Shown are Western blots of time courses of fIX (100 nM) activated by fXIa-WT (2 nM active sites) in the presence of vehicle control (C) or 1 μM FXIa-HC (HC). Samples collected at various times (shown at bottom) into nonreducing SD sample buffer were size fractionated by SDS-PAGE. Detection was with a polyclonal anti-human fIX antibody and chemiluminescence. Positions of standards for fIX, fIXα, and fIXαβ are shown between the images.
TABLE 3
Affinity for FXa binding to FIX, fIXα, and fIXαβ

| Analyte                     | Factor IX  | Factor IXα | Factor IXαβ |
|-----------------------------|------------|------------|-------------|
| FXa-WT                      | 130 ± 20   | 140 ± 30   | 60 ± 10     |
| FXa-HC                      | 90 ± 20    | 70 ± 10    | 70 ± 10     |
| FXa-CD                      | >5000      | >5000      | >5000       |
| FXa/PrCa3                   | >3000      | >3000      | >3000       |
| FXa-CD/PK-HC                | >5000      | >5000      | >5000       |
| FXa/PrCa2-Ser140            | 40 ± 10    | 40 ± 10    | ND          |

* ND, not done.

Affinity for FXa binding to FIX, fIXα, and fIXαβ

Using surface plasmon resonance, FXa species (1 to 5000 nM) in Ca2+ containing HBS-P buffer were perfused across sensor chips coated with FXa, fIXα, or fIXαβ. HBS-P buffer without FXa was then perfused for 10 min to follow dissociation. Data were corrected for nonspecific binding by subtracting signals obtained with analytes infused through a flow cell without coupled protein. Binding was analyzed using a bivalent binding model for all species except FXa-CD/PK-HC and FXa-CD, which were evaluated with a 1:1 binding model. Kd values were calculated from the quotient of the derived dissociation (kd) and association (ka) rate constants.

**FIGURE 6. Model for the mechanism of fIX activation by FXa.** In the schematic images representing FXa, apple domains are shown as four clustered gray circles with the exosite on A3 indicated in black (E). The FXa catalytic domain is a white ellipse with the active site indicated by a black circle (A). For fIX, the catalytic domain (dark gray ellipse) and light chain (light gray ellipse) are connected by a line representing a disulfide bond. The fIX activation peptide is the white oval between the heavy and light chains. Bi-directional arrows represent reversible binding, and uni-directional arrows represent proteolytic cleavage. FIX is activated by a FXa subunit by sequential cleavage after Arg145 and Arg180, with the intermediate fIXα released and then rebound to the FXa A3 domain. Details of the model are described in the text. The dashed lines indicate the possibility that some fraction of fIXα may be converted to fIXαβ without release from the FXa A3 domain.

In our data, we simulated a model that includes conformational repositioning of fIXα-fXa to the productive complex fIXα-C-fXa without release (represented by dashed arrows in Fig. 6), in parallel with release rebinding. Because the true ratio of fIXα-fXa to fIXα-C-fXa is unknown, we examined a range of values for the ratio (0.05–1), and found that the model fits data sets for cleavage after Arg180 for both nascent and purified fIXα by FXa-WT reasonably well at a ratio of 0.5, a Kd of ~100 nM for binding of fIXα to FXa in a productive complex, and a kcat of ~20 min⁻¹. Independently determined parameters for cleavage of the first bond, obtained by Michaelis-Menten analysis, and K values for surface plasmon resonance binding of fIX, fIXα, and fIXαβ to active site-blocked FXa-WT were kept constant. The results suggest a possible upper limit of ~40% (the difference between kcat of 35 min⁻¹ and 20 min⁻¹) of fIXα proceeding to fIXαβ through repositioning without release-rebinding. If the mechanism only involved repositioning of fIXα without release, no transient intermediate should be observed in time courses with FXa-WT, no fIXα would competitively bind to active site-blocked FXa, and kcat for the second cleavage would have to be exceedingly large (up to 60 to 150 min⁻¹). Our data indicate this latter scenario is not likely.

The 3-fold greater efficiency of cleavage of fIXα generated in situ compared with purified fIXα recruited from the aqueous phase in the release-rebind model deserves comment. Although it must be recognized that this result may simply reflect structural perturbations in fIXα acquired during purification, the difference in catalytic efficiency disappears in reactions with exosite-impaired proteases, perhaps weakening this argument somewhat. The difference in catalytic efficiency may actually reflect some degree of fIXα repositioning without release, or perhaps induced fit related to conformational changes in FXa, fIXα, or both triggered by FX cleavage after Arg145.

Coagulation proteases are trypsin-like enzymes, but with more restricted substrate specificity than trypsin. Binding interactions at exosites on the proteases distinct from the active site are key to the mechanisms of action of these enzymes, and have been shown to be primary determinants of substrate affin-
Factor IX Activation Mechanism

ity and specificity during activation of prothrombin (29–33) and factor X (34). Binding of the substrate at the exosite precedes substrate docking with the active site and catalysis (31, 33). In the current study, high affinity binding between fIX or fIXα and fXla was largely, if not completely, due to a Ca\(^{2+}\)-dependent interaction with the A3 domain. fXla/PKA3 and the isolated fXla protease domain (fXla-CD) did not engage fIX or fIXα with high affinity, resulting in ~25-fold increases in \(K_m\) for cleavage after both Arg\(^{145}\) and Arg\(^{180}\). These results are not consistent with a model based on the fXia catalytic domain containing a high affinity fIX binding exosite. Sinha et al. (17) reported that the slow rate of fIX conversion to fIXαβ by an isolated fXla protease domain was due to reduced \(k_{cat}\), with \(K_m\) for the overall reaction similar to that for fXla-WT. It was concluded that fIX, but not fIXα, bound to an exosite on the catalytic domain in a Ca\(^{2+}\)-independent manner. A mechanism was proposed that required fIX to bind to both the catalytic domain and heavy chain exosites, although \(K_m\) data imply that the putative catalytic domain site would dominate the binding interaction. Our results do not support this model. Although we noted that fXla-CD activates fIX similarly in the presence or absence of Ca\(^{2+}\), the kinetic and binding data fail to support the presence of a fIX binding site on fXla-CD. Furthermore, our results for fIX activation by fXla-WT and direct binding studies do not indicate that fIX binds with high affinity to fXla-WT in the absence of Ca\(^{2+}\). The reason for the differences between our results and those of Sinha et al. (17) are not clear; however, their study used a chromogenic assay to examine fIX activation, which would not facilitate examination of the initial cleavage converting fIX to fIXα with the detail we were able to achieve with our densitometry-based approach.

The same group posited that catalytic domain amino acids Glu\(^{458}\) and Lys\(^{550}\) (Glu\(^{98}\) and Lys\(^{192}\)) in chymotrypsin numbering may be part of a fIX binding exosite (35). However, substitutions for these residues resulted in >100-fold reductions in \(k_{cat}\) for fIX conversion to fIXαβ, more consistent with a catalytic defect. Preliminary work from our group indicated that fXla with a Lys\(^{550}\) substitution activates fIX with normal \(K_m\), with the reduction in \(K_m\) likely due to disruption of the interaction between the Lys\(^{550}\) side chain and the substrate P3\(^{\prime}\) residue (36). Recently, Marcinkiewicz et al. (37) reported that cross-talk between the fXla catalytic domain and heavy chain is required for expression of the fIX binding site on the heavy chain, based on observing that isolated fXla heavy chain (fXla-HC) does not bind to fIX. In the current study, in contrast, fXla-HC and fXla-WT bound to fIX and fIXα with similar affinity in the presence of Ca\(^{2+}\). Furthermore, fXla-HC inhibited fIX activation by fXia. These findings are most consistent with a mechanism in which fIX binds initially to the fXia heavy chain, with binding being independent of a contribution from the catalytic domain.

Although our data do not support the presence of a fIX-binding exosite on the fXia catalytic domain that is required for initial substrate recognition, they do not rule out the possibility that such a binding site is expressed as the result of initial binding of fIX to the A3 exosite (although such a process would not explain the differences between our results and published work). Previously, we noted that binding of fIX to fXia results in a mixed-type inhibition of cleavage of a tripeptide substrate by fXia, consistent with fIX binding to A3 causing changes in the protease domain, in addition to competing with the tripeptide at the active site (18).

The Gla domains of the coagulation proteases fVIIa, fIXa, and factor Xa contain \(\gamma\)-carboxylated glutamic acid residues that bind Ca\(^{2+}\) and facilitate binding to phosphatidylserine-rich membranes. \(K_m\) for reactions involving these proteases are strongly influenced by the Gla domain phospholipid interaction. fXia lacks a Gla domain (10–12), and fXla activation of fIX is not influenced appreciably by phospholipid. However, the fIX Gla domain is required for binding to fXia (25), and may be the structure that interacts with the A3 domain. Supporting this is the observation that the kinetic parameters of fIX activation by fXla-WT in the absence of Ca\(^{2+}\) similar to those for activation by fXla/PKA3 (Table 1), and that an antibody to the fIX Gla domain results in accumulation of fIXα (Fig. 4C). It is possible, therefore, that during fIX activation by fXia, the A3 domain performs the Gla domain binding role that phospholipid performs during fIX activation by fVIIa.

FXI is a relatively new component of the blood coagulation mechanism, making its appearance during mammalian evolution as the result of a duplication of the PK gene (38). Human PK and fXI share a high degree of structural homology, and are 58% identical in amino acid sequence (10). However, the sequence around the putative fIX binding site in the fXI A3 domain, which is highly conserved in mammals, is distinctly different from the corresponding sequence in PK (38), supporting the conclusion that changes to the A3 domain were critical to the ability of fXla to engage fIX as a substrate.

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