Productive Recognition of Factor IX by Factor XIa Exosites Requires Disulfide Linkage between Heavy and Light Chains of Factor XIa

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In the intrinsic pathway of blood coagulation factor XIa (FXIa) activates factor IX (FIX) by cleaving the zymogen at Arg145-Ala146 and Arg180-Val181 bonds releasing an 11-kDa activation peptide. FXIa and its isolated light chain (FXIa-LC) cleave S-2366 at comparable rates, but FXIa-LC is a very poor activator of FIX, possibly because FIX undergoes allosteric modification on binding to an exosite on the heavy chain of FXIa (FXIa-HC) required for optimal cleavage rates of the two scissile bonds of FIX. However, preincubation of FIX with a saturating concentration of isolated FXIa-HC did not result in any potentiation in the rate of FIX cleavage by FXIa-LC. Furthermore, if FIX binding via the heavy chain exosite of FXIa determines the affinity of the enzyme-substrate interaction, then the isolated FXIa-HC should inhibit the rate of FIX activation by depleting the substrate. However, whereas FXIa/S557A inhibited FIX activation of by FXIa, FXIa-HC did not. Therefore, we examined FIX binding to FXIa/S557A, FXIa-HC, FXIa-LC, FXIa/C362S/C482S, and FXIa/S557A/C362S/C482S. The heavy and light chains are disulfide-linked in FXIa/S557A but not in FXIa/C362S/C482S and FXIa/S557A/C362S/C482S. In an ELISA assay only FXIa/S557A ligated FIX with high affinity. Partial reduction of FXIa/S557A to produce heavy and light chains resulted in decreased FIX binding, and this function was regained upon reformation of the disulfide linkage between the heavy and the light chains. Therefore, we conclude that substrate recognition by the FXIa exosite(s) requires disulfide-linked heavy and light chains.

Significance: Covalent linkage between FXIa-HC and FXIa-LC is required to form the substrate binding exosite.

Results: Efficient FIX activation by FXIa occurred only when FXIa-HC and FXIa-LC were disulfide-linked.

Conclusion: Substrate recognition by FXIa exosites requires disulfide-linked FXIa-HC and FXIa-LC.

Factor IX (FIX), an essential component of the blood coagulation cascade, can be activated by the factor VIIa (FVIIa)/tissue factor (TF) complex in the extrinsic pathway (1–4) and by factor Xa (FXIa) in the intrinsic pathway of blood coagulation (5–7). Conversion of zymogen FIX to its activated form (FIXa) is achieved by cleavages at two peptide bonds one at Arg145-Ala146 and the other at Arg180-Val181. FXIa, generated by proteolytic cleavage of zymogen FXI by FXIIa, thrombin, or FXIa (8, 9), is an essential component of the consolodation phase of blood coagulation because tissue factor pathway inhibitor in complex with factor Xa shuts down the extrinsic pathway by inhibiting FVIIa/TF (10, 11). FXIa exists in human plasma at a concentration of ~30 nM as a homodimer consisting of two identical subunits linked by a disulfide bond formed by Cys321 in each heavy chain (12, 13). Thus, each subunit consists of one N-terminal heavy chain joined to the C-terminal light chain (or catalytic domain) by a disulfide bond formed between Cys362 in the heavy chain and Cys482 in the light chain. It was originally established in our laboratory (14, 15) and later confirmed by other investigators (16–18) that binding of FIX to an exosite on the heavy chain of FXIa is essential for optimal activation of FIX. In addition, a second exosite on the light chain of FXIa has recently been identified from kinetic analyses of FIX activation by FXIa-LC (19).

This investigation is aimed at understanding the structural and functional features within FXIa required for the interaction of FIX with these two FXIa exosites in the generation of FIXa. We and others have shown earlier that the catalytic domain of FXIa (FXIa-LC) is a poor activator of the macromolecular substrate FIX, although both intact FXIa and the FXIa-LC hydrolyze the chromogenic substrate S-2366 at comparable rates (14–16, 20). Thus, the isolated catalytic domain of FXIa possesses normal enzymatic activity against small peptide substrates but deficient activity against the normal macromolecular substrate FIX. There are several plausible explanations for this discrepancy, one of which is that FIX undergoes an allo-

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teric modification upon binding to the heavy chain of FXIa that facilitates access of the two FIX scissile bonds for cleavages by FXIa. Validation of this mechanism would predict that preincubation of FIX with the isolated heavy chain of FIXa would potentiate rates of FIX activation by the isolated catalytic domain of FXIa. Alternatively, if the affinity of the enzyme-substrate interaction is determined solely by the binding of FIX to the heavy chain of FXIa, then FIX activation by FXIa should be inhibited in the presence of excess of FXIa-HC because of substrate depletion. These two hypotheses were examined in experiments, the results of which are reported in this manuscript.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human FIX, FIXa, FXI, and FXIa were purchased from Hematologic Technologies (Essex Junction, VT). FXIIa, Corn trypsin inhibitor-coupled agarose beads, high molecular weight kininogen (HK), polyclonal antibodies against FIX and FXI, and ELISA kits were from Enzyme Research Laboratories (South Bend, IN). Chromogenic substrates S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide) and S-2765 (N-α-benzyloxycarbonyl-D-arginyl-glycyl-L-arginine-p-nitroanilide) were obtained from DiaPharma (Westchester, OH). Spectrozyme FxXa (methyl-sulfonyl-D-cyclohexylglycyl-glycyl-arginine-p-nitroanilide) was purchased from American Diagnostics (Greenwich, CT). Transformed human embryonic kidney cells (HEK293) were obtained from the ATCC (CRL1573). DMEM was purchased from Mediatech (Herndon, VA). Vitamin K1 (2-methyl-3-phytyl-1,4-naphthoquinone) was purchased from Abbott Laboratories (Chicago, IL). The irreversible serine protease inhibitor 4-amidinophenylmethanesulfonyl fluoride (APMSF), HEPES, Tris, L-phosphatidylcholine, L-phosphatidylserine, Q-Sepharose anion exchange resin, fatty acid-free BSA, benzamidine, and other reagents were purchased from Sigma.

**Preparation of Constructs for FXI/S557A, FXI/C362S/C482S, and FXI/S557A/C362S/C482S**—The cDNA constructs of FXI mutants were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with wild-type FXI cDNA inserted into the expression vector pJVCVMV (12, 18, 19) serving as the template. Each mutant was sequenced using both reverse and forward primers to confirm the presence of the desired mutation and to ensure the absence of any polymerase-induced errors.

**Expression and Purification of FXI Mutants**—Stable cell lines expressing the wild type and mutants of FXI were prepared by transfection of HEK293 cells as described earlier (19, 21, 22). Cells were grown in DMEM with 10% fetal bovine serum for 500 h and then switched to the same medium supplemented with 500 µg/ml of G418. Expression of FXI in the medium was determined by enzyme-linked immunosorbent assay as described previously (19, 21, 22). Cell lines exhibiting maximal expression of FXI proteins were expanded in roller bottles (Nunc, Naperville, IL). After reaching confluence, cells were washed, and the media were replaced by serum-free DMEM supplemented with ITS (insulin-transferrin-selenium-A, Invitrogen), 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) and EDTA (5 mM) until ready to be purified.

Expressed wtFXI, FXI/S557A, FXI/C362S/C482S and FXI/S557A/C362S/C482S proteins in the conditioned medium were purified using a monoclonal antibody (5F7) affinity column according to the protocol described earlier (19, 21, 22). 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 TBS followed by elution of the protein using 2 M potassium thiocyanate. The eluted protein was dialyzed and concentrated using a Centricon Plus-20, 30,000 NMWL ultrafiltration unit (Millipore, Bedford, MA). To prepare FXI-HC and FXI-LC, the concentrated and purified FXI/C362S/C482S and FXI/S557A/C362S/C482S were activated with FIXa 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 FIXa using corn trypsin inhibitor-coupled agarose beads, and the HC bound to the 5F7 column was eluted and purified as described for FXI/C362S/C462S (21).

Protein concentration was estimated using the BCA assay (Pierce). The purified proteins were size-fractionated by 4–15% SDS-PAGE under reducing and non-reducing conditions and stained using GelCode blue stain (Pierce). An Odyssey infrared imager (LI-COR, Inc., Lincoln, NE) was used for visualization and densitometric analysis of the bands.

**Kinetic Assay of FIX Activation by FXIa-LC after Preincubation of FIX with the Heavy Chain of FXIa**—FIX activation by FXIa-LC was carried out using a modification (19) of a coupled assay described originally by Wagenvoord et al. (23). In this assay, FIX is first activated by FXIa-LC to FIXa, which is then used to activate FX in the presence of FVIIlla, phospholipids, and calcium. FXa thus generated was estimated using the chromogenic substrate S-2765. All reagents were diluted in TBS containing 0.1% BSA (TBSA). Varying concentrations of FIX (0.2–2.2 µM) were incubated with a fixed concentration (2.88 µM) of FXIa-HC or buffer for 20 min, followed by fixation activation by FXIa-LC (15–20 nM) for 5–10 min in a microtiter plate at 37 °C in a total volume of 50 µl of TBSA supplemented with 5 mM CaCl₂. Adding EDTA to 25 mM and chilling on ice stopped the reactions. 5 µl of the reaction mixture was then added to chilled TBSA containing 25 mM EDTA to make a total volume of 200 µl. 5 µl from the diluted reaction mixture was added to 60 µl of TBSA containing 10 mM CaCl₂, followed by addition of 10 µl of 10 µM phospholipid vesicles (phosphatidylcholine/phosphatidylserine) and incubation for 2 min at 37 °C. 10 µl of freshly activated FVIIlla (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 FVIIlla, FX, and phosphatidylcholine/phosphatidylserine in the reaction mixture were 6.6 units/ml, 0.2 µM, and 1 µM, respectively. Adding EDTA to 25 mM and placing it on ice stopped the activation of FX by FIXa. 50 µl of each reaction was mixed with 50 µl of 1 mM S-2765, and the change in absorbance at 405 nm was followed on a ThermoMax microtiter plate reader. The concentration of FIXa generated was obtained from a standard curve constructed using known concentrations of purified FIXa.
Western Blot Analyses of FIX Activation by FXIa-LC after Preincubation of FIX with the Heavy Chain of FXIa—FIX (1 μM) was incubated with buffer or FXIa-HC (2 μM) for 15 min at room temperature and then activated using FXIa-LC (15 nM) as described above. In a separate experiment, we examined the activation of FIX using a higher concentration of FXIa-LC (50 nM) after preincubating FIX (1 μM) with varying concentrations of FXIa-HC (1–5 μM). The reaction products were fractionated by SDS-PAGE. The protein bands were transferred to a nitrocellulose membrane using a semidymer transfer apparatus (Bio-Rad). The membrane, after thorough washing, was incubated with goat anti-human FIX polyclonal antibody (ERL, South Bend, IN), and the bound antibody was detected by HRP-conjugated anti-goat IgG (Sigma-Aldrich, St. Louis, MO) and visualized by chemiluminescence.

Inhibition of FIX Cleavages by FXIa in the Presence of FXIa/S557A or FXIa-HC—FIX (1 μM) was incubated with TBSA buffer, FXIa/S557A (200 nM), or FXI-HC (200 nM) for 15 min at room temperature and then activated using FXIa (1 nM). At specified time points, aliquots were drawn into SDS sample buffer for size fractionation. In a separate experiment, FIX (1 μM) was incubated with different concentrations of FXIa/S557A (200 nM–1 μM) or FXI-HC (1–4 μM) for 15 min at room temperature followed by activation by FIXa/LC (1 μM). FIX cleavage products were analyzed by Western blot analysis as described above.

Factor IX Binding to the Active Site Inhibited FXIa (FXIai), Isolated Heavy Chain (FXI-HC), Isolated Light Chain (FXIa-LC), and to Zymogen FXI—To prevent activation of FIX by FXIa or by FXIa-LC during prolonged incubation in the ELISA assay, the two proteins were irreversibly inhibited by incubation with 4-amidinophenylmethanesulfonyl fluoride by making five sequential additions of 5 μl of 10 mM 4-amidinophenylmethanesulfonyl fluoride to 50 μl of the protein solution (2–5 μM) and allowing 5 min after each addition for the inhibitor to interact with the protein. The inactivated proteins were then dialyzed extensively before being used in the ELISA assay. The proteins were inactivated by >97%, as judged by their ability to hydrolyze S-2366. 100 μl of FIX (10 μg/ml) in 50 mM carbonate buffer (pH 9.6) was added to wells of a microtiter plate and incubated overnight at 4°C. Uncoated sites of the wells were blocked by incubation with 200 μl of BSA at a concentration of 1 mg/ml. FIX coated to the wells was allowed to interact with varying concentrations (as indicated in the figures) of FXIa, FXIa/LC, FXI-HC, or FXI in TBS containing Ca²⁺ (5 mM) for 90 min at room temperature. The wells were thoroughly washed with TBS containing Ca²⁺ (5 mM) and Tween 20 (0.02%) to remove the unbound proteins, followed by detection of FIX-bound FXIa, FXIa/LC, FXI-HC, or FXI using HRP-conjugated polyclonal antibody made against FXI (Enzyme Research Laboratories) according to the protocol provided by the supplier.

Comparison of FIX Binding to FXIa/S557A and FXIa/S557A/C362S/C482S—Like plasma FXIa, the mutant FXIa/S557A is a dimer of two identical subunits joined by a disulfide linkage through Cys³²¹ in the heavy chain, and each subunit of the enzyme is a two-chain molecule joined by Cys³⁶² in the heavy chain and Cys⁴⁸² in the light chain. Because both Cys³⁶² and Cys⁴⁸² are mutated in FXIa/S557A/C362S/C482S, the activated triple mutant produces a dimeric heavy chain (one heavy chain from each subunit joined by the interchain disulfide bond at Cys³²¹) of ~90 kDa and a free light chain of ~35 kDa. ELISA assays of FXIa/S557A and FXIa/S557A/C362S/C482S for FIX binding were performed as described above.

In a separate experiment a fixed concentration of FIX (5 nM) was incubated with varying concentrations (15 nM–1 μM) of either FXIa/S557A or FXIa/S557A/C362S/C482S in solution for 15 min at room temperature. These preincubated samples (100 μl) were then added to the wells of a microtiter plate coated with FXIa/S557A. FIX bound to the wells was detected using HRP-conjugated polyclonal antibody to FIX.

Comparison of HK Binding to FXIa/S557A and FXIa/S557A/C362S/C482S—FXIa/FXI a binds to HK through its heavy chain (24, 25). To examine whether FXIa-HC retains its capacity to bind to HK we compared binding of HK to FXIa/S557A and FXIa/S557A/C362S/C482S. For this purpose, the wells of a microtiter plate were coated with HK by incubation of 100 μl of HK at a concentration of 10 μg/ml in 50 mM carbonate buffer (pH 9.6) as for the ELISA assays described above. After blocking free sites with BSA, either FXIa/S557A or FXIa/S557A/C362S/C482S at concentrations designated in the figure were added and incubated for 90 min followed by extensive washing. HK-bound proteins were then detected using peroxidase-conjugated polyclonal antibody to FXI as described above.

Comparison of FXIa and FXIa/S557A/C362S/C482S in the S-2366 Hydrolysis Assay—To assess whether the light chain of FXIa free from the heavy chain retains its active site integrity, we compared FXIa and FXIa/S557A/C362S/C482S in their ability to hydrolyze the small peptide substrate S-2366. FXIa or FXIa/S557A/C362S/C482S at a concentration of 3 nM in TBS containing 1 mg/ml BSA was added to varying concentrations (0–1.5 mM) of S-2366 in TBS, and the rate of hydrolysis was measured by monitoring generation of p-nitroanilene at 405 nm in a plate reader.

Comparison of Plasma FXI (pFXI), FXI/S557A, and FXI/S557A/C362S/C482S Binding to Monospecific Polyclonal Antibody Raised against FXI—To examine whether any major structural alteration resulted from mutations at Cys³⁶ and Cys⁴⁸, we compared plasma FXI, FXI/S557A, and FXI/S557A/C362S/C482S in their capacity to bind to a monospecific polyclonal antibody raised against plasma FXI (FXI ELISA kit, Enzyme Research Laboratories) using the protocol provided by the manufacturer.

Partial Reduction of FXIa/S557A Followed by Reoxidation—200 μl of FXIa/S557A (3.75 μM) was partially reduced using 150 μM DTT by incubation for 20 min at room temperature as described previously (14). Half of the reduced product was alkylated by further incubation for 30 min with 300 μl of iodoacetamide, and both the alkylated and non-alkylated samples were dialyzed for 48 h with two changes of buffer containing 0.02% Tween 20, EDTA (2 mM), and benzamidine (5 mM) and a final change of buffer without EDTA. After dialysis, each sample was estimated for protein concentration and used for FIX binding as described above.
A plausible explanation for the observation that the isolated catalytic domain of FXIa is a poor activator of FIX compared with wild-type FXIa, despite its intact enzymatic activity against small peptide substrates, is that FIX, bound to the exosite in the heavy chain of FXIa, undergoes a conformational change that facilitates optimal cleavage rates of the two FIX peptide bonds, leading to the formation of FIXa. If this hypothesis is correct, then activation of FIX by FXIa-LC should be potentiated by preincubation of FIX with FXIa-HC. This hypothesis was tested by examining the rate of activation of FIX preincubated with buffer or FXIa-HC. As shown in Fig. 1A, preincubation of FIX with the isolated heavy chain of FXIa does not potentiate the rate of FIX activation by FXIa-LC. Cleavage patterns of FIX activation by FXIa-LC in the presence and absence of FXIa-HC were also examined by Western blot analysis and are shown in Fig. 1B. We also examined activation of FIX using a higher concentration of FXIa-LC (50 nM) after preincubating FIX (1 μM) with varying concentrations of FXIa-HC (1–5 μM) for 15 min at room temperature. The results of these experiments (Fig. 1C) demonstrate no discernible difference in the cleavage rate or cleavage pattern of FIX activation by the catalytic domain of FXIa in the presence or absence of the FXIa heavy chain.

**Effects of FXIa/S557A and FXIa-HC on the Rate of FIX Cleavages by FXIa**—If the affinity of the substrate-enzyme interaction is solely determined through interaction of FIX with the heavy chain of FXIa, then a saturating concentration of isolated FXIa-HC should deplete the substrate, making it unavailable for binding to the enzyme, and thereby inhibiting the rate of FIX activation. We therefore compared the cleavage of FIX (1 μM) by FXIa (1.0 μM) in the presence or absence of a 200-fold molar excess of the FXIa-HC (200 nM) (Fig. 2B). The result of this experiment demonstrated no discernible effect of the isolated FXIa heavy chain on the rate or cleavage pattern of FIX activation. On the contrary; the intact molecule with the active site Ser mutated to Ala (FXIa/S557A) and, at a similar concentration (200 nM), was able to inhibit the cleavage rate of FIX activation by FXIa (Fig. 2A). The effects of higher concentrations of FXIa-HC as well as of FXIa/S557A were also examined, as shown in Fig. 2, D and C, respectively. Although FXIa/S557A inhibited cleavage of the substrate almost completely at 500 nM, no demonstrable inhibition could be observed, even at concentrations of FXIa-HC as high as 4 μM.

**Factor IX Binding to FXIai, Isolated FXIa-HC, FXIa-LCi, and Zymogen FXI**—Because FXIa-HC was ineffective in potentiating FIX activation by FXIa-LC or inhibiting the rate of FIXa generation by full-length FXIa, we examined binding of the substrate, FIX to FXIa and to the isolated heavy and light chains of FXIa in an ELISA assay. Because FXIa or FXIa-LC would cleave FIX during long incubation on the plate, both enzymes were inactivated by the irreversible serine protease inhibitor 4-amidinophenylmethanesulfonyl fluoride, followed by dialysis before being used in the ELISA assay. In this assay, FIX was coated on the wells of a microtiter plate, followed by incubation with FXIa, FXIa-HC, FXIa-LCi, or zymogen FXI. Binding of these proteins to immobilized FIX were then detected as described under “Experimental Procedures.” The results of these assays are shown in Fig. 3A. Although FXIa was found to bind to FIX with high affinity (Kd ~0.25–0.5 nM), no detectable binding to FIX of the zymogen FXI or the isolated heavy or light chain could be demonstrated. In a separate experiment, binding to FIX was examined using high concentrations of FXIa, FXIa-HC, and FXIa-LCi. As shown in Fig. 3B, binding of these proteins was extremely poor, even at a concentration of ~2000-fold higher than the apparent Kd value of FXIai binding to FIX.

**Comparison of Binding to FIX of FXIa/S557A and FXIa/S557A/C362S/C482S**—To rule out the possibility that the isolation procedure may have been responsible for the loss of the capacity of FIX to recognize FXIa-HC and FXIa-LC, we investigated the binding to FIX of FXIa/S557A and FXIa/
S557A/C362S/C482S without isolation of the heavy or light chains. The proteins resulting from these mutations are represented schematically in Fig. 4A. Zymogen plasma FXI (Fig. 4B, lane 2), FXI/S557A (lane 3), and FXI/S557A/C362S/C482S (lane 4) all migrated, as expected, as single protein bands at 160 kDa by non-reduced SDS-PAGE. The heavy and the light chains of FXIa/S557A (Fig. 4B, lane 6) are disulfide linked as in FXIa (lane 5) and, therefore, run as single bands by non-reduced SDS-PAGE. Because the mutant FXIa/S557A/C362S/C482S lacks the disulfide bond between the heavy chain and the light chain, the activated mutant migrates as two protein bands, one comprising the heavy chain from each subunit disulfide linkage in FXIa Required for FIX Recognition

FIGURE 2. Inhibition by FXIa/S557A and FXIa-HC of FIX activation by FXIa. A, FIX (1 µM) was preincubated with buffer or FXIa/S557A (200 nM) for 15 min at room temperature, followed by activation using FXIa (1.0 nM) and size fractionation. B, same as in A, except FXIa-HC (200 nM) instead of FXIa/S557A was used as the inhibitor. The last lane represents FIX incubated with FXIa-HC (1 µM) alone for 60 min before fractionation to examine whether or not FXIa-HC has any contamination of FXIa that may cleave FIX. C, FIX (1 µM) was preincubated with buffer (lane 2) or varying concentrations of FXIa/S557A (lane 3, 200 nM; lane 4, 500 nM; lane 5, 1 µM) for 15 min at room temperature, followed by activation using FXIa (1.0 nM) for 25 min before size fractionation. Unactivated FIX is shown in lane 1. D, same as in C except FIX was preincubated with buffer (lane 2) or varying concentrations of FXI-HC (lane 3, 1 µM; lane 4, 2 µM; lane 5, 4 µM). Unactivated FIX is shown in lane 1. FIX cleavage products were size-fractionated and visualized by Western blot analysis as described under “Experimental Procedures” section.

FIGURE 3. Comparison of FIX binding to in an ELISA assay. A, wells of a microtiter plate were coated with FIX by incubating the wells with 100 µl of 50 mM carbonate buffer (pH 9.6) containing 1 µg of protein. After blocking the remaining sites of the well with BSA, bound protein was allowed to interact with varying concentrations of FXIa (■), FXIa-HC (○), FXIa-LC (●), or zymogen FXI (▲) as indicated. After removing unbound protein by extensive washing, FIX-bound FXIa, FXIa-HC, FXIa-LC, or FIX was detected using HRP-conjugated FXI polyclonal antibody. B, binding to FIX of FXIa-HC (■), FXIa-LC (●), or FIX (▲) at higher concentrations (15 nM-1 µM) of the three proteins were performed exactly the same way as described in A. The results are representative of three separate experiments. Each data point is the mean of duplicate determinations, and the error bars are S.D.
linked at Cys\textsuperscript{321} and the other consisting of the isolated light chain (Fig. 4B, lane 7). The results of binding to FIX of FXIa/S557A and FXIa/S557A/C362S/C482S are shown in Fig. 4C. Although FXIa/S557A, like FXIai, binds to FIX with high affinity ($K_i$ \approx 0.25–0.5 nM), the FXIa/S557A/C362S/C482S mutant demonstrated no detectable binding.

It is possible that FXIa/S557A/C362S/C482S does indeed bind to FIX in solution but not to FIX coated to the surface of a microtiter plate. To examine this possibility we designed the binding assay as follows. A fixed concentration of FIX (5 nM) was incubated with varying concentrations (15 nM–1 \mu M) of FXIa/S557A (□) or FXIa/S557A/C362S/C482S (○) and then added to the wells of a microtiter plate precoated with FIX/S557A. FIX bound to the wells was detected using HRP-conjugated FIX polyclonal antibody. The results depicted are representative of two separate experiments, and each data point is the mean of duplicate determinations. The error bars are S.D.

Comparison of HK Binding to FXIa and FXIa/S557A/C362S/C482S—To rule out the possibility that the isolated heavy chain and light chain of FXIa or the free heavy and light chains formed in FXIa/C362S/C482S/S557A may be non-functional, we examined binding of FXIa and FXIa/S557A/C362S/C482S to HK. The results, shown in supplemental Fig. 1A, demonstrate that the binding to HK of FXIa/S557A/C362S/C482S was identical to that of FXIa/S557A. Thus, FXIa/S557A/C362S/C482S containing the free heavy chain of FXIa retains its functional property of HK binding.
Partial Reduction of FXIa/S557A with 150 μM DTT produced M DTT produced 2-fold molar excess of iodoacetamide to block the reduced cysteine residues. Both of the blocked and unblocked samples were dialyzed as described under “Experimental Procedures.” It is clear that although the reduced and blocked protein FXIa/S557A-R/B (supplemental Fig. 2B, lane 3) was unable to form any dimeric FXIa, the unblocked protein FXIa/S557A-R/O (supplemental Fig. 2B, lane 2) produced a significant amount of dimeric FXIa, some of which was formed by oxidation of the intersubunit free Cys321 bonds of the monomeric form and some by disulfide bond formation between the free Cys362 and Cys482 in the heavy chain and the free Cys482 in the light chain. In addition to dimeric FXIa, two new bands appeared below the dimeric protein (supplemental Fig. 2B, lane 2). Because the reduced gel (supplemental Fig. 2B, lane 5) showed only two bands comprising the heavy and light chains of FXIa, the band at ~90–100 kDa most likely represents the dimeric heavy chain, and the band at ~125–130 kDa represents one monomeric FXIa (80 kDa) joined through Cys321 to one heavy chain (50 kDa). Total protein concentrations of the dialyzed FXIa/S557A-R/B and FXIa/S557A-R/O samples were then determined and examined in our FIX binding assay. The results (supplemental Fig. 2C) clearly demonstrate that at all three concentrations of the proteins tested, binding to FIX of FXIa/S557A-R/O was significantly greater than that of FXIa/S557A-R/B. Thus, formation of the disulfide bond between Cys362 and Cys482 within the heavy and the light chains promotes enhanced FIX binding, whereas prevention of this disulfide bond formation inhibits FIX binding. These results support the conclusion that the binding of FXIa to FIX and the efficient activation of FIX by FXIa requires that the heavy and light chains of FXIa be joined together by the disulfide bond between Cys362 and Cys482.

**DISCUSSION**

We showed previously that the macromolecular substrate (FIX) binds to the enzyme (FXIa) at an exosite remote from its active site (14, 15). This suggests that FXIa is unique among coagulation enzymes by possessing an endogenous substrate-binding site within the non-catalytic domain that serves a function similar to that of various cofactor molecules (e.g. FVIIIa, FVa, and TF) that potentiate substrate activation by the enzyme. Subsequently, numerous studies have demonstrated that macromolecular substrates in the coagulation cascade interact with various enzymes to determine the affinity and specificity of the Michaelis complex. These extended interactive sites are distinct from active site residues and have been appropriately termed “exosites” (26–28). During FIX activation either by the FVIIa-TF complex or by FXIa, the zymogen is cleaved at Arg1435-Ala1446 and Arg180-Val181 bonds forming FXa, a two-chain molecule joined by a disulfide bond, and releasing an activation peptide with M, ~11 kDa (5–7, 29, 30). This study was undertaken to examine the interaction of FIX with exosites in FXIa in the generation of FXa. It has been documented (14–16, 18–20) that, compared with FXIa, the isolated light chain is a poor activator of FIX, although hydrolysis rates of the small peptidyl substrate S-2366 by the two enzymes are similar. The weak enzymatic activity of the isolated light chain against FIX is not due to allosteric modification of FIX upon binding to the FXIa-HC because our data (Fig. 1) clearly demonstrate that preincubation of FIX with saturating concentrations of FXIa-HC did not potentiate the cleavage rate of FIX by FXIa-LC.
**Cys{superscript}362}-Cys{superscript}482 Linkage in FXIa Required for FIX Recognition**

Does the interaction of FIX with the isolated FXIa-HC alone determine the affinity of the enzyme-substrate interaction? If this were the case, then the rate of activation of FIX by FXIa should be inhibited in the presence of FXIa-HC as a result of substrate depletion because of complex formation of FIX with the isolated FXIa-HC. However, although the rate of FIX cleavage by FXIa was inhibited in the presence of FXIa/S557A (Fig. 2, A and C), it was unaltered in the presence of FXIa-HC (Fig. 2, B and D).

Although kinetic studies examining the activation of FIX by FXIa strongly suggest the presence in the FXIa-HC of an enzyme-substrate interactive site remote from the active site, the kinetic data do not provide information on the nature and architecture of the enzyme-substrate interactive site(s). Therefore, we examined the direct binding to FIX in an ELISA assay of FXIai, compared with the isolated heavy chain or light chain. Although FXIai bound to FIX with high affinity (K<sub>d</sub> ~0.25–0.5 nM), no binding could be demonstrated for FXIa-HC or FXIa-LCi (Fig. 3A). When FIX binding was examined at high concentrations (15 nM-1 µM, i.e. 30–2000-fold in excess of the K<sub>d</sub>), only FXIa-HC and FXI demonstrated very low level binding (Fig. 3B).

To examine whether the isolation procedure contributes to the loss of the FIX binding properties of the isolated heavy and light chains of FXIa, ELISA assays were carried out to examine the binding to FIX of FXIa/S557A and of the triple mutant, FXIa/S557A/C362A/C482A, without isolation of the heavy or the light chain. The results (Fig. 4C) demonstrate that although FXIa/S557A binds to FIX with high affinity (K<sub>d</sub> ~0.25–0.5 nM), the activated triple mutant fails to bind FIX. The impaired binding of the triple mutant is not an artifact resulting from the solid phase assay because FIX, after preincubation in solution with varying concentrations of FXIa/S557A, inhibited FIX binding dramatically, whereas FXIa/S557A/C362S/C482S demonstrated only minor (< 15%) inhibition at very high concentrations (1 µM) (Fig. 4D). Therefore, once the light chain and the heavy chain of FIXa are separated from one another by the absence of the covalent bond between the heavy and light chains, the recognition site for FIX is lost.

Mild reduction and alklylation of FXIa/S557A produced a single chain subunit as well as free heavy and light chains (supplemental Fig. 2B, lane 3). Each subunit of FXIa interacts with FIX as an independent enzyme, accounting for the fact that binding to FIX of the reduced and alkylated FXIa/S557A is diminished but not abolished (supplemental Fig. 2C). In contrast, the reduced and oxidized sample (supplemental Fig. 2B, lane 2) demonstrated binding to FIX that was consistently higher than that of the reduced and alkylated sample (supplemental Fig. 2C). Thus, although blocking disulfide bond formation results in loss of FIX binding, its reformation restores the interaction between FIX and FXIa.

Why do the heavy and the light chains of FIXa need to be disulfide-linked for interaction with FIX? FIXa, unlike other coagulation enzymes, is a homodimer, and it has been suggested that the relative paucity of accumulation of FIXa (FIX cleaved only at Arg<sup>145</sup>) during FXIa-catalyzed FIX activation might be explained by the simultaneous cleavage of the two scissile bonds of a single FIX molecule by the two active sites of dimeric FXIa (31, 32). This hypothesis is rendered extremely unlikely because FXIa/G326C (22) and several FXIa/C321A mutants (33) that exist predominantly as monomers can cleave FIX, generating FIXa at rates identical to those catalyzed by dimeric wtFXIa. Recently, FXIa with only one subunit in the activated form has been shown to activate FIX as efficiently as FXIa with two active sites (34). Therefore, it is reasonable to conclude that each subunit of FXIa acts as an independent enzyme and that (a) FIX interactive site(s) is/are present in each subunit of FIXa, provided the heavy chain and light chain are joined by a disulfide bond.

Thus, the following facts have been established concerning the activation of FIX by FXIa. 1) Each subunit of FIXa acts as an independent enzyme, cleaving FIX at Arg<sup>145</sup>-Ala<sup>146</sup> and Arg<sup>180</sup>-Val<sup>181</sup>. 2) FXIa and not FXI binds to FIX and, therefore, the FIX recognition site is formed only when the Arg<sup>169</sup>-Ile<sup>370</sup> bond of each subunit is cleaved. 3) (A) FIX binding exosite(s) is/are present both on the heavy and the light chain of each subunit of FIXa. 4) The FIX interactive site within FXIa is formed only when the heavy and the light chains are disulfide-linked.

The most logical hypothesis that is consistent with the above facts is that the covalent attachment of the heavy and light chains of FIXa is required to bring the two exosites, one in the heavy chain and the other in the light chain, into close proximity to form a single substrate binding exosite for efficient FIX activation. Detailed information on the structure of the enzyme, FIXa, would be required to confirm or refute this hypothesis. The crystal structures of FXI (35) and catalytic domain of FIXa (36, 37) have been published, but no such data are available for the full-length enzyme. However, small angle x-ray scattering and ultrastructural (electron microscopic) data on FIXa suggested that FXI activation is accompanied by a major conformational change from an elongated to a more compact arrangement of the domains (32). These data and our present studies suggest that upon activation of FXI to FXIa, a cryptic or a newly formed FIX binding exosite, possibly consisting of residues from both the heavy and light chains, is made available for interaction with the substrate. Alternatively, such a substrate-binding exosite could consist of residues involving or in close proximity to the disulfide bond (Cys<sup>362</sup>-Cys<sup>482</sup>) connecting the heavy and light chains of FIXa. In conclusion, the endogenous cofactor function of the heavy chain requires its covalent linkage to the active-site containing FIXa light chain.

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