# **Characterization of Novel Forms of Coagulation Factor XIa** INDEPENDENCE OF FACTOR XIa SUBUNITS IN FACTOR IX ACTIVATION\*

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Factor XI is the zymogen of a dimeric plasma protease, factor XIa, with two active sites. In solution, and during contact activation in plasma, conversion of factor XI to factor XIa proceeds through an intermediate with one active site (1/2-FXIa). Factor XIa and 1/2-FXIa activate the substrate factor IX, with similar kinetic parameters in purified and plasma systems. During hemostasis, factor IX is activated by factors XIa or VIIa, by cleavage of the peptide bonds after Arg<sup>145</sup> and Arg<sup>180</sup>. Factor VIIa cleaves these bonds sequentially, with accumulation of factor IX $\alpha$ , an intermediate cleaved after Arg<sup>145</sup>. Factor XIa also cleaves factor IX preferentially after Arg<sup>145</sup>, but little intermediate is detected. It has been postulated that the two factor XIa active sites cleave both factor IX peptide bonds prior to releasing factor IXa $\beta$ . To test this, we examined cleavage of factor IX by four single active site factor XIa proteases. Little intermediate formation was detected with 1/2-FXIa, factor XIa with one inhibited active site, or a recombinant factor XIa monomer. However, factor IX $\alpha$  accumulated during activation by the factor XIa catalytic domain, demonstrating the importance of the factor XIa heavy chain. Fluorescence titration of active site-labeled factor XIa revealed a binding stoichiometry of  $1.9 \pm 0.4$ mol of factor IX/mol of factor XIa ( $K_d = 70 \pm 40$  nm). The results indicate that two forms of activated factor XI are generated during coagulation, and that each half of a factor XIa dimer behaves as an independent enzyme with respect to factor IX.

Factor XI (FXI),<sup>3</sup> the zymogen of the blood coagulation serine protease factor XIa (FXIa), is comprised of two identical disulfide-linked 80-kDa subunits (1–5). All other coagulation serine proteases are monomers (1, 2). The proteases factor XIIa (FXIIa) and  $\alpha$ -thrombin are likely physiologic activators of FXI, cleaving the Arg<sup>369</sup>–Ile<sup>370</sup> bond to form FXIa (3–7). Whereas it is assumed that FXIa generated during coagulation has both subunits of the dimer cleaved (two active sites per molecule), this has not been studied in detail, and, hypothetically, FXI with only one cleaved subunit may be generated.

During coagulation, FXIa converts factor IX (FIX) to the protease factor IXa $\beta$  (FIXa $\beta$ ), by cleaving the Arg<sup>145</sup>–Ala<sup>146</sup> and Arg<sup>180</sup>–Val<sup>181</sup> peptide bonds (8–12). FIX is also activated by factor VIIa (FVIIa) by cleavage at these same bonds (12). FVIIa bound to the membrane protein tissue factor (TF) initially cleaves FIX after Arg<sup>145</sup>, generating the intermediate FIX $\alpha$ , prior to cleavage after Arg<sup>180</sup> to generate FIXa $\beta$  (9, 12–14). In contrast, little intermediate appears to be generated during activation by FXIa (8, 15). It has been proposed that the dimeric structure of FXIa may account for its capacity to activate FIX without intermediate formation (15, 16), with the two protease domains each cleaving one FIX activation site prior to releasing FIXa $\beta$ .

Here, we report that FXI activation by factor XIIa or  $\alpha$ -thrombin proceeds through an intermediate in which only one subunit of the dimer is cleaved, and that this intermediate species is formed in plasma during contact activation-induced coagulation. We purified and characterized the intermediate and used it, along with other forms of FXIa with one active site per molecule, to address the importance of the dimeric structure of FXIa to FIX activation.

# **EXPERIMENTAL PROCEDURES**

*Materials*—FXIIa, FIXa $\alpha$ , high molecular weight kininogen (HK) and corn trypsin inhibitor were from Enzyme Research Labs (South Bend, IN). Human  $\alpha$ -thrombin, FVIIa, FXIa, FIX, FIXa $\beta$ , and fluorescein-Phe-Pro-Arg-CH<sub>2</sub>Cl were from Hematologic Technologies (Essex Junction, VT). Hirudin (Lepirudin) was from Berlex (Wayne, NJ), soybean trypsin inhibitor-agarose from Sigma, and benzamidine-Sepharose from Amersham Biosciences. L-Pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2366) was from DiaPharma (West Chester, OH), 1,5-Dansyl-Glu-Gly-Arg-CH<sub>2</sub>Cl was from Calbiochem (La Jolla), and methyl-sulfonyl-D-cyclo-hexyl-glycyl-glycyl-arginine-*p*-nitroanilide (S-299) from American Diagnostics (Greenwich, CT).

*Purification of Plasma FXI*—Frozen plasma (2 liters) collected in acid-citrate-dextrose was thawed at 4 °C, and supplemented with benzamidine (20 mM). FXI was purified from the cryosupernatant by affinity chromatography using the anti-human FXI antibody 1G5.12 (17). After loading, the column was washed with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM benzamidine and eluted with 2 M NaSCN in the same buffer.



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<sup>&</sup>lt;sup>3</sup> The abbreviations used are: FIX, factor IX; FIXaα, factor IXaα; FIXaβ, factor IXaβ; FXI, factor XI; FXIa, factor XIa; 1/2-FXIa-, factor XIa with only one subunit activated; FXIa-1/2i, FXIa with one active site inhibited by a chloromethyl ketone inhibitor; FXIa/PKA4, recombinant FXIa with the apple 4 domain replaced with the corresponding domain from plasma prekallikrein; FVIIa, factor VIIa; TF, tissue factor; HK, high molecular weight kininogen; S-2366, pyro-Glu-Pro-Arg-*p*-nitroanilide; CD, catalytic domain; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PK, plasma prekallikrein.

The eluate was concentrated by ultrafiltration and dialyzed against 50 mM Hepes, pH 7.4, 125 mM NaCl, 20 mM benzamidine. Purity was assessed by SDS-PAGE and concentration by colorimetric assay (Bio-Rad).

Western Blots of FXI Activation in Plasma—Human plasmas with 0.38% sodium citrate (George King, Overland Park, KS) were mixed with equal volumes of PTT A reagent (Diagnostica Stago, Asnières-sur-Seine, France) at 37 °C. At various times, 9  $\mu$ l of reactions were mixed with 6  $\mu$ l of non-reducing sample buffer (233 mM Tris-Cl, pH 6.8, 138 mM SDS, 19% glycerol, 0.01% bromphenol blue), fractionated on 6% polyacrylamide-SDS gels, and transferred to nitrocellulose. The primary antibody was goat anti-human FXI IgG (Enzyme Research Laboratories, South Bend, IN) and secondary antibody was horseradish peroxidase-conjugated anti-goat IgG. Detection was by chemiluminescence.

Preparation of FXI with a Single Catalytic Active Site (1/2-FXIa)—As will be shown, FXI activation proceeds through an intermediate with one activated subunit (1/2-FXIa). Plasma FXI (0.6-12 μм) in 50 mM Hepes, pH 7.4, 125 mM NaCl, underwent limited digestion by incubating with FXIIa (625 nm) or thrombin (860 nm) for 1 h at 24 °C. Reactions were terminated by addition of corn trypsin inhibitor (8.6  $\mu$ M) or hirudin (20  $\mu$ M), respectively. The mixture was chromatographed on benzamidine-Sepharose. Elution was with 50 mM Hepes, pH 7.4, 125 mM NaCl, 50 mM benzamidine. FXI was found in the flow through and 1/2-FXIa in the eluate. 1/2-FXIa was also prepared by chromatography on soybean trypsin inhibitor-agarose. Elution was with 50 mM Hepes, pH 7.4, 1 M NaCl, 1 M benzamidine, 1 mM EDTA. The active site concentration for 1/2-FXIa was determined by complete inhibition with fluorescein-Phe-Pro-Arg-CH<sub>2</sub>Cl, followed by dialysis to remove free inhibitor. The protein concentration was determined by absorbance at 280 nm (corrected for absorbance of the fluorophore) with  $\epsilon$  of 214,400  $M^{-1}$  cm<sup>-1</sup>, and the fluorescein concentration was determined by absorbance at 491 nm with  $\epsilon$  of 79,000 M<sup>-1</sup> cm<sup>-1</sup>.

Preparation of FXIa with One Inhibited Active Site (FXIa-1/2i)—1/2-FXIa (2  $\mu$ M) in 50 mM Hepes, 125 mM NaCl, 1 mg/ml polyethylene glycol 8000, pH 7.4, was incubated with 20  $\mu$ M D-Phe-Pro-Arg-CH<sub>2</sub>Cl at 23 °C to irreversibly inhibit active sites. Residual inhibitor was removed by dialysis against the same buffer. The unactivated subunit of inhibited 1/2-FXIa was activated by incubation with FXIIa (500 nM) for 7 h at 23 °C. Complete conversion to FXIa was demonstrated by SDS-PAGE. FXIa-1/2i was separated from residual inhibited FXIa by chromatography on benzamidine-Sepharose.

*Preparation of FXIa Bound to Polyacrylamide Beads*—FXIa bound to UltraLink Iodoacetyl Resin (Pierce) was prepared by a modification of the method of Dharmawardana and Bock (18). 1/2-FXIa (1 μM) in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 8000, pH 7.4 (coupling buffer), was incubated with 20 μM ATA-Phe-Pro-Arg-CH<sub>2</sub>Cl (18, 19) to inhibit active sites. After dialysis to remove free inhibitor, dialysate (500 μl) was mixed with 100 μl of packed UltraLink Resin, previously washed with coupling buffer. Protein coupling was initiated by addition of NH<sub>2</sub>OH to 0.1 M, and incubation at 24 °C for 4 h with rocking. Beads with bound protein

showed no activity in a chromogenic assay using S-2366, indicating the active sites of bound 1/2-FXIa were blocked. To generate an active site on the uncleaved 1/2-FXIa subunit, the beads were incubated with FXIIa (600 nM) at 37 °C for 6 h with frequent mixing. Unreacted sites were blocked by rocking with monomeric bovine serum albumin (18) (20 mg/ml) for 12 h at 24 °C. Specific activity of the reacted beads was determined by cleavage of S-2366 using FXIa as a standard (Table 1). The activity of the packed resin was 370 nm FXIa active sites.

Preparation of Recombinant FXIa Monomer (FXIa/PKA4) and FXIa Catalytic Domain (FXIa<sup>CD</sup>)—Recombinant FXI with the A4 domain replaced by the A4 domain from plasma prekallikrein (FXI/PKA4) (20, 21), or FXI with Cys<sup>362</sup> and Cys<sup>482</sup> changed to Ser (FXI-Ser<sup>362,482</sup>) (22), were expressed in HEK 293 cells as described. Protein from stably transfected clones was purified from conditioned media (Cellgro Complete, Mediatech, Herndon, VA) by chromatography using anti-human factor XI-IgG 1G5.12 (17). The column was eluted with 2 M sodium thiocyanate in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl (Tris/NaCl). Protein containing fractions were pooled and concentrated by ultrafiltration, dialyzed against Tris/NaCl, and stored at -80 °C. FXI/PKA4 and FXI-Ser<sup>362,482</sup> (100–300  $\mu$ g/ml) were activated by incubation with 2  $\mu$ g/ml FXIIa at 37 °C. Complete activation was confirmed by reducing SDS-PAGE. FXI-Ser<sup>362,482</sup>, which lacks the disulfide bond that connects the FXIa heavy chain to the catalytic domain, was reapplied to the 1G5.12 column. The catalytic domain (FXIa<sup>CD</sup>) binds to the column, whereas the heavy chain is found in the flow through. FXIa<sup>CD</sup> was eluted as described above and dialyzed against Tris/NaCl.

*Preparation of Recombinant FIX Cleavage Site Mutants*— The nucleotides coding for Arg<sup>145</sup> or Arg<sup>180</sup> in the human FIX cDNA were changed to Ala (GCG) using a Chameleon kit (Stratagene, La Jolla). The constructs encode proteins designated FIX-Ala<sup>145</sup> and FIX-Ala<sup>180</sup>. cDNAs were ligated into vector pJVCMV, and stably expressing HEK293 cell lines (ATCC CRL 1573) were prepared as described (23). Proteins were purified from conditioned media (Cellgro Complete supplemented with 10 µg/ml vitamin K<sub>1</sub>) by chromatography using a calciumdependent monoclonal IgG (SB 249417) that recognizes the properly γ-carboxylated FIX Gla domain, as previously described (23).

*FXIa Hydrolysis of S*-2366—FXIa (6 nM active sites, 3 nM protein), 1/2-FXIa (6 nM active sites, 6 nM protein), or FXIa-1/2i (6 nM active sites 6 nM protein) were diluted in assay buffer (50 mM Hepes, 125 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.1 mg/ml bovine serum albumin) containing S-2366 (15.6–2000  $\mu$ M). Initial rates of generation of free *p*-nitroaniline in 100- $\mu$ l reaction volumes were measured by continuous monitoring of absorbance at 405 nm (3 mm path length) in a SpectraMax 340 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA).

FIX Activation by FXIa Followed by Chromogenic Substrate Cleavage (22)—FIX (25–2000 nM) in assay buffer was activated by FXIa (1–6 nM active sites), 1/2-FXIa (1–4 nM active sites), or FXIa-1/2i (2.5–10 nM active sites) at 24 °C. At various time points between 0 and 120 min, 60- $\mu$ l aliquots were removed and mixed with 6  $\mu$ l of assay buffer containing 150  $\mu$ M aprotinin. Aprotinin completely inhibited FXIa without affecting



FIXa $\beta$  activity. Sixty-six microliters of 1 mm S299 in assay buffer with 66% ethylene glycol was added to the quenched sample, and substrate hydrolysis was followed by measuring the change in absorbance at 405 nm. Generation of FIXa $\beta$  as a function of time was determined by interpolation of the linear dependence of the initial rate of S299 hydrolysis on known concentrations of FIXa $\beta$ .

Initial rates for progress curves of FIXa $\beta$  generation were obtained by analyzing the first 5 min of each curve with a second order polynomial equation. Resulting  $v_o$  values were fit by the Michaelis-Menten equation, and values for  $K_m$  and  $k_{cat}$ were obtained from direct non-linear least squares analysis. The initial 5 min of FIX activation are minimally influenced by product inhibition, and defined the  $K_m$  and  $k_{cat}$  values adequately. The values for  $K_m$  and  $k_{cat}$  were used to analyze complete progress curves by the integrated Michaelis-Menten equation. With values for  $K_m$  and  $k_{cat}$  fixed, full progress curves were fitted simultaneously by the integrated rate equation with product inhibition to obtain estimates of  $K_i$  for FIXa $\beta$ .

Activity of FXIa in Plasma Clotting Assays—FXIa enzymes were diluted to 5  $\mu$ g/ml in 20 mM Tris-Cl, 100 mM NaCl, 1 mg/ml bovine serum albumin, pH 7.4, and serial 1:2 dilutions were prepared in the same buffer. Sixty  $\mu$ l of each dilution was mixed with an equal volume of FXI-deficient plasma, and rabbit brain cephalin, followed by incubation for 30 s at 37 °C. Sixty  $\mu$ l of 25 mM CaCl<sub>2</sub> was added and the time to clot formation was determined on a Dataclot 2 fibrometer (Helena Laboratories, Beaumont, TX). Clotting times were plotted against enzyme concentration on a log-log plot, and FXIa activity was determined as a percent of control by comparison to a control curve constructed with plasma FXIa.

FIX Activation Followed by Western Blot-Plasma or recombinant FIX (100 nM) were incubated in assay buffer at 24 °C with various FXIa species, or with FVIIa in the presence of saturating human TF (Innovin, Dade-Behring, Miami, FL). In some reactions, FXIa inhibited by a tripeptide chloromethyl ketone (FXIai) was included. At various times,  $7-\mu$ l samples were mixed with 7  $\mu$ l of reducing sample buffer (233 mM Tris-Cl, 138 mM SDS, 19% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue, pH 6.8), fractionated on 12% polyacrylamide-SDS gels, and then transferred to nitrocellulose. The primary antibody was goat anti-human FIX polyclonal IgG (Enzyme Research Laboratories), and the secondary antibody was horseradish peroxidase-conjugated anti-goat IgG. Detection was by chemiluminescence. The relative positions of bands representing FIX, FIX $\alpha$ , FIX $a\alpha$ , and FIX $a\beta$  were determined by Western blots of standards for each protein.

Titration of Active Site-labeled FXIa with FIX and FIXa $\beta$ — FXIa (6.25  $\mu$ M) was diluted in titration buffer (50 mM Hepes, 125 mM CaCl<sub>2</sub>, 1 mg/ml polyethylene glycol 8000, pH 7.4) and inhibited with a 10-fold molar excess of 1,5-dansyl-Glu-Gly-Arg-CH<sub>2</sub>Cl at 24 °C. Residual FXIa activity was determined by diluting aliquots to 20 nM FXIa in 120  $\mu$ l of titration buffer with 500  $\mu$ M S-2366, and monitoring changes in absorbance at 405 nm until FXIa activity was reduced  $\geq$ 99.9%. The concentration of inhibited active sites per mol of FXIa was determined by measuring dansyl concentration by absorbance at 335 nm with  $\epsilon$  of 8,000 M<sup>-1</sup> cm<sup>-1</sup>, and FXIa concentration by absorbance at



FIGURE 1. Time course of FXI activation by FXIIa monitored by SDS-gel electrophoresis. FXI (12  $\mu$ M) in assay buffer was incubated at 24 °C with FXIIa (860 nM) as described under "Experimental Procedures." At various times, duplicate aliquots were removed into reducing and non-reducing sample buffer, and fractionated on (A) a 12% polyacrylamide-SDS gel (reducing) or (B) a 6% polyacrylamide gel run (non-reducing), and stained with GelCode Blue (Pierce). Migration of protein standards for unreduced FXI (FXI), unreduced FXIa (FXIa), reduced FXIa (Z), and the heavy chains (HC) and catalytic domains (CD) of reduced FXIa are shown. The position of migration of the reaction intermediate is indicated by INT.

280 nm (corrected for absorbance of the fluorophore) with  $\epsilon$  of 214,400 m<sup>-1</sup> cm<sup>-1</sup>. Probe incorporation was 1.98 moles per mole of FXIa. Fluorescence titrations were performed with an SLM 8100 fluorometer, using acrylic cuvettes coated with polyethylene glycol 20,000. Fluorescence intensity titrations of dansyllabeled FXIa were performed with 335 nm excitation (16-nm band pass) and 552 nm emission (16-nm band pass) in titration buffer supplemented with 2  $\mu$ M D-Phe-Pro-Arg-CH<sub>2</sub>Cl at 24 °C. The quadratic binding equation was fit to fluorescence changes (( $F_{obs} - F_o$ )/ $F_o = \Delta F/F_o$ ) as a function of total FIX concentration, to determine the maximum change in fluorescence ( $\Delta F_{max}/F_o$ ), dissociation constant ( $K_d$ ), and stoichiometry (n) using SCIEN-TIST software (MicroMath Scientific Software, Salt Lake City, UT). Parameter errors represent 95% confidence intervals.

#### RESULTS

Activation of FXI by Factor XIIa and Thrombin—The conversion of the 80-kDa subunits of FXI to the 50-kDa heavy chains and 30-kDa catalytic domains of FXIa is evident on reducing polyacrylamide gels (Fig. 1*A*). On non-reducing gels, the 160-kDa FXI dimer migrates slightly more rapidly than FXIa (Fig. 1*B*). During time course experiments, a species migrating in an intermediate position between FXI and FXIa was observed on non-reducing gels (Fig. 1*B*). The PTT assay is used in clinical laboratories to assess plasma coagulation initiated by surface-dependent activation of FXII (contact activation). Western blots of human plasma exposed to a silica-containing PTT reagent (Fig. 2) demonstrated a band migrating between FXI and FXIa in normal plasma (Fig. 2*A*). Generation of FXIa and the intermediate were dependent on FXIIa (Fig. 2*B*) and the plasma protein HK (Fig. 2*C*), which is required for FXI binding to the contact surface.

An intermediate was also observed during FXI activation by  $\alpha$ -thrombin (Fig. 3*A*). When a mixture of FXI and the intermediate was chromatographed on benzamidine-Sepharose, the intermediate bound (Fig. 3*B*), whereas FXI eluted in the flow-through, indicating the intermediate was an active protease. Unlike FXIa, the intermediate contains uncleaved subunits (Fig. 3*B*) and is, therefore, a species with only one subunit of the dimer cleaved at the Arg<sup>369</sup>–Ile<sup>370</sup> bond (1/2-FXIa). The num-





FIGURE 2. **Time course of FXI activation in plasma by contact activation monitored by Western blotting.** *A*, normal human plasma, and human plasmas lacking Factor XII (*B*) or HK (*C*) were mixed with equal volumes of PTT reagent at 37 °C. At various times, samples were removed into non-reducing sample buffer and fractionated on 6% polyacrylamide-SDS gels, followed by Western blotting as described under "Experimental Procedures." Migration of protein standards for FXI and FXIa are shown, and migration of the reaction intermediate is indicated by *INT*. Note that the time course is longer for factor XII-deficient plasma than for normal or HK-deficient plasma.

ber of moles of active sites per mol of 1/2-FXIa protein was 0.93, compared with 1.98 for FXIa. 1/2-FXIa cleaves a tripeptide substrate at about half of the rate (58%) of an equimolar concentration of FXIa (Fig. 3*C*). Whereas FXIa is not seen in the sample in Fig. 3*B*, we observed traces of FXIa in some 1/2-FXIa preparations.

FXIa with One Inhibited Active Site (FXIa-1/2i)—We prepared an additional single active site FXIa species, FXIa-1/2i, using the procedure shown in Fig. 3A. 1/2-FXIa was incubated with a tripeptide chloromethyl ketone to irreversibly inhibit the active sites. After dialysis, the uncleaved subunit of 1/2-FXIa was activated with FXIIa. The only new active sites formed in this step are due to cleavage of this subunit. No new fully active FXIa was generated because zymogen FXI was removed during preparation of 1/2-FXIa, and any FXIa in the 1/2-FXIa preparation was inactivated by the chloromethyl ketone. The product, FXIa-1/2i, has two active sites per molecule, one of which is blocked by the inhibitor, and was separated from traces of inhibited FXIa, by benzamidine-Sepharose chromatography, as shown (Fig. 3A).

Activities of Single Active Site FXIa Species in Chromogenic Substrate Assays—The kinetic parameters for cleavage of the tripeptide substrate S-2366 by FXIa, 1/2-FXIa, and FXIa-1/2i were determined, and the Michaelis-Menten equation was fit to the data (Table 1), treating each active FXIa subunit as an independent enzyme. Substrate affinity ( $K_m$ ) and catalytic efficiency ( $k_{cat}$ ) were similar for the three proteases. Activation of FIX was studied by progress curve analysis and from the FIX dependence of the initial rate of FIXa $\beta$  formation, using a chromogenic assay (Fig. 4) (22). The results, summarized in Table 1, indicate that FXIa and its singly active derivatives have similar apparent affinities ( $K_m$ ) and turnover numbers ( $k_{cat}$ ) for FIX. The results



FIGURE 3. Time course of FXI activation by  $\alpha$ -thrombin monitored by SDSgel electrophoresis, and a schematic diagram of the generation and purification of 1/2-FXIa and FXIa-1/2i from plasma FXI. A, FXI (625 nm) in assay buffer was incubated at 37 °C with  $\alpha$ -thrombin (625 nm). At various times, aliquots were removed into non-reducing sample buffer and fractionated on SDS-6% polyacrylamide gels. Migration of protein standards for FXI and FXIa are shown, and migration of the reaction intermediate is indicated by 1/2-FXIa. Below the 1-h time point on the gel are schematic diagrams of FXI/FXIa dimers, with ellipses representing heavy chains, and circles representing catalytic domains. A filled circle represents unactivated FXI polypeptide, an open circle represents activated polypeptide, and a circle with an X represents activated polypeptide in which the active site is inhibited with a chloromethyl ketone (CMK). To prepare 1/2-FXIa, FXI undergoes limited activation by thrombin, followed by separation of the active species from residual FXI by chromatography on benzamidine-Sepharose as described under "Experimental Procedures." FXIa with one inhibited active site per dimer (FXIa-1/2i) was prepared by treating 1/2-FXIa with CMK to inhibit all active sites. After dialysis, the unactivated chain of inhibited 1/2-FXIa was activated by incubation with FXIIa. The final product, FXIa-1/2i, was separated from traces of inhibited FXIa by chromatography on benzamidine-Sepharose. B, samples of purified FXI, 1/2-FXIa, and FXIa were fractionated under nonreducing conditions on a SDS-6% polyacrylamide (top) or reducing conditions on a SDS-12% polyacrylamide gel (bottom), and stained with GelCode blue. C, FXIa (○) or 1/2-FXIa (●) (6 nм) in assay buffer containing 500 mм S-2366 was incubated at 24 °C and changes in absorbance at 405 nm were followed as described under "Experimental Procedures."

for 1/2-FXIa are unlikely to be due to contaminating FXIa. If 1/2-FXIa (the vast majority of protease in the preparation) did not cleave FIX, and all FIX activation was due to traces of FXIa, the turnover number ( $k_{cat}$ ) would be very low relative to the FXIa control. Previously, we demonstrated that FIX and FIXa $\beta$  bind to FXIa in a mutually exclusive manner, and that the  $K_i$  for product inhibition and the  $K_m$  for FIX activation were similar (22). In the present studies, the  $K_i$  values for product inhibition

#### TABLE 1

# Kinetic parameters for cleavage of S-2366 and activation of FIX by FXIa, 1/2-FXIa, and FXIa-1/2i

Values for  $K_m$  and  $k_{cat}$  for S-2366 cleavage were determined by fitting the Michaelis-Menten equation to substrate dependence curves using eight concentrations of S-2366.  $K_m$  and  $k_{cat}$  for FIX activation were based on initial rates for FIX activation from full progress curves (Fig. 4) as described under "Experimental Procedures." The resulting  $v_o$  values were analyzed by fitting the Michaelis-Menten equation, and values for  $K_m$  and  $k_{cat}$  were obtained from direct non-linear least squares analysis. Values for  $K_m$  and  $k_{cat}$  were used to analyze the complete progress curves by the integrated Michaelis-Menten equation. With values for  $K_m$  and  $k_{cat}$  fixed, full progress curves were fitted simultaneously by the integrated rate equation with product inhibition to obtain estimates of  $K_i$  for FIXa $\beta$ . Errors in parameters represent 95% confidence intervals.

Substrate	Enzyme	$K_m$	k <sub>cat</sub>	$K_i$
			$min^{-1}$	им
S2366	FXIa	$400\pm20~\mu{ m M}$	$16.1 \pm 0.3$	$ND^{a}$
	1/2-FXIa	$300 \pm 20 \mu M$	$12.4 \pm 0.3$	ND
	FXIa-1/2i	$340 \pm 7 \ \mu M$	$21.6 \pm 0.2$	ND
FIX	FXIa	53 ± 8 пм	$33 \pm 1$	$23 \pm 46$
	1/2-FXIa	90 ± 40 nм	$28 \pm 3$	$23 \pm 26$
	FXIa-1/2i	$90 \pm 30 \mathrm{nM}$	$23 \pm 2$	$18 \pm 23$

<sup>a</sup> ND, not determined.



FIGURE 4. **FIX activation by FXIa**, **1/2-FXIa**, **and FXIa-1/2i followed by chromogenic substrate assay.** FIX at 25 ( $\triangle$ ), 50 ( $\blacktriangle$ ), 100 ( $\square$ ), 250 ( $\blacksquare$ ), 500 ( $\bigcirc$ ), or 1000 nM (O) in assay buffer was incubated at 24 °C with 1–10 nM active sites of FXIa (A), 1/2-FXIa (B), or FXIa-1/2i ( $\bigcirc$ ). At appropriate time points, aliquots were assayed for FIXa $\beta$  activity as described under "Experimental Procedures." D, the initial rates for activation of FIX (25–1000 nM) by FXIa (O), 1/2-FXIa ( $\bigcirc$ ), and FXIa-1/2i ( $\blacksquare$ ) were determined from the progress curves in A-C as described under "Experimental Procedures," and are plotted as a function of initial FIX concentration in each reaction.

were similar for FXIa, 1/2-FXIa, and FXIa-1/2i (Table 1), and were reasonably similar to  $K_m$  values, given the techniques used.

Activities of Single Active Site FXIa Species in Plasma Coagulation Assays—To determine whether 1/2-FXIa and FXIa-1/2i activate FIX in plasma, the enzymes were compared with FXIa in an assay that requires FXIa to activate FIX during generation of a fibrin clot. The relative activities of 1/2-FXIa (102%) and FXIa-1/2i (130%) were comparable with fully active FXIa (activity arbitrarily set at 100%) when corrected for the number of active sites per molecule.

Cleavage of FIX by Factor VIIa/TF and FXIa—During FIX activation by FVIIa/TF, the  $Arg^{145}$ —Ala<sup>146</sup> bond is cleaved initially, resulting in formation of the intermediate FIX $\alpha$  (Fig. 5, A and B) (13–15). In contrast, FIX cleavage by FXIa generates little intermediate (Fig. 5*C*). In our hands (Fig. 5*D*), and others



FIGURE 5. Time courses of FIX activation by FVIIa and FXIa monitored by Western blotting. A, FIX is cleaved after Arg<sup>145</sup> and Arg<sup>180</sup> to release the activation peptide (AP) and produce the active protease FIXa $\beta$ . Initial cleavage after Arg<sup>145</sup> separates the light chain (LC) and the N terminus of the AP, producing the intermediate FIX $\alpha$ . This is the preferred pathway for FIX activation by FVIIa/TF (bold solid arrows). Cleavage initially after Arg<sup>180</sup> results in separation of the FIX heavy chain (catalytic domain) from the C terminus of the AP, generating a partially active intermediate (FIXa $\alpha$ ). This is a minor reaction during FIX activation by FVIIa/TF (small solid arrows). Activation of FIX by FXIa generates relatively little intermediate (broken arrow). B–D, FIX (100 пм) in assay buffer was incubated at 24 °C with (B) 1 nm FVIIa in the presence of saturating TF or (C and D) 1 nm active sites of two different preparations of FXIa. Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Migration of protein standards are shown for zymogen FIX (FIX), the large chain of FIX $\alpha$  (FIX $\alpha$ ), the heavy chain of FIXa $\beta$  (HC), and the light chain of FIXa $\beta$ /FIX $\alpha$ (LC).

(15), traces of intermediate migrating in the position of FIX $\alpha$  are seen in some time courses with FXIa. FXI circulates in plasma as a complex with HK (24). The apparent rate and pattern of FIX cleavage by FXIa was not changed by a saturating concentration (500 nM) of HK under identical conditions to those in Fig. 5 (data not shown).

Cleavage of FIX by FXIa in the Presence of FXIai—The initial interaction of FIX with FXIa involves exosites on the FXIa heavy chain that are available on active site inhibited FXIa (FXIai, see below) (22), and FXIai is expected to behave as a competitive inhibitor of FIX activation by FXIa. The rate of FIX cleavage is significantly reduced when FIX is activated by FXIa in the presence of a 1000-fold molar excess of FXIai (Fig. 6). Interestingly, accumulation of FIX $\alpha$  is also observed, suggesting that 1) FXIa initially cleaves the FIX Arg<sup>145</sup>–Ala<sup>146</sup> bond to form FIX $\alpha$ , and 2) that FIX $\alpha$  is released from FXIa and is avail-



FIGURE 6. Time course of FIX activation by FXIa in the presence of FXIai monitored by Western blotting. FIX (100 nM) in assay buffer was incubated at 24 °C with 2 nM active sites of FXIa in the absence (A) or presence (B) of 1000 nM FXIai. Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Note that the sampling times differ for the two reactions. Migration of protein standards is shown for zymogen FIX (FIX), the large chain of FIXa (HC), and the light chain of FIXa /FIXa (LC).



FIGURE 7. **Time courses of recombinant FIX activation by FXIa monitored by Western blotting.** Recombinant (*A*) wild type FIX, (*B*) FIX-Ala<sup>180</sup>, and (*C*) FIX-Ala<sup>145</sup> (100 nm) in assay buffer were incubated at 24 °C with FXIa (0.2 nm active sites). Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Migration of protein standards is shown for zymogen FIX (FIX), the large chain of FIXa $\alpha$  (HC), and the light chain of FIXa $\beta$ /FIX $\alpha$  (LC).

able to bind to FXIai. To complete conversion to FIXa $\beta$ , FIX $\alpha$  must rebind to FXIa, followed by cleavage of the Arg<sup>180</sup>–Val<sup>181</sup> bond. Therefore, in addition to competing with FXIa for binding to FIX, FXIai also appears to compete for binding to FIX $\alpha$ . The result argues strongly against a mechanism for FIX activation where both activation sites on FIX are cleaved without release of an intermediate.

*FXIa* Cleavage of *FIX-Ala*<sup>145</sup> and *FIX-Ala*<sup>180</sup>—To further demonstrate that *FXIa* initially cleaves *FIX* at the Arg<sup>145</sup>–Ala<sup>146</sup> bond, we used recombinant *FIX* in which Arg<sup>145</sup> or Arg<sup>180</sup> was changed to Ala (Fig. 7). *FIX-Ala*<sup>145</sup> and *FIX-Ala*<sup>180</sup> can only be cleaved to *FIXaa* and *FIXa*, respectively. *FXIa* clearly shows a preference for cleavage after Arg<sup>145</sup> (Fig. 7*B*), readily converting



FIGURE 8. Time courses of FIX activation by single active site FXIa species monitored by Western blotting. FIX (100 nM) in assay buffer was incubated at 24 °C with 2 nM (active sites) (A) 1/2-XIa, (B) FXIa-1/2i, or (C) FXIa/PKA4. Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Migration of protein standards is shown for zymogen FIX (*FIX*), the large chain of FIX $\alpha$  (*FIX* $\alpha$ ), the heavy chain of FIX $\alpha\beta$  (*HC*), and the light chain of FIX $\alpha\beta$ /FIX $\alpha$ (*LC*). There was a defect in the well for the 3-min sample for 1/2-FXIa, which resulted in an abnormal pattern of electrophoresis.

FIX-Ala<sup>180</sup> to the expected intermediate. In comparison, cleavage of Arg<sup>180</sup> in FIX-Ala<sup>145</sup> (Fig. 7*C*) is substantially slower, with only a trace of the large fragment of FIXa $\alpha$  evident on the blot. The data show that FXIa preferentially cleaves FIX at Arg<sup>145</sup>–Ala<sup>146</sup> prior to Arg<sup>180</sup>–Val<sup>181</sup>.

Cleavage of FIX by Single Active Site FXIa Species—Cleavage of FIX by 1/2-FXIa was studied to determine whether FXIa requires two active sites per molecule to cleave FIX without intermediate accumulation. Little intermediate was observed on Western blots (Fig. 8A), showing that one active site per FXIa molecule is sufficient for normal FIX cleavage. The results are not explained by traces of FXIa in the 1/2-FXIa preparation, as demonstrated by consideration of the following scenarios. If two FXIa active sites are required for normal FIX cleavage, intermediate will accumulate when 1/2-FXIa cleaves FIX and should be seen on Western blots, even if a trace of FXIa activates some FIX, because the vast majority of protease is 1/2-FXIa. Alternatively, 1/2-FXIa may not cleave FIX, and all FIX activation is by FXIa. We would not expect the intermediate to form; however, the rate of FIX activation would be significantly reduced relative to the FXIa control. This was not evident in Fig. 8A, and was not observed in the kinetic studies (Fig. 4).

FXIa-1/2i is a dimer with one functional and one blocked active site. The method used to prepare this enzyme makes contamination with FXI or FXIa unlikely. Activation of FIX by FXIa-1/2i generated FIXa $\beta$  with little intermediate accumulation (Fig. 8*B*). We also examined FIX activation by FXIa bound to polyacrylamide beads through an inhibitor occupying one of the FXIa active sites. Functional FXIa active sites are generated after protein was bound to the bead, and it is not possible for fully active FXIa to be present. Intermediate accumulation was not observed when bound FXIa activated FIX (Fig. 9), showing that FXIa with a single active subunit cleaves the FIX activation sites normally when the other subunit is tethered to a surface.

Activation of FIX by FXIa/PKA4—Availability of monomeric FXIa would offer another approach to address the importance





FIGURE 9. Time course of FIX activation by FXIa immobilized on polyacrylamide beads monitored by Western blotting. FIX (100 nM) in assay buffer was incubated at 24 °C with 2 nM active sites of (A) FXIa or (B) FXIa linked to polyacrylamide beads through a chloromethyl ketone inhibitor occupying one of the active sites. Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Migration of protein standards is shown for zymogen FIX (*FIX*), the heavy chain of FIXa $\beta$  (*HC*), and the light chain of FIXa $\beta$  (*LC*).

of the dimeric structure to FIX activation. Removing the interchain disulfide bond involving Cys<sup>321</sup> in the FXI fourth apple (A4) domain does not produce a pure monomeric protein because of non-covalent interactions between the two FXI subunits (21, 25). FXI has a high degree of structural homology to plasma prekallikrein (PK) (4, 26), which is a monomer. Previously, we described recombinant FXIa in which the A4 domain is replaced with the PK A4 domain (20). The chimera, FXIa/ PKA4, is a monomer on size exclusion chromatography (21), and cleaves S-2366 and FIX with similar kinetic parameters to FXIa (20). As with the single active site plasma FXIa species, little intermediate was formed during FIX activation by FXIa/ PKA4 (Fig. 8*C*).

Activation of FIX by FXIa<sup>CD</sup>-Previously we described recombinant FXIa catalytic domain (FXIa<sup>CD</sup>), prepared by activating a FXI variant lacking the Cys<sup>362</sup>–Cys<sup>482</sup> disulfide bond that connects the heavy chain and catalytic domain in FXIa (22). FXIa<sup>CD</sup> cleaves a chromogenic substrate similarly to FXIa, but is a poor activator of FIX (Fig. 10A), likely due to the loss of substrate binding exosites on the heavy chain (22). Sinha et al. (27) showed that FXIa<sup>CD</sup> cleaved FIX with accumulation of FIX $\alpha$ . In these studies, which used stained gels and high substrate concentrations, FIX $\alpha$  was also evident during FIX activation by wild type FXIa. We activated a physiologic concentration of FIX (100 nm) with a high concentration of FXIa<sup>CD</sup> and also observed significant accumulation of FIX $\alpha$  (Fig. 10*B*). The result is distinctly different from those for other FXIa single active site species, and indicates that activation of FIX with limited intermediate accumulation requires the FXIa heavy chain.

The Stoichiometry of FIX and FIXa $\beta$  Binding to FXIa—The data presented so far strongly indicate that each FXIa subunit behaved as a complete enzyme toward FIX. Each FXIa dimer, therefore, should bind two FIX molecules. FXIa was inhibited with a tripeptide chloromethyl ketone linked to a fluorescent



FIGURE 10. Time courses of FIX activation by FXIa<sup>CD</sup> monitored by Western blotting. FIX (100 nM) in assay buffer was incubated at 24 °C with FXIa<sup>CD</sup> 2 nM active sites (*A*) or 50 nM active sites (*B*). Aliquots were removed at the indicated reaction times into denaturing reducing buffer, fractionated on SDS-12% polyacrylamide gels, and analyzed by Western blotting as described under "Experimental Procedures." Note that the sampling times differ for the two reactions. Migration of protein standards is shown for zymogen FIX (*FIX*), the large chain of FIX $\alpha$  (*FIX* $\alpha$ ), heavy chain of FIX $\alpha$  (*HC*), and light chain of FIX $\alpha$  (*LC*).

dansyl group that functions as a reporter of change in the microenvironment around the FXIa active site (19, 28). The fluorescence probe is covalently linked via the chloromethyl ketone to the active site catalytic histidine and serine residues (Fig. 11*A*). Because initial binding of FIX to FXIa involves interactions with exosites remote from the active site (20, 22), blocking the active site does not significantly affect FIX binding.

Binding of FIX to labeled FXIa increased the dansyl fluorescence (Fig. 11*B*), with a maximum enhancement at saturation of  $43 \pm 3\%$ , and a stoichiometry of  $1.9 \pm 0.4$  mol of FIX per mol of FXIa. The  $K_d$  for the interaction (70 nm  $\pm 40$  nm) was consistent with published results (22, 23). Earlier work showed that FIX and FIXa $\beta$  have similar affinities for FXIa (22). Binding of FIXa $\beta$  to labeled FXIa increased the fluorescence to a maximum of  $23 \pm 1\%$  (Fig. 11*C*). The stoichiometry of FIXa $\beta$  binding to FXIa was ~2:1 (2.2  $\pm$  0.4), with a  $K_d$  of 100  $\pm$  50 nm. The results support the conclusion that each half of the FXIa dimer functions as a complete enzyme toward FIX.

# DISCUSSION

In 1977, Bouma and Griffin (3) reported that human FXI was comprised of two disulfide bond-linked polypeptides. FXI is the only coagulation protease that is a dimer (1, 2). An interchain bond involving Cys<sup>321</sup> links the identical 80-kDa FXI subunits in all mammalian species studied, with the exception of the rabbit (His<sup>321</sup>) (29). Rabbit FXI is, however, a non-covalently associated dimer (29). Activation of each FXI subunit requires cleavage of the Arg<sup>369</sup>-Ile<sup>370</sup> bond (3-5). It has been assumed that FXIa formed during coagulation has two cleaved subunits, and essentially all studies of the kinetic and binding properties of plasma FXIa have been conducted with this type of protease. Bouma and Griffin (3) proposed the existence of a form of FXIa with one cleaved subunit, but to date it has not been described in either purified or plasma systems. Indeed, the standard practice of following FXI activation with reducing gels will not allow fully and partially activated species to be distinguished.



FIGURE 11. **Titration of active site-labeled FXIa with FIX and FIXa**, A,  $2 \mu g$  of (*U*) unlabeled FXIa or (*L*) FXIa inhibited with 1,5-dansyl-Glu-Gly-Arg-CH<sub>2</sub>Cl were fractionated on SDS-12% polyacrylamide gels under non-reducing (*left*) or reducing (*center*) conditions, and stained with GelCode Blue. Prior to staining, gels were photographed under ultraviolet light (*right*). Note that the fluorescent dansyl probe has been appropriately incorporated into the FXIa catalytic domain. Migration of protein standards for unreduced FXIa (XIa), heavy chain (*HC*), and catalytic domain (*CD*) of reduced FXIa are shown. *B* and *C*, fluorescence titrations of dansyl-labeled FXIa (100 nm ( $\bigcirc$ ), 550 nm ( $\bigcirc$ ), or 1000 nm ( $\triangle$ )) with FIX (*A*) or FIXa $\beta$  (*B*) were performed as described under "Experimental Procedures." The *solid lines* represent the nonlinear least squares fits by the quadratic binding equation with the parameters given in the text.

Activation of FXI results in formation of functional exosites and active sites that facilitate FIX binding and cleavage (3, 4, 22). The accompanying conformational changes cause FXIa to migrate slower than FXI on non-reducing polyacrylamide gels. When FXI is activated in solution by FXIIa or  $\alpha$ -thrombin, or in plasma by contact activation, a species migrating in an intermediate position between FXI and FXIa is evident. FXI activation clearly goes through this intermediate, and conversion of FXI to the intermediate is relatively rapid compared with conversion of intermediate to FXIa. Generation of the intermediate in plasma requires FXII and HK, similar to the requirements for contact-mediated generation of FXIa. The intermediate, which contains one cleaved and one uncleaved subunit (1/2-FXIa), is an active protease as demonstrated by its capacity to 1) bind to benzamidine and soybean trypsin inhibitor, 2) cleave the substrates S-2366 and FIX, and 3) promote clot formation in plasma.

As discussed, it is unlikely that the activities of 1/2-FXIa are attributable to contaminating FXIa. However, to address this issue we prepared FXIa-1/2i, which has one functional and one inhibited active site per dimer, using techniques that remove FXI and FXIa. FXIa-1/2i in solution, or bound to the surface of a bead performs similarly to FXIa and 1/2-FXIa, providing further support for the concept that one active site is sufficient for FXIa activation of FIX.

These data raise questions concerning the predominant FXIa species during coagulation. While fully activated FXIa forms in plasma during contact activation, a relatively large amount of FXIa is required to initiate coagulation through this mechanism. It is postulated, however, that fibrin formation *in vivo* is initiated by factor VIIa/TF, with FXIa serving a secondary role in clot maintenance (30). Models of this process indicate that very low concentrations of FXIa activity (subpicomolar) can affect fibrin stability (31–33). In the absence of an artificial surface, both FXIIa and  $\alpha$ -thrombin activate FXI to fully activated FXIa slowly, and 1/2-FXIa may be a major active FXI species in plasma.

In current models of hemostasis, FIX activation by FVIIa/TF is required for sustained generation of factor Xa (1, 2, 30), whereas activation by FXIa contributes to consolidation of coagulation in tissues with high fibrinolytic activity (34). In both cases, FIX is cleaved after Arg<sup>145</sup> and Arg<sup>180</sup> to generate FIXa $\beta$  (9–11). FVIIa/TF initially cleaves FIX after Arg<sup>145</sup>, forming FIX $\alpha$ , which accumulates prior to formation of FIXa $\beta$  (9, 12–14). This suggests that cleavage at Arg<sup>180</sup> is rate-limiting, and that FIX $\alpha$  dissociates from FVIIa/TF and is reacquired prior to cleavage after Arg<sup>180</sup>. Interestingly, FXIa also cleaves FIX preferentially after Arg<sup>145</sup>, as shown in studies with FIX-Ala<sup>145</sup> and FIX-Ala<sup>180</sup>, however, an intermediate does not accumulate appreciably (9-11, 15). Two mechanisms could explain these findings. FXIa may activate FIX by sequentially cleaving  $\operatorname{Arg}^{145}$  and  $\operatorname{Arg}^{180}$  prior to releasing FIXa $\beta$ , and without release of an intermediate (a processive mechanism). Alternatively, FIX $\alpha$  may dissociate from FXIa, and rebind to facilitate cleavage at  $\operatorname{Arg}^{180}$ . In this case, the rate of conversion of FIX $\alpha$  to FIXa $\beta$  would be faster than for FIX to FIX $\alpha$  to explain the lack of intermediate accumulation. Activation of prothrombin to  $\alpha$ -thrombin by factor Xa in the prothrombinase complex is a well characterized example of the latter mechanism (35–37).

Wolberg *et al.* (15) proposed a processive mechanism for FIX activation by FXIa, based on the absence of intermediate on Western blots accumulation, and the observation that FIX and the intermediates FIX $\alpha$  and FIXa $\alpha$  are converted to FIXa $\beta$  by FXIa at approximately similar rates. They postulated that the two protease domains of FXIa may cleave the activation sites of one FIX molecule, either simultaneously or sequentially, prior to releasing FIXa $\beta$  (15). In the crystal structure of zymogen FXI, the catalytic domains are at opposite ends of the molecule, and would be unable to interact simultaneously with one FIX molecule.

ecule (38). However, work on the structure of the FXI A4 domain by Samuel *et al.* (16) suggests that conformational changes occur during FXI activation that bring the catalytic domains into closer proximity. In the structure for FXI A4 dimer, an  $\alpha$ -helix not present in the zymogen crystal structure is observed. It is postulated that this  $\alpha$ -helix forms after cleavage of Arg<sup>369</sup>–Ile<sup>370</sup>, and alters interdomain contacts with the opposite A4 domain. This reorients the two halves of the dimer so that the catalytic domains are closer together, permitting them to interact with a single FIX molecule. The availability of the FXIa species with single active sites provided us with a means to address these intriguing proposals.

The results presented here demonstrate that the mechanism involved in FIX activation by FXIa applies to catalysis by individual subunits of the FXIa dimer, and do not support a model in which two catalytic domains are required for normal FIX cleavage. Each FXIa subunit, therefore, can be considered a separate enzyme. Of the more than one hundred trypsin-like proteases described, only FXIa (3-5, 38) and the T-lymphocyte apoptotic protease granzyme A (39, 40) are homodimers. Granzyme A must be a dimer for proper cleavage of macromolecular substrates. The substrate binding pockets of granzyme A are near the dimer interface, and substrate binding exosites on the adjacent subunit extend the active site clefts across the interface (39, 40). The exosites are not available in the protease monomer, resulting in poor substrate recognition. Recognition of FIX by FXIa also involves exosite interactions (17, 20, 22, 27, 41), however, the current results indicate that the active site and exosites required for FIX activation reside on the same FXIa subunit.

Furthermore, the results are not supportive of a processive mechanism in which both factor IX activation sites are cleaved without release of an intermediate. Instead, the data strongly indicate that FIX is cleaved initially at the Arg<sup>145</sup>-Ala<sup>146</sup> bond, forming FIX $\alpha$ , which is released from FXIa. FIX $\alpha$  must then rebind to FXIa, probably in a new conformation facilitating cleavage of the Arg<sup>180</sup>–Val<sup>181</sup> bond, to complete conversion to FIXa $\beta$ . It is clear that the FXIa heavy chain is crucial for this process. In addition to a marked decrease in rate of FIX activation (27, 42, 43), substantial accumulation of FIX $\alpha$ prior to formation of FIXa $\beta$  is observed when FIX is incubated with the single active site species FXIa<sup>CD</sup>, which lacks a heavy chain. The loss of FIX binding exosites on the heavy chain appears to have a greater effect on cleavage after Arg<sup>180</sup> than Arg<sup>145</sup> (27). One FXIa heavy chain exosite likely interacts with the FIX-Gla domain (23). Sinha et al. (27) showed that FIX $\alpha$  accumulated during FIX activation by FXIa in the absence of calcium, supporting the notion that loss of the Gla-FXIa heavy chain interaction disproportionately affects cleavage at Arg<sup>180</sup>.

The observation that FXI is a dimer in all species examined strongly suggests that this is important for some aspect of protease function. Other coagulation serine proteases have vitamin K-dependent modifications to the Gla-domain that facilitate binding to platelets and phospholipid (1, 2, 44). FXI has no Gla-domain, but binds to platelets through platelet glycoprotein 1b (45–47). Previously, we proposed a model where one FXIa subunit tethers the molecule to a platelet, whereas the

other interacts with FIX (48). The observation that 1/2-FXIa has full activity toward FIX despite having only one cleaved subunit raises the possibility that FXI can be activated to 1/2-FXIa on the platelet, with the unactivated subunit remaining bound to glycoprotein 1b. This hypothesis is supported by the finding that FXIa linked to beads through one active site activates FIX with minimal intermediate generation.

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