Macromolecular Substrate-Binding Exosites on Both the Heavy and Light Chains of Factor XIa Mediate the Formation of the Michaelis Complex Required for Factor IX-Activation†

Dipali Sinha,*,‡ Mariola Marcinkiewicz,‡ Duraiswamy Navaneetham,‡ and Peter N. Walsh‡,§

Sol Sherry Thrombosis Research Center, and the Departments of Biochemistry and Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received November 6, 2006; Revised Manuscript Received May 18, 2007

ABSTRACT: Binding of factor IX (FIX) to an exosite on the heavy chain of factor XIa (FXIa) is essential for the optimal activation of FIX (Sinha, D., Seaman, F. S., and Walsh, P. N. (1987) *Biochemistry 26*, 3768−3775). To gain further insight into the mechanisms of activation of FIX by FXIa, we have investigated the kinetic properties of FXIa-light chain (FXIa-LC) with its active site occupied by either a reversible inhibitor of serine proteases (*p*-aminobenzamidine, PAB) or a small peptidyl substrate (S-2366) and have examined FIX cleavage products resulting from activation by FXIa or FXIa-LC. PAB inhibited the hydrolysis of S-2366 by FXIa-LC in a classically competitive fashion. In contrast, PAB was found to be a noncompetitive inhibitor of the activation of the macromolecular substrate FIX. Occupancy of the active site of the FXIa-LC by S-2366 also resulted in noncompetitive inhibition of FIX activation. These results demonstrate the presence of an exosite for FIX binding on the FXIa-LC remote from its active site. Furthermore, examination of the cleavage products of FIX indicated that in the absence of either Ca^{2+} or the heavy chain of FXIa there was substantial accumulation of the inactive intermediate FIXα, indicating a slower rate of cleavage of the scissile bond Arg^{180}−Val^{181}. We conclude that binding to two substrate-binding exosites one on the heavy chain and the other on the light chain of FXIa is required to mediate the formation of the Michaelis complex and efficient cleavages of the two spatially separated scissile bonds of FIX.

Initiation of the blood coagulation cascade at the site of a vascular injury is thought to be triggered through the activation of factor X (FX)[1] by FVIIa in complex with the tissue factor that is exposed at the site of blood vessel damage (*1*−*4*). Activated FX (FXa) in the presence of FV and a phospholipid surface converts prothrombin to thrombin, the enzyme responsible for the conversion of fibrinogen to fibrin (*5*, *6*). Tissue factor pathway inhibitor, however, shuts down the formation of FXa through this extrinsic pathway (*7*−*11*), and further generation of thrombin probably occurs through the intrinsic pathway whereby FXI activation by thrombin generates FXIa, which then activates FIX to its active form FIXa that in turn activates FX to FXa. Activation of FIX to FIXa is achieved through proteolytic cleavages at two specific sites, one after Arg^{145} and the other after Arg^{180}, releasing an 11 kDa activation peptide (*12*−*14*). FXIa, FIXa, FXa, and thrombin are members of the family known as serine proteases. The catalytic domains of all these enzymes are highly homologous to each other as well as to trypsin, the archetypical arginine-specific serine proteinase (*15*, *16*). FXI, however, differs from FIX, FX, and prothrombin in a number of aspects. The N-terminus of the vitamin-K-dependent proteins, FIX, FX, and prothrombin consists of a γ-carboxyglutamic acid domain followed by epidermal growth factor domains in FX and FIX and kringle domains in prothrombin, whereas in contrast, FXI has four homologous domains called apple domains (*17*, *18*). In solution phase, the k_{cat} values for activation of FX by FIXa (0.07 min^{-1}) (*19*) or of prothrombin by FXa (0.0044 min^{-1}) (*20*) are extremely low, whereas the generation of FIXa by FXIa is reasonably fast (k_{cat} ∼20−30 min^{-1}) (*21*−*24*). The presence of a phospholipid surface potentiates the activity of both FIXa and FXa by several orders of magnitude (*19*, *25*, *26*), while the same surface has hardly any effect on the activity of FXIa (*13*, *27*, *28*). It is quite conceivable that the mechanism of activation of FIX by FXIa is different from that of any of the protease substrates downstream in the cascade.

We have previously shown that a FIX-substrate binding exosite is present on the heavy chain of FXIa (*29*), an observation later proved by other investigators (*24*, *30*). To study the mechanism of FIX activation by FXIa in further

† This study was supported by research grant HL74124 from the National Institutes of Health (to P.N.W.).

* To whom correspondence may be addressed. Dipali Sinha, Ph.D., Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, Pennsylvania 19140. Tel: 215-707-4458. Fax: 215-707-3005. E-mail: dipali@temple.edu.

‡ Sol Sherry Thrombosis Research Center.

§ Departments of Biochemistry and Medicine.

[1] Abbreviations: FIX, factor IX; FXIa, factor XIa; FX, factor X; LC, light chain; LCi, inhibited light chain; PAB, *p*-aminobenzamidine; L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline, chromogenic substrate S-2366; *N*-α-benzoxycarbonyl-D-arginyl-glycyl-L-arginine-*p*-nitroaniline, chromogenic substrate S-2765; methylsulfonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide, Spectrozyme fIXa; APMSF, *p*-amidinophenylmethanesulfonyl fluoride.

10.1021/bi062296c CCC: $37.00
Published on Web 08/04/2007

detail, we have now isolated the FXIa catalytic domain or light chain (FXIa-LC) devoid of the heavy chain to compare its functional properties with those of the parent molecule FXIa. We predicted that a reversible active site inhibitor such as *p*-aminobenzamidine (PAB) would inhibit FIX activation by FXIa-LC in a competitive fashion since the substrate-binding exosite that resides in the heavy chain is missing in this enzyme. However, contrary to this expectation, PAB was found to inhibit FIX activation by FXIa-LC in a noncompetitive fashion, suggesting the presence of an additional exosite in the catalytic domain of FXIa. This observation prompted us to compare the kinetic properties of FXIa and FXIa-LC with respect to cleavages of small peptidyl as well as macromolecular substrates. The results presented here demonstrate the presence of an extended macromolecular substrate recognition exosite in the catalytic domain in addition to the one identified previously on the apple domain containing heavy chain.

EXPERIMENTAL PROCEDURES

Pooled normal plasma and FXI deficient plasma were obtained from George King Biomedical (Overland Park, KS). Human FXI, FXIa, FXIIa, FIX, FX, and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant FVIII was generously provided as a gift from Baxter/Hyland (Glendale, CA). Chromogenic substrates S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline) and S-2765 (*N*-α-benzoxycarbonyl-D-arginyl-glycyl-L-arginine-*p*-nitroaniline) were from DiaPharma (Westchester, OH). Spectrozyme fIXa (methylsulfonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide) was from American Diagnostics (Greenwich, CT). 4-Aminobenzamidine (PAB) was from Aldrich (Milwaukee, WI). HEPES, L-α-phosphatidylcholine, L-α-phosphatidylserine, and *p*-amidinophenylmethanesulfonyl fluoride (APMSF) were from Sigma.

Expression of FXI/C362S/C482S. The cDNA construct of the mutant FXI/C362S/C482S was made using the QuikChange site-directed mutagenesis kit (Stratagene, LaJolla, CA), with wild type FXI cDNA inserted into the expression vector pJVCMV serving as the template. Human embryonic kidney 293 (HEK293) cells were cotransfected with 40 μg of the cDNA construct of the mutant FXI/C362S/C482S and 2 μg of the plasmid vector RSVneo using lipofectamine (GIBCO, Carlsbad, CA). RSVneo contains a gene that confers resistance to neomycin and thereby allows for the selection of positive clones. Cells were grown in DMEM with 10% fetal bovine serum for 48 h and then switched to the same medium supplemented with 500 μg/mL of G418. Stable clones expressing the FXI mutant as assessed by ELISA using a polyclonal goat anti-FXI antibody (Affinity Biologicals, Hamilton, Ontario) were expanded in roller bottles (NUNC, Naperville, IL). After reaching confluence, cells were washed, and the media were replaced by serum free Cellgro complete media (Mediatech, Herndon) supplemented with soybean trypsin inhibitor (10 μg/mL) and lima bean trypsin inhibitor (10 μg/mL). Conditioned media were removed every 48 h and kept frozen after supplementing with benzamidine (5 mM) until ready to be purified.

FXI/C362S/C482S from the cell supernatant was purified using monoclonal antibody 5F7 according to the protocol described earlier (*31*, *32*). Briefly, the cell supernatant was passed through the affinity column at a rate of 0.5 mL/min. The column was washed with 10 column volumes of Tris buffered saline followed by elution of the protein using 2 M KSCN. The eluted protein was dialyzed and concentrated using Centricon Plus-20, 30,000 NMWL (Millipore Corporation, Bedford, MA). To prepare FXI-heavy chain and FXI-LC, the concentrated and purified FXI/C362S/C482S was activated with FXIIa at a substrate to enzyme ratio of 20:1 at 37 °C for 24 h. The incubation mixture was passed through a 5F7 antibody column, the unbound LC was purified from FXIIa using a corn trypsin inhibitor agarose column, and the heavy chain bound to the 5F7 column was eluted and purified as described for FXI/C362S/C462S. Protein concentration was estimated using the BCA assay (Pierce, Rockford, IL). The purified proteins were size fractionated on a 4−15% SDS−PAGE under nonreducing conditions and stained using GelCode blue stain (Pierce, Rockford, IL).

Expression of FXI/E361-V607 Using Yeast Expression Vector pPICZαA. A peptide comprising 237 amino acids at the C-terminal end of FXI (FXI/E361-V607) was expressed in yeast following the protocol currently described for the expression of the kunitz domain of protease nexin 2 (*33*). The full-length human FXI cDNA in pJVCMV vector (*30*) served as the template for the synthesis by polymerase chain reaction of the catalytic domain of FXIa. An Xho I site followed by a Kex2 signal cleavage site was introduced at the 5′-end, whereas the 3′-end of the product received a stop codon followed by a Not I site. The polymerase chain reaction product was introduced into the yeast expression vector, pPICZαA (Invitrogen, USA), using the Xho I and Not I sites and propagated in XL1-Blue bacteria. The pPICZαA plasmid containing the FXI/E361-V607 insert was sequenced from 5′ to the α-mating factor secretion signal using an AOX5 primer to confirm mutations at desired sites as well as the integrity of the reading frame. The plasmid construct pPICZαA with the desired insert was linearized at the Sac I site and cloned into the methylotrophic yeast *Pichia pastoris* X33 competent cell genome (Invitrogen, USA). Selected yeast clones were lysed either by repeated heat−thaw cycles or by lyticase (Sigma, USA), and the lysates were analyzed by polymerase chain reaction using specific forward and reverse primers for correct insert size. Successful clones were grown in yeast growth medium containing glycerol, BMGY (buffered medium containing glycerol and yeast nitrogen base), at 30 °C until sufficient cell density was obtained. These cells were transferred to methanol containing expression medium BMMY (buffered medium containing methanol and yeast nitrogen base) and incubated at 30 °C for 96 h under vigorous agitation and supplemented with 0.5% methanol every 24 h. FXI/E361-V607 from the supernatant was purified by ammonium sulfate precipitation followed by ion exchange chromatography. FXIa-LC was obtained by activation of FXI/E361-V607 by incubation with FXIIa at 37 °C for 24 h at a substrate/enzyme molar ratio of 20:1. FXIIa was removed from the preparation using corn trypsin inhibitor/agarose beads (Sigma).

Preparation of Active Site Inhibited Light Chain (LCi) of FXIa. The catalytically inactive light chain of FXIa (LCi) was made by treating the purified FXI-LC with APMSF. FXI-LC at a concentration of 1.3 mg/mL was inactivated by sequential additions of 100 μM APMSF, followed by

incubation at room temperature for 5 min after each addition. Residual enzyme activity was measured using S-2366. After six sequential incubations with APMSF, residual enzyme activity was found to be undetectable. The preparation was dialyzed vs Tris buffered saline and stored at −20 °C until use.

Hydrolysis of S-2366 by FXIa and FXIa-LC in the Presence and Absence of PAB. For kinetic studies of S-2366 hydrolysis, FXIa (3.0 nM) or FXIa-LC (5.3 nM) in Tris buffered saline with 0.1% BSA was added to varying concentrations of S-2366 (0−1.5 mM) at 37 °C, and the change in absorbance at 405 nm was followed on a microtiter plate reader. When PAB was used as the inhibitor, the experiment was done essentially the same way except that the enzyme mixed with the desired concentration of PAB was added to the substrate. Michaelis−Menten constants (K_m and V_{max}) were determined by standard methods using the average of duplicate measurements of initial rates at varying substrate concentrations. Two separate experiments were carried out with similar results. Values of V_{max} were converted to nM pNA/s using an extinction coefficient of 9800 optical density units at 405 nm per mole of pNA. Turnover number (k_{cat}) was calculated from the ratio of V_{max} to enzyme concentration.

Activation of FIX by FXIa and FXIa-LC in the Presence and Absence of PAB. Activation of FIX by FXIa or FXIa-LC was assessed by a modification (*23*) of the method of Wagenvoord et al. (*34*). This is a coupled assay in which FIX is first activated by FXIa or FXIa-LC to FIXa, which is then used to activate FX in the presence of FVIIIa, phospholipids, and calcium. The FXa thus generated was estimated using the chromogenic substrate S-2765. All reagents were diluted in Hepes buffered saline containing 0.1% BSA. FIX (0.2−2.2 μM) was incubated with 5 mM $CaCl_2$ and FXIa (0.7 nM) for 3 min or FXIa-LC (15−20 nM) for 5−10 min in a microtiter plate at 37 °C in a total volume of 50 μL. Five microliters of the reaction mixture was then added to chilled Tris buffered saline with 0.1% BSA containing 25 mM EDTA to make a total volume of 200 μL to prevent further activation of FIX. Control experiments demonstrated that 40-fold dilution of the substrate and enzyme in 25 mM EDTA and lowering the temperature to ∼4 °C was completely effective in curtailing further activation of FIX by FXIa or by FXIa-LC. Five microliters from the diluted reaction mixture was then added to 60 μL Tris buffered saline with 0.1% BSA containing 10 mM $CaCl_2$ followed by the addition of 10 μL of phosphatidylcholine/phosphatidylserine (PC/PS) (10 μM) and was incubated for 2 min at 37 °C. Ten microliters of freshly activated FVIIIa (66 units/mL) followed by 10 μL of FX (2 μM) were then added, and the reaction mixture was incubated for 3 min at 37 °C. Final concentrations of FVIIIa, FX, and PC/PS in the reaction mixture were 6.6 units/mL, 0.2 μM, and 1 μM respectively. Adding EDTA to 25 mM and placing on ice stopped the activation of FX by FIXa. Fifty microliters of each reaction was mixed with 50 μL of 2 mM S-2765, and the change in absorbance at 405 nm was followed on the ThermoMax microtiter plate reader. Activation in the presence of PAB was done in essentially the same way except that FXIa or FXIa-LC with the desired concentration of the inhibitor was added to FIX in the first step. The effect of the residual concentration of PAB (500 nM for the highest concentration of the inhibitor used) in the Xa generation step was found to be negligible (i.e., <0.5% inhibition). The concentration of FIXa generated was obtained from a standard curve constructed using known concentrations of purified FIXa. Michaelis−Menten constants were determined using averages from three separate experiments.

Activation of FIX by FXIa or FXIa-LC in the Presence of S-2366. This assay was similar to the one described above except that different concentrations of FIX (0−2.0 μM) in the microtiter well received a fixed concentration of S-2366 before the addition of the enzyme in the first step of the coupled assay. No detectable cleavage of S-2366 (2 mM) could be demonstrated by FIXa at a concentration as high as 100 nM, which is much higher than the maximum concentration of enzyme generated in the FIXa generation step. The highest concentration of S-2366 in the FXa generation step was 30 μM when FXIa-LC was the activator, and it was 1.5 μM for activation with FXIa. The contribution toward mOD/min of pNA due to cleavage of S-2366 by Xa was <0.5% when FXIa-LC was the activator, and it was <0.05% when FXIa was used as the enzyme. Therefore, the observed inhibition was strictly due to the occupation of the active site of FXIa or FXIa-LC by the peptidyl substrate.

Effect of LCi on the Activation of FIX by FXIa. Different concentrations of FIX (0−1000 nM) in Tris buffered saline with 0.1% BSA containing 5 mM Ca^{2+} in the wells of a microtiter plate at 37 °C received a fixed concentration (0 or 1 μM) of LCi, and activation was initiated by the addition of FXIa (0.25 nM). After incubation for 5 min at 37 °C, the reaction was stopped by the addition of aprotinin at a final concentration of 20 μM to neutralize FXIa. The reaction mixture was made 33% in ethylene glycol, and the generated FIXa was measured by changes in absorbance at 405 nM after the addition of Spectrozyme fIXa at a final concentration of 2 mM. The concentration of FIXa in the reaction mixture was determined from a standard curve generated using known concentrations of FIXa. In a separate experiment, different concentrations of LCi (0−2.5 μM) were added to a fixed concentration of FIX (400 nM), and the FXIa generated was determined as described above. LCi, even at the highest concentration used, did not affect the hydrolysis of Spectrozyme fIXa by FIXa.

Data Analysis. Kinetic parameters were derived by fitting the initial velocity data to the established steady-state rate expressions for classical competitive, classical noncompetitive, or mixed-type inhibition (*35*) using KaleidaGraph (Synergy Software, PCS Inc., Reading, PA), and the results with only the best fits are described. As indicated below (see Results), we observed the formation of the inactive intermediate FIXα due to initial cleavage of the Arg^{145}−Ala^{146} bond of FIX when activated either by FXIa or FXIa-LC. It has been demonstrated by Lu et al. that initial velocity studies of FIXa formation from either FIX or FIXα by FVIIa/TF could be described by the Henri−Michaelis−Menten equation with essentially equivalent steady-state kinetic constants (*7*). Furthermore, our present studies demonstrated linear initial velocities of the formation of FIXa using a wide range of substrate concentrations whether the enzyme was FXIa or FXIa-LC. Since FIXa is the only enzymatically active species in the pathway of FIX activation, these observations permitted the analysis of formation of FIXa by a single set of steady-state kinetic constants. The double reciprocal plots

Table 1: Kinetic Parameters of S-2366 Hydrolysis and FIX Activation by FXIa and FXIa-LC[a]

enzyme	substrate	inhibitor	inhibition type	$K_m \pm$ S.D. (μM)	$k_{cat} \pm$ S.D (min^{-1})	$K_i \pm$ S.D. (μM)
FXIa	S-2366	PAB	competitive	225 ± 25	6780 ± 240	60 ± 7
	FIX	PAB	noncompetitive	0.210 ± 0.029	20 ± 1	95 ± 20
	FIX	S-2366	noncompetitive	0.200 ± 0.020	19.5 ± 2.5	425 ± 52
FXIa-LC	S-2366	PAB	competitive	357 ± 80	7260 ± 240	85 ± 10
	FIX	PAB	noncompetitive	0.385 ± 0.052	0.043 ± 0.007	86 ± 9
	FIX	S-2366	noncompetitive	0.470 ± 0.033	0.035 ± 0.006	420 ± 45

[a] The values were derived from initial velocity measurements using 7 to 8 different substrate concentrations in the presence or absence of 2 to 3 different concentrations of the inhibitor.

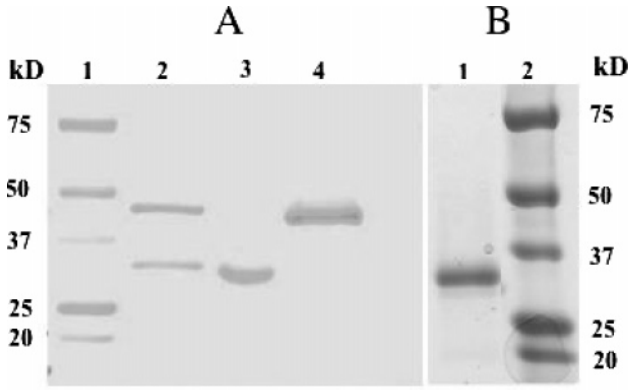

FIGURE 1: SDS−PAGE of purified light and heavy chains of FXIa under reducing conditions. The heavy and light chains of FXIa prepared as described in the Experimental Procedures section were size fractionated on SDS−PAGE (4−15% gradient gel), and the bands were visualized using GelCode Blue. (A) Lane 1, molecular weight marker; lane 2, plasma FXIa; lane 3, purified light chain (from mammalian expression system); lane 4, purified heavy chain. (B) Lane 1, purified light chain made in yeast expression system; lane 2, molecular weight marker.

shown as inserts are provided solely for the purpose of visual presentation to support the conclusions drawn from nonlinear least-square analysis. The values of the kinetic constants provided in the legend of each graph and in Table 1 are averages of two or more experiments.

Characterization of FIX Cleavage Products upon Activation with FXIa or FXIa-LC. FIX was incubated with FXIa or FXIa-LC in the presence of either Ca^{2+} (5 mM) or EDTA (2 mM) at 37 °C at a substrate to active site ratio of 50:1, and at different intervals, aliquots were transferred to SDS−PAGE sample buffer followed by fractionation under reducing conditions and visualization of the bands by staining with GelCode blue. Cleavage of only $Arg^{145}-Ala^{146}$ should result in generating two bands of Mr 18 kDa (light chain, LC, of FIXa) and 38 kDa (heavy chain, HC, of FIXa + the activation peptide), whereas cleavage of only $Arg^{180}-Val^{181}$ should result in generating a single band of Mr 28 kDa (LC of FIXa + activation peptide or heavy chain of FIXa). Cleavages of both bonds produce three bands of Mr 28 kDa (HC), Mr 18 kDa (LC), and Mr 10 kDa (activation peptide).

RESULTS

Characterization of the Expressed Proteins. Purity of the heavy and the light chain preparations was judged by their fractionation on SDS−PAGE (Figure 1). The isolated light chain prepared using the mammalian expression system had no detectable contamination with the heavy chain of FXIa as confirmed by ELISA using two different heavy chain specific monoclonal capture antibodies, and this preparation was indistinguishable from FXIa-LC expressed in the yeast expression system with respect to its size and functional activities toward both the small peptidyl substrate S-2366 and the macromolecular substrate FIX. The data shown in Figures 2−4 were obtained using yeast FXIa-LC, whereas those shown in Figures 5 and 6 were obtained from the mammalian expression system.

Kinetic Analysis of the Hydrolysis of S-2366 by FXIa and FXIa-LC in the Presence and Absence of PAB. PAB is a reversible inhibitor of serine proteases. The mechanism of inhibition of several coagulation enzymes by PAB has been extensively investigated, and it has been shown that PAB competes for the binding of the small peptidyl substrate to the active site of the enzyme (*36*−*41*). Thus, PAB has been shown to inhibit FVIIa with a K_i of 60−70 μM (*36*) and prothrombinase with a K_i of ~32 μM (*42*). Therefore, PAB is expected to inhibit the amidolytic activity of FXIa or FXIa-LC in a competitive fashion. Initial velocity measurements of the hydrolysis of the chromogenic substrate S-2366 in the presence of different fixed concentrations of the inhibitor showed that PAB is a classical competitive inhibitor of FXIa and FXIa-LC (Figure 2). Increasing concentrations of the inhibitor systematically increased the K_m without any significant change in V_{max}, and the same observation was also reflected in the double reciprocal plots (inset of Figure 2). Thus, PAB inhibits the amidolytic activity of both FXIa and FXIa-LC by competing with the peptidyl substrate for the binding site on the enzymes. The inhibition constant K_i was found to be 60 ± 7 μM for FXIa and 85 ± 10 μM FXIa-LC (see Table 1).

Kinetic Analysis of the Activation of FIX by FXIa and FXIa-LC in the Presence and Absence of PAB. The results of the effect of PAB on FIX activation by FXIa and FXIa-LC are shown in Figure 3. Analysis of the initial velocity measurements demonstrated that in contrast to small peptidyl substrate hydrolysis (Figure 2), increasing concentrations of PAB systematically decreased V_{max} without altering the K_m, an example of classical noncompetitive inhibition. Double reciprocal plots of the initial velocity data (inset of Figure 3) also indicated a change in V_{max} without any significant alteration in K_m, indicating that in either case PAB inhibited FIXa generation by a noncompetitive mechanism. Since FIX activation was studied using a coupled assay and PAB is known to inhibit all serine proteases, we had to make sure that the inhibitory effect seen in our assay was not due to the effect of residual PAB on generated FIXa and FXa. Two parallel sets of experiments were therefore carried out. In one experiment, PAB along with FXIa or FXIa-LC was added to FIX in the first stage, and in the other experiment,

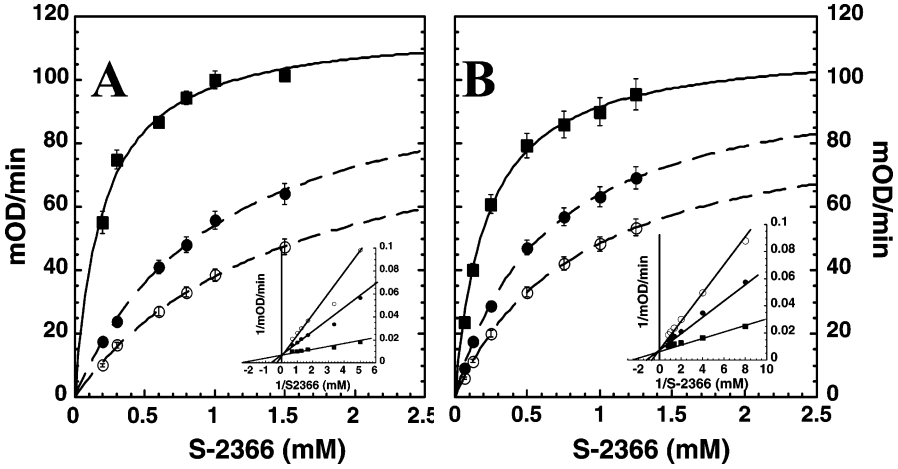

FIGURE 2: Hydrolysis of S-2366 by FXIa and FXIa-LC in the presence and absence of PAB. (A) Increasing concentrations of S-2366 (0−1.5 mM) were incubated with FXIa (3 nM) in the presence of a fixed concentration (■, 0 μM; ●, 220 μM; ○, 440 μM) of PAB, and the rate of pNA generation was measured and analyzed according to a competitive inhibition mechanism as described in the Experimental Procedures section with the following fitted constants: $K_m = 225 \pm 25$ μM, $k_{cat} = 6780 \pm 240$ min^{-1}, and $K_i = 60 \pm 7$ μM. Inset: Double reciprocal plots of the data and fitted lines. (B) Increasing concentrations of S-2366 (0−1.25 mM) were incubated with FXIa-LC (5.3 nM) in the presence of a fixed concentration (■, 0 μM; ●, 143 μM; ○, 286 μM) of PAB, and the rate of pNA generation was determined and analyzed as described above with the following fitted constants: $K_m = 357 \pm 80$ μM, $k_{cat} = 6780 \pm 240$ min^{-1}, and $K_i = 85 \pm 10$ μM. Inset: Double reciprocal plots of the data and the fitted lines.

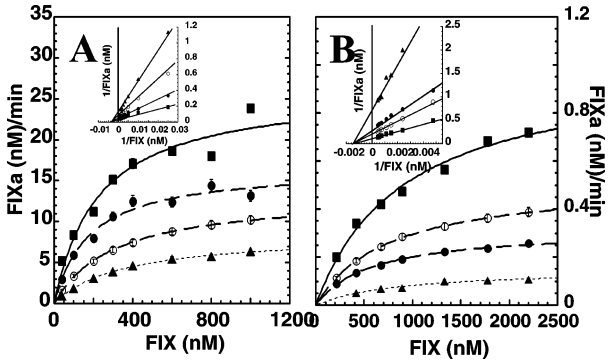

FIGURE 3: Activation of FIX by FXIa or FXIa-LC in the presence and absence of PAB. (A) Increasing concentrations of FIX (0−1000 nM) were incubated with FXIa (0.7 nM) for 3 min in the presence of a fixed concentration (■, 0 μM; ●, 150 μM; ○, 250 μM; ▲ 400 μM) of PAB, and the rate of FIXa generation was determined using the coupled assay as described in the Experimental Procedures section. Titration curves were generated according to classical noncompetitive inhibition with the following fitted parameters: $K_m = 210 \pm 29$ nM, $k_{cat} = 20 \pm 1$ min^{-1}, and $K_i = 95 \pm 20$ μM. Inset: Double reciprocal plots of the data and the fitted lines. (B) Increasing concentrations of FIX (0−2200 nM) were incubated with FXIa-LC (20 nM) for 10 min in the presence of a fixed concentration (■, 0 μM; ○, 150 μM; ● 250 μM; ▲ 400 μM) of PAB, and the rate of FIXa generation was determined using the coupled assay as described above. The data were analyzed according to a classical noncompetitive inhibition model with the following fitted kinetic constants: $K_m = 385 \pm 52$ nM, $k_{cat} = 0.043 \pm 0.007$ min^{-1}, and $K_i = 86 \pm 9$ μM. Inset: Double reciprocal plots of the data and the fitted lines.

PAB was added at the FXa generation stage at the same final concentration as that achieved at this step of the first set. In the FXa generation stage, the concentration of PAB used in the first stage is diluted by 800-fold, and its inhibitory effect at this low concentration was found to be negligible (i.e., <0.5%). The noncompetitive inhibition by PAB seen for FXIa was not surprising since the FIX binding site is known to reside on the heavy chain of FXIa. What was surprising was that inhibition by PAB of FIX activation by FXIa-LC was best described by a fit of the data to a noncompetitive inhibition model. We expected that PAB should inhibit FXIa-LC in a competitive fashion since the FIX-binding exosite is missing because of the absence of the heavy chain of FXIa. This unexpected result demonstrated the presence of a second FIX substrate-binding exosite on the light chain of FXIa that is remote from the active site. The inhibition constant K_i was found to be 95 + 20 μM for FXIa and 86 + 9 μM for FXIa-LC (see Table 1).

Kinetic Analysis of the Activation of FIX by FXIa and FXIa-LC in the Presence and Absence of S-2366. As shown in Figure 4A and B, the small peptidyl substrate S-2366 inhibited FIX activation both by FXIa and FXIa-LC, and from data analysis (Table 1), the type of inhibition was found to be noncompetitive in both cases. The noncompetitive inhibition for FIX activation by FXIa was expected since it confirms further the existence of an exosite on the heavy chain of the enzyme reported previously (*29*, *30*). If the small peptidyl substrate S-2366 and FIX binding sites on FXIa-LC are mutually exclusive (i.e., identical), then S-2366 should inhibit FIXa generation in a competitive fashion. However, as shown in Figure 4B, the inhibition by S-2366 of FIX activation by FXIa-LC was found to be purely noncompetitive, demonstrating that FIX binds to a site on the FXIa-LC remote from the active site.

Effect of LCi on the Activation of FIX by FXIa. As shown in Figure 5A and B, LCi inhibited FIX activation by FXIa. In the absence of LCi, the values of K_m and k_{cat} corresponded closely with those recorded in Table 1. Since the active site of the light chain is inactivated in LCi, and hydrolysis of Spectrozyme fIXa by FIXa is unaffected by LCi, the only way the inhibition can be explained is that the substrate is binding to the inhibitor forming a nonproductive SI complex. In a system with an enzyme with one active site and a substrate with a single cleavage site, such inhibition should produce a sigmoid curve (*43*). It is clear in Figure 5A that the curve in the presence of 1 μM LCi is not perfectly sigmoid, although inhibition is evident both from Figure 5A and B. The reason for such deviation from ideality is probably due to the fact that FXIa has two active sites and that the substrate FIX is cleaved at two separate sites. The

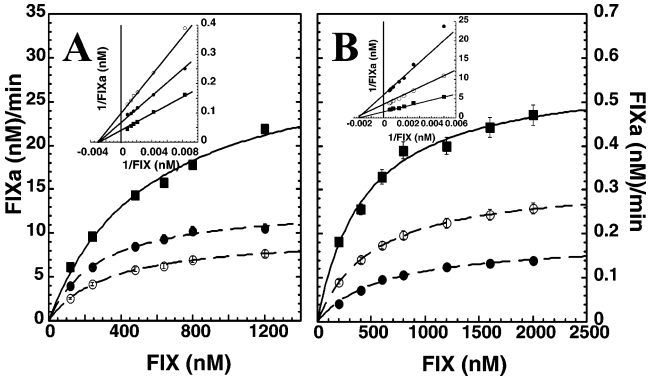

FIGURE 4: Activation of FIX by FXIa and FXIa-LC in the presence and absence of S-2366. (A) Increasing concentrations of FIX (0−1000 nM) were incubated with FXIa (0.7 nM) for 3 min in the presence of a fixed concentration (■, 0 mM; ●, 600 μM; ○, 1200 μM) of S-2366, and the rate of FIXa generation was determined using the coupled assay. Titration curves were generated according to complete noncompetitive inhibition with the following parameters: $K_m = 200 \pm 20$ nM, $k_{cat} = 19.5 \pm 2.5$ min^{-1}, and $K_i = 425 \pm 52$ μM. Inset: Double reciprocal plots of the data and the fitted lines. (B) Increasing concentrations of FIX (0−2000 nM) was incubated with FXIa-LC (15 nM) for 5 min in the presence of a fixed concentration (■, 0 mM; ○, 500 μM; ●, 1500 μM) of S-2366, and the rate of FIXa generation was determined using the coupled assay as described above, and the data were analyzed according to a classical noncompetitive inhibition model with the following fitted kinetic parameters: $K_m = 470 \pm 33$ nM, $k_{cat} = 0.035 \pm 0.006$ min^{-1}, and $K_i = 420 \pm 45$ μM. Inset: Double reciprocal plots of the data and the fitted lines.

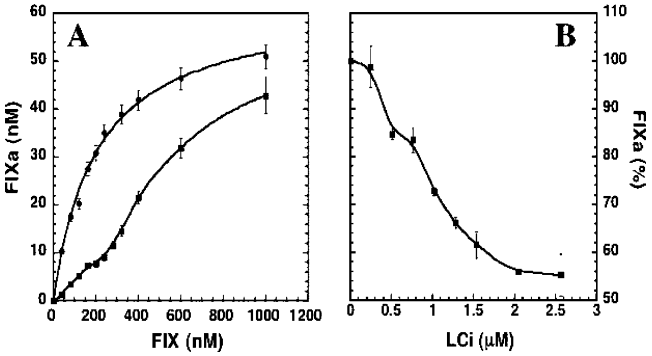

FIGURE 5: Effect of LCi on the activation of FIX by FXIa. (A) Increasing concentrations of FIX (0−1200 nM) were incubated with FXIa (0.25 nM) in the presence (■) and absence (●) of a fixed concentration (0 or 1 μM) of LCi for 5 min at 37 °C. FXIa was neutralized by the addition of aprotinin at a final concentration of 20 μM. The reaction mixture was made 33% in ethylene glycol, and the generated FIXa was measured using Spectrozyme fIXa as described in the Experimental Procedures section. (B) A fixed concentration of FIX (400 nM) was incubated with FXIa (0.25 nM) in the presence of varying concentrations (0 to 2.56 μM) of LCi for 5 min at 37 °C, and the FIXa generated was determined as described above. The amount of FIXa generated in the absence of LCi (41 nM) is expressed as 100% in panel B, and all other data points are expressed as percentages of this control.

only way to better understand this inhibition is to use monomeric FXIa and a single cleavage site mutant of FIX.

Activation of FIX by FXIa and FXIa-LC and Size Fractionation of the Cleavage Products. FIX was activated by FXIa or FXIa-LC at a substrate to active site ratio of 50:1 in the presence of Ca^{2+} (Figure 6, panels A and B) or in the presence of EDTA (Figure 6, panels C and D), and the cleavage products were size fractionated on SDS−PAGE under reducing conditions. The activation peptide is not visible on SDS−PAGE because it is heavily glycosylated and is therefore not adequately stained. Examination of the cleavage products clearly demonstrates that in the presence of Ca^{2+}, activation of FIX is much faster by FXIa (panel A) than by FXIa-LC (panel B), confirming the data obtained by kinetic analysis (Table 1). It is also evident that there is a distinct difference between the Ca^{2+}-dependent (panel A) and the Ca^{2+}-independent (panel C) activation of FIX by FXIa. The formation of FIXa resulting from cleavages of both of the scissile bonds of FIX was much faster in the presence of Ca^{2+} (panel A) than in its absence (panel C). Furthermore, in the presence of Ca^{2+} (panel A), the major activation product even at the earliest time point studied was FIXa, although there was slight (<10% at 30 s) accumulation of the inactive intermediate FIXα (*M*r ~38 kD, i.e., the FIX catalytic domain linked to the activation peptide). Initial accumulation of this intermediate was evident at other substrate to active site ratios of 100:1, 200:1, and 250:1 that we have studied (data not shown). In the absence of Ca^{2+} (panel C), however, the accumulation of inactive FIXα is much more pronounced. Interestingly, whereas Ca^{2+} had a major effect on the activation of FIX by FXIa (panels A and C), it did not have any effect when FXIa-LC was the enzyme (panels B and D), and the major cleavage product that appeared initially was the inactive intermediate FIXα. Thus, the rate of cleavage of the scissile bond $Arg^{180}-Val^{181}$ of FIX is slower when activated by FXIa in the absence of Ca^{2+} or by FXIa-LC in the presence or absence of Ca^{2+}. Further details of these two separate bond cleavages in FIX by FXIa are presented in the Discussion section.

DISCUSSION

In the intrinsic pathway of blood coagulation, FIX is activated to FIXa by FXIa in the presence of calcium ions. The formation of fully active FIXa requires the cleavage of two peptide bonds, one at $Arg^{145}-Ala^{146}$ and the other at $Arg^{180}-Val^{181}$ of the zymogen (*13*). The mechanism of interaction of the substrate with the enzyme to facilitate these two peptide bond cleavages is incompletely understood. FXIa is unique in its homodimeric structure among the trypsin-like serine proteases of the blood coagulation cascade. In addition, FXIa-catalyzed FIX-activation is the only enzymatic reaction in blood coagulation for which there is no defined exogenous protein cofactor; therefore, it is particularly interesting that the heavy chain of FXIa is required for the optimal activation of FIX, although the active site of the enzyme resides within the light chain. Thus, the heavy chain can be regarded as containing an endogenous cofactor in the form of an extended macromolecular substrate recognition exosite. To gain further insight into this enzymatic process, we have in the present study investigated the functional properties of the isolated monomeric catalytic domain (FXIa-LC) and compared them with those of the full-length dimeric enzyme FXIa. Our initial experimental observation was a surprise. A reversible active site inhibitor PAB was found to be a noncompetitive inhibitor of FIX activation by FXIa-LC. We expected that PAB would prove to be a classical competitive inhibitor of FXIa-LC since the FIX-binding exosite that resides in the heavy chain of FXIa is missing in FXIa-LC. This observation led us to investigate FXIa-LC in further detail and to examine the cleavage products of FIX resulting from activation by FXIa and FXIa-LC.

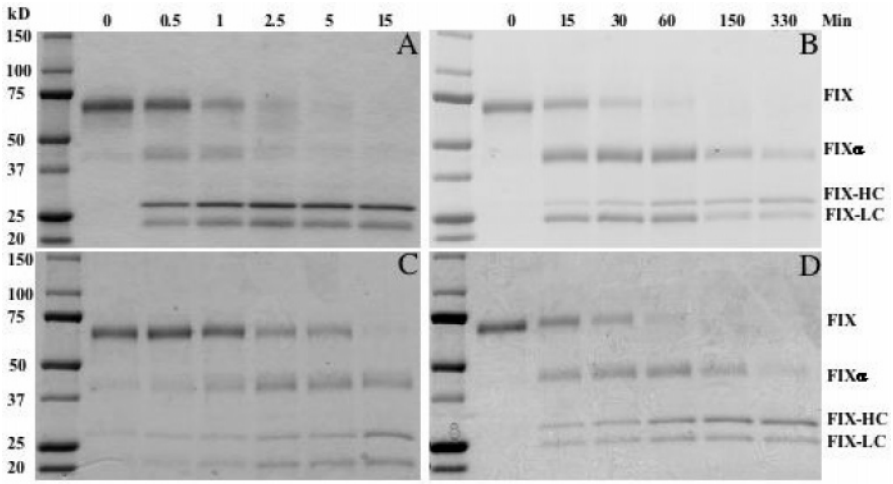

FIGURE 6: FIX cleavage by FXIa or FXIa-LC in the presence or absence of Ca^{2+}. FIX was incubated with FXIa or FXIa-LC at a substrate to active site ratio of 50:1 in the presence of 5 mM Ca^{2+} or in the presence of 2 mM EDTA. At different intervals, as indicated, aliquots were boiled in SDS−PAGE buffer and fractionated under reducing conditions using a 4−15% gradient gel. The bands were visualized using GelCode Blue. (A) Activation of FIX by FXIa in the presence of 5 mM Ca^{2+}. (B) Activation of FIX by FXIa-LC in the presence of 5 mM Ca^{2+}. (C) Activation of FIX by FXIa in the presence of 2 mM EDTA. (D) Activation of FIX by FXIa-LC in the presence of 2 mM EDTA.

A reversible active-site inhibitor of serine proteases such as PAB is expected to inhibit hydrolysis of S-2366 or activation of FIX by FXIa or by FXIa-LC in a purely competitive fashion if the substrate and the inhibitor binding sites on the enzymes are mutually exclusive, i.e., identical. This in fact was the case when PAB was used as an inhibitor for the hydrolysis of S-2366 by FXIa or FXIa-LC (Figure 2). Inhibition by PAB of FIX activation by FXIa was, however, found to be noncompetitive (Figure 3A). A similar result has recently been reported when aprotinin, another reversible inhibitor of FXIa, was used instead of PAB (*24*). This result was not surprising considering the fact that previous studies identified the presence of a substrate-binding exosite on the heavy chain of FXIa (*29*, *30*). If the substrate binding exosite is present only in the heavy chain of FXIa, then PAB should inhibit the activation of FIX by FXIa-LC in a competitive fashion, but it is evident from our results that the mechanism is purely noncompetitive (Figure 3B). Activation of the macromolecular substrate FIX by FXIa-LC was further examined with the active site of the enzyme occupied by a peptidyl substrate. It is quite clear from the results (Figure 4B) that the inhibition resulting from occupation of the active site by the small peptidyl substrate S-2366 was noncompetitive, that is, a decrease in V_{max} without any significant change in K_m, further confirming that the binding sites for the macromolecular and the peptidyl substrates are separate and distinct. Therefore, during activation of FIX by FXIa-LC, the macromolecular substrate FIX binds to FXIa-LC at a site remote from its active site. FXIa-LC activates FIX with a K_m of ~386 nM, which is quite comparable to the ~210 nM obtained for the activation of FIX by FXIa. The turnover number (k_{cat}) for FXIa-LC is ~465-fold impaired, and the catalytic efficiency (k_{cat}/K_m) is ~850-fold impaired compared with intact dimeric FXIa (Table 1), suggesting that the exosite on the light chain is necessary but not sufficient for optimal catalysis. However, this impairment serves to emphasize the kinetic contribution of the heavy chain exosite and in no way implies that the light chain exosite makes a minor contribution. In fact, an observation from the experiment shown in Figure 6 is that the rate of bond cleavage at R145 in FIX to generate FIXα is only ~15-fold slower for the isolated monomeric catalytic domain than for the dimeric full-length FXIa (compare the 1-min time point for FXIa in panel 6A with the 15-min time point for FXIa-LC in panel 6B). Thus, it can be concluded that FXIa-LC is only 15-fold less efficient than FXIa in cleaving FIX at R145, and that the major advantage of the presence of the heavy chain substrate-binding site is to promote efficient cleavage of FIX at R180. Our hypothesis is that these two exosites, located in the heavy chain and the light chain act in concert to position the macromolecular substrate for efficient cleavage by the enzymatic active sites. However, we cannot understand the contribution of the LC exosite until the mechanism of cleavages of the two scissile bonds of FIX by full length dimeric FXIa is elucidated, and the location and functional contribution of the two exosites acting in concert are defined.

If the exosite on the heavy chain of FXIa were the only substrate−enzyme binding site required for optimal functional activity of FXIa, then FIX activation by FXIa would remain unaffected in the presence of LCi. However, LCi was found to inhibit FIXa generation as demonstrated in Figure 5A and B. This result demonstrates that LCi by binding to FIX through an exosite forms a nonproductive complex and thereby depletes the substrate. The formation of a productive complex at higher substrate concentrations overcomes this inhibitory effect (*43*). A plot of *V* vs *S* for such an inhibition should produce a sigmoidal curve when an enzyme with one active site and a substrate with a single cleavage site are involved. However, the data in Figure 5A depicts the proteolytic cleavage of a substrate with two scissile bonds by an enzyme with two active sites, whereas LCi is a monomer. Therefore, although the curve depicted in Figure 5A demonstrates some sigmoidicity, we did not attempt a formal analysis of these data by models that assume a single enzyme active site and a single substrate cleavage site. Studies are planned in the future to re-examine the inhibitory effects of LCi on the cleavage of FIX mutants with a single cleavage site by monomeric FXIa. Nonetheless, since LCi had no effect on the hydrolysis of Spectrozyme fIXa by FIXa,

the observed effects shown in Figure 5A and B must be due to the formation of a nonproductive SI complex. This result confirms further that FIX binds to the FXIa light chain at a site remote from the active site. The results of these studies demonstrate that the mechanism by which FXIa or FXIa-LC hydrolyzes the small peptidyl substrate S-2366 is quite different from the way it activates its macromolecular substrate FIX. Similar conclusions have previously been drawn for the activation of FX by the extrinsic Xase complex and for activation of prothrombin by prothrombinase (*36*, *37*).

Using an ELISA-based system for FIX activation by FXIa, Pedicord et al. (*44*) reported mixed type inhibition for active site inhibitors such as leupeptin or aprotonin, and they concluded that the active site of FXIa does not contribute significantly to the affinity of FXIa toward FIX. A similar conclusion was reached by Ogawa et al. (*24*) using aprotinin as an inhibitor and a peptidyl substrate to measure FIXa formation. Our data showing noncompetitive inhibition by PAB of FIX activation by FXIa further confirm the existence of an exosite outside the active site of FXIa for its macromolecular substrate FIX. The interesting new observation that we are reporting is the existence of a second exosite on the catalytic domain of FXIa. Ogawa et al. (*24*) demonstrated that whereas FIX inhibits S-2366 hydrolysis by FXIa no inhibition by FIX of S-2366 hydrolysis by FXIa-LC could be observed, and they attributed this result as being due to the loss of a FIX binding site in the heavy chain of FXIa. These results do not contradict the existence of a FIX substrate binding exosite within the light chain or catalytic domain of FXIa that is distinct and separate from that occupied by the same peptidyl substrate, S2366.

It has been well documented that when FIX is activated by FVIIa/tissue factor in the extrinsic pathway, initial cleavage occurs at the $Arg^{145}-Ala^{146}$ bond followed by $Arg^{180}-Val^{181}$ that results in accumulation of the intermediate FIXα. Subsequent cleavage of $Arg^{180}-Val^{181}$ in FIXα generates FIXaβ (*13*, *21*, *22*, *45*). However, for activation of FIX by FXIa, there are controversial reports regarding whether the two peptide bonds are simultaneously or sequentially cleaved (*12*−*14*, *21*, *22*, *27*). Since our kinetic data demonstrated that FXIa-LC is comparable to the parent enzyme in its effectiveness to hydrolyze the small peptidyl substrate but is a poor activator of the macromolecular substrate FIX, we investigated how FXIa-LC hydrolyzes the two scissile bonds of FIX compared to FXIa. Examination of the cleavage products of FIX activation in the presence of Ca^{2+} by FXIa (Figure 6, panel A) and by FXIa-LC (Figure 6, panel B) clearly demonstrated that the formation of FIXa resulting from the cleavages of both of the scissile bonds is much faster when FXIa was the enzyme. Moreover, for activation of FIX by FXIa-LC (Figure 6, panel B), the rate of cleavage of the $Arg^{145}-Ala^{146}$ peptide bond in FIX was much faster than that of $Arg^{180}-Val^{181}$, resulting in the accumulation of the inactive intermediate FIXα. This explains why FIX activation by FXIa-LC is characterized by a severely reduced k_{cat}. Examination of the cleavage products of FIX activation generated in the presence and absence of Ca^{2+} demonstrated very clearly that while there is a distinct difference between the Ca^{2+}-dependent (Figure 6, panel A) and the Ca^{2+}-independent (Figure 6, panel C) activation of FIX by FXIa, Ca^{2+} did not seem to play any role when FXIa-LC was the enzyme (Figure 6, panels B and D). For the activation of FIX by FXIa in the presence of Ca^{2+} (Figure 6, panel A), the major activation product even at earlier time points was FIXa, although minor (<10%) accumulation of FIXα was evident. When the cleavage patterns in panels A and C of Figure 6 are compared, it is clear that in the absence of Ca^{2+} the rate of cleavage of the scissile bond $Arg^{180}-Val^{181}$ of FIX by FXIa is much slower compared to that of the $Arg^{145}-Ala^{146}$ bond that results in the accumulation of inactive intermediate FIXα. When FXIa-LC was the enzyme (panels B and D of Figure 6) the rate of cleavage of the scissile bond $Arg^{180}-Val^{181}$ is slow, irrespective of the presence or absence of Ca^{2+}. This result explains why FIX-activating activities of FXIa and FXIa-LC were found to be very similar in the absence of Ca^{2+} (*29*).

In the present study, we have examined the cleavage products of FIX resulting from activation by FXIa at several different substrate to enzyme ratios (50:1−250:1), and some accumulation of FIXα was detected consistently at earlier time points. This result is in contrast to that reported by Wolberg et al. (*22*). We believe that the discrepancy is due to the use of different detection systems. We used a direct staining method, whereas Wolberg et al. used western blot analysis. This observation, however, does not rule out the possessive mechanism that the authors proposed for the activation of FIX by FXIa. At this point, we are investigating whether cleavage of the scissile bond $Arg^{145}-Ala^{146}$ is a requirement for the subsequent cleavage of the $Arg^{180}-Val^{181}$ bond to generate fully active FIXa.

Since FIX has been shown to bind to the heavy chain of FXI through its γ-carboxyglutamic acid domain (*46*), the lack of an ordered structure of the γ-carboxyglutamic acid domain in the absence of Ca^{2+} may prevent it from binding to the heavy chain of FXIa. Therefore, the absence of Ca^{2+} or the heavy chain produces similar effects. We speculate that binding to the two exosites one on the heavy chain and the other on the light chain is likely to be required to overcome the geometric constraints for active site docking before cleavage of the two spatially distinct peptide bonds. On the basis of these results, we propose a model for the activation of FIX by FXIa and FXIa-LC as shown in Figure 7. Binding of FIX to two exosites, one on the heavy chain and the other in the light chain of FXIa, brings the two scissile bonds into favorable positions to be docked and cleaved by FXIa in rapid succession (Figure 7A). Lack of either the heavy chain (Figure 7C) or of Ca^{2+} (Figure 7B) allows the binding of the substrate to occur only through the light chain, which results in reduced rates of cleavage of the $Arg^{180}-Val^{181}$ bond and consequent accumulation of FIXα. The absence of the heavy chain in FXIa-LC is not the only difference between FXIa and FXIa-LC since FXIa is a dimer with two active sites, whereas FXIa-LC is a monomer. Therefore, the presence of two active sites in FXIa might contribute to the accelerated rate of cleavages of the two scissile bonds in FIX, as suggested by Wolberg et al. (*22*).

The presence of substrate binding exosites has been well documented in thrombin (*47*), prothrombinase, and the FX activating complex (*36*, *37*, *39*), but FXIa is unique by possessing two substrate binding exosites for its macromolecular substrate FIX. The heavy chain of FXIa in this respect can be compared to the cofactor FVa in prothrombinase, to FVIIIa in Xase in the intrinsic pathway, or to tissue factor

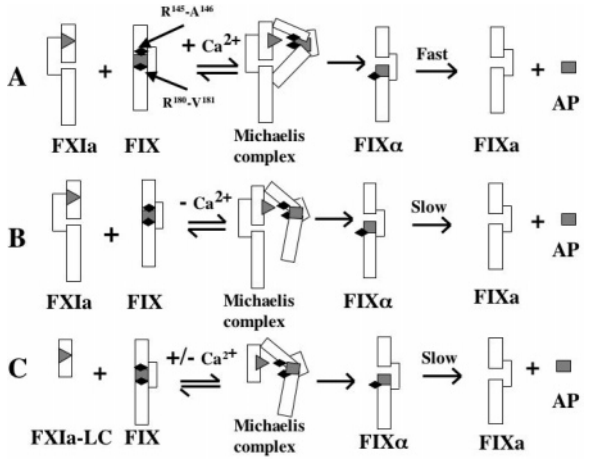

FIGURE 7: Proposed model illustrating the cleavages of the two spatially separated scissile bonds of FIX. (A) In the presence of Ca^{2+}, FXIa binds to FIX through both its heavy and light chains that brings the two spatially separated scissile bonds closer together to be docked and cleaved almost simultaneously by the enzyme to form FIXa with very little accumulation of FIXα since the subsequent cleavage of the scissile bond $Arg^{180}-Val^{181}$ is fast. (B) FIX binds only to the exosite in the light chain of FXIa since in the absence of Ca^{2+}, the γ-carboxyglutamic acid-domain of FIX is either unable or binds with low affinity to the heavy chain of FXIa, resulting in sequential cleavages with substantial accumulation of FIXα resulting from the slower cleavage of the scissile bond $Arg^{180}-Val^{181}$. (C) FIX can bind only to the light chain of the enzyme in the absence of its heavy chain, and there is substantial accumulation of FIXα as in the case of Ca^{2+}-independent activation of FIX by FXIa.

in the extrinsic pathway. Prothrombinase cleaves the two spatially distinct peptide bonds in prothrombin at different rates, which results in the accumulation of meizothrombin, and Boskovic and Krishnaswamy have clearly demonstrated that the difference in these rates arises from differences in the intramolecular binding step for docking to elements around the scissile bonds (*42*). Whether the same is true for FIX activation by FXIa-LC remains to be investigated.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Sriram Krishnaswamy for his critical comments and helpful suggestions, and we thank Patricia Pileggi for help in manuscript preparation.

REFERENCES

1. Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation, *Biochemistry 30*, 10363−10370.
2. Broze, G. J., Jr. (1995) Tissue factor pathway inhibitor and the revised theory of coagulation, *Annu. Rev. Med. 46*, 103−112.
3. Mann, K. G., Butenas, S., and Brummel, K. (2003) The dynamics of thrombin formation, *Arterioscler., Thromb., Vasc. Biol. 23*, 17−25.
4. Edgington, T. S., Dickinson, C. D., and Ruf, W. (1997) The structural basis of function of the TF. VIIa complex in the cellular initiation of coagulation, *Thromb. Haemost. 78*, 401−405.
5. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes, *Annu. Rev. Biochem. 57*, 915−956.
6. Marsh, H. C., Jr., Meinwald, Y. C., Thannhauser, T. W., and Scheraga, H. A. (1983) Mechanism of action of thrombin on fibrinogen. Kinetic evidence for involvement of aspartic acid at position P10, *Biochemistry 22*, 4170−4174.
7. Lu, G., Broze, G. J., Jr., and Krishnaswamy, S. (2004) Formation of factors IXa and Xa by the extrinsic pathway: differential regulation by tissue factor pathway inhibitor and antithrombin III, *J. Biol. Chem. 279*, 17241−17249.
8. Baugh, R. J., Broze, G. J., Jr., and Krishnaswamy, S. (1998) Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor, *J. Biol. Chem. 273*, 4378−4386.
9. Lindhout, T., Franssen, J., and Willems, G. (1995) Kinetics of the inhibition of tissue factor-factor VIIa by tissue factor pathway inhibitor, *Thromb. Haemost. 74*, 910−915.
10. Jesty, J., Wun, T. C., and Lorenz, A. (1994) Kinetics of the inhibition of factor Xa and the tissue factor-factor VIIa complex by the tissue factor pathway inhibitor in the presence and absence of heparin, *Biochemistry 33*, 12686−12694.
11. Broze, G. J., Jr., Warren, L. A., Novotny, W. F., Higuchi, D. A., Girard, J. J., and Miletich, J. P. (1988) The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of activation, *Blood 71*, 335−343.
12. Fujikawa, K., Legaz, M. E., Kato, H., and Davie, E. W. (1974) The mechanism of activation of bovine factor IX (Christmas factor) by bovine factor XIa (activated plasma thromboplastin antecedent), *Biochemistry 13*, 4508−4516.
13. Di, Scipio, R. G., Kurachi, K., and Davie, E. W. (1978) Activation of human factor IX (Christmas factor), *J. Clin. Invest. 61*, 1528−1538.
14. Lindquist, P. A., Fujikawa, K., and Davie, E. W. (1978) Activation of bovine factor IX (Christmas factor) by factor XIa (activated plasma thromboplastin antecedent) and a protease from Russell's viper venom, *J. Biol. Chem. 253*, 1902−1909.
15. Bode, W., Brandstetter, H., Mather, T., and Stubbs, M. T. (1997) Comparative analysis of haemostatic proteinases: structural aspects of thrombin, factor Xa, factor IXa and protein C, *Thromb. Haemost. 78*, 501−511.
16. Neurath, H. (1984) Evolution of proteolytic enzymes, *Science 224*, 350−357.
17. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) Location of the disulfide bonds in human coagulation factor XI: the presence of tandem apple domains, *Biochemistry 30*, 2056−2060.
18. Fujikawa, K., Chung, D. W., Hendrickson, L. E., and Davie, E. W. (1986) Amino acid sequence of human factor XI, a blood coagulation factor with four tandem repeats that are highly homologous with plasma prekallikrein, *Biochemistry 25*, 2417−2424.
19. Rawala-Sheikh, R., Ahmad, S. S., Ashby, B., and Walsh, P. N. (1990) Kinetics of coagulation factor X activation by platelet-bound factor IXa, *Biochemistry 29*, 2606−2611.
20. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) The contribution of bovine Factor V and Factor Va to the activity of prothrombinase, *J. Biol. Chem. 254*, 10952−10962.
21. Bajaj, S. P., Rapaport, S. I., and Russell, W. A. (1983) Redetermination of the rate-limiting step in the activation of factor IX by factor XIa and by factor VIIa/tissue factor. Explanation for different electrophoretic radioactivity profiles obtained on activation of 3H- and 125I-labeled factor IX, *Biochemistry 22*, 4047−4053.
22. Wolberg, A. S., Morris, D. P., and Stafford, D. W. (1997) Factor IX activation by factor XIa proceeds without release of a free intermediate, *Biochemistry 36*, 4074−4079.
23. Sinha, D., Marcinkiewicz, M., Gailani, D., and Walsh, P. N. (2002) Molecular cloning and biochemical characterization of rabbit factor XI, *Biochem. J. 367*, 49−56.
24. Ogawa, T., Verhamme, I. M., Sun, M. F., Bock, P. E., and Gailani, D. (2005) Exosite-mediated substrate recognition of factor IX by factor XIa. The factor XIa heavy chain is required for initial recognition of factor IX, *J. Biol. Chem. 280*, 23523−23530.
25. Mertens, K., van Wijngaarden, A., and Bertina, R. M. (1985) The role of factor VIII in the activation of human blood coagulation factor X by activated factor IX, *Thromb. Haemost. 54*, 654−660.
26. Tracy, P. B., Eide, L. L., and Mann, K. G. (1985) Human prothrombinase complex assembly and function on isolated peripheral blood cell populations, *J. Biol. Chem. 260*, 2119−2124.
27. Osterud, B., Bouma, B. N., and Griffin, J. H. (1978) Human blood coagulation factor IX. Purification, properties, and mechanism of activation by activated factor XI, *J. Biol. Chem. 253*, 5946−5951.
28. Mannhalter, C., Schiffman, S., and Deutsch, E. (1984) Phospholipids accelerate factor IX activation by surface bound factor XIa. *Br. J. Haematol. 56*, 261−271.
29. Sinha, D., Seaman, F. S., and Walsh, P. N. (1987) Role of calcium ions and the heavy chain of factor XIa in the activation of human coagulation factor IX, *Biochemistry 26*, 3768−3775.

30. Sun, Y., and Gailani, D. (1996) Identification of a factor IX binding site on the third apple domain of activated factor XI, *J. Biol. Chem. 271*, 29023−29028.
31. Sinha, D., Koshy, A., Seaman, F. S., and Walsh, P. N. (1985) Functional characterization of human blood coagulation factor XIa using hybridoma antibodies, *J. Biol. Chem. 260*, 10714−10719.
32. Sinha, D., Marcinkiewicz, M., Lear, J. D., and Walsh, P. N. (2005) Factor XIa dimer in the activation of factor IX, *Biochemistry 44*, 10416−10422.
33. Navaneetham, D., Jin, L., Pandey, P., Strickler, J. E., Babine, R. E., Abdel-Meguid, S. S., and Walsh, P. N. (2005) Structural and mutational analyses of the molecular interactions between the catalytic domain of Factor XIa and the Kunitz protease inhibitor domain of protease Nexin 2, *J. Biol. Chem. 280*, 36165−36175.
34. Wagenvoord, R., Hendrix, H., Tran, T., and Hemker, H. C. (1990) Development of a sensitive and rapid chromogenic factor IX assay for clinical use, *Haemostasis 20*, 276−288.
35. Segel, I. H. (1975) Simple Inhiition Systems, in *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (Segel, I. H., Ed.) pp 100−176, John Wiley & Sons, New York.
36. Baugh, R. J., Dickinson, C. D., Ruf, W., and Krishnaswamy, S. (2000) Exosite interactions determine the affinity of factor X for the extrinsic Xase complex, *J. Biol. Chem. 275*, 28826−28833.
37. Krishnaswamy, S., and Betz, A. (1997) Exosites determine macromolecular substrate recognition by prothrombinase, *Biochemistry 36*, 12080−12086.
38. Evans, S. A., Olson, S. T., and Shore, J. D. (1982) p-Aminobenzamidine as a fluorescent probe for the active site of serine proteases, *J. Biol. Chem. 257*, 3014−3017.
39. Betz, A., Vlasuk, G. P., Bergum, P. W., and Krishnaswamy, S. (1997) Selective inhibition of the prothrombinase complex: factor Va alters macromolecular recognition of a tick anticoagulant peptide mutant by factor Xa, *Biochemistry 36*, 181−191.
40. Craig, P. A., Olson, S. T., and Shore, J. D. (1989) Transient kinetics of heparin-catalyzed protease inactivation by antithrombin III. Characterization of assembly, product formation, and heparin dissociation steps in the factor Xa reaction, *J. Biol. Chem. 264*, 5452−5461.
41. Olson, S. T. (1988) Transient kinetics of heparin-catalyzed protease inactivation by antithrombin III. Linkage of protease-inhibitor-heparin interactions in the reaction with thrombin, *J. Biol. Chem. 263*, 1698−1708.
42. Boskovic, D. S., and Krishnaswamy, S. (2000) Exosite binding tethers the macromolecular substrate to the prothrombinase complex and directs cleavage at two spatially distinct sites, *J. Biol. Chem. 275*, 38561−38570.
43. Segel, I. H. (1975) Rapid Equilibrium Partial and Mixed-Typed Inhibition, in *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (Segel, I. H., Ed.) pp 161−226, John Wiley & Sons, New York.
44. Pedicord, D. L., Seiffert, D., and Blat, Y. (2004) Substrate-dependent modulation of the mechanism of factor XIa inhibition, *Biochemistry 43*, 11883−11888.
45. Lawson, J. H., and Mann, K. G. (1991) Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation, *J. Biol. Chem. 266*, 11317−11327.
46. Aktimur, A., Gabriel, M. A., Gailani, D., and Toomey, J. R. (2003) The factor IX gamma-carboxyglutamic acid (Gla) domain is involved in interactions between factor IX and factor XIa, *J. Biol. Chem. 278*, 7981−7987.
47. Pospisil, C. H., Stafford, A. R., Fredenburgh, J. C., and Weitz, J. I. (2003) Evidence that both exosites on thrombin participate in its high affinity interaction with fibrin, *J. Biol. Chem. 278*, 21584−21591.

BI062296C