Functional Domains in the Heavy-Chain Region of Factor XI: A High Molecular Weight Kinogen-Binding Site and a Substrate-Binding Site for Factor IX

By Frank A. Baglia, Dipali Sinha, and Peter N. Walsh

To probe the molecular interactions of factor XI we have prepared two monoclonal antibodies (MoAbs; 5F7 and 3C1), each of which binds the heavy chain of reduced and alkylated factor XIa. Competitive solid phase radioimmunoassay (RIA) binding studies revealed that 5F7 and 3C1 are directed against different epitopes within factor XI. One antibody (5F7) blocked the surface-mediated proteolytic activation of factor XI and its binding to HMW kinogen, but had no effect on factor-Xla-catalyzed factor IX activation. The other antibody (3C1) is a competitive inhibitor of factor-IX activation by factor XIa, but blocked factor-XI binding to HMW kinogen only at 1,000-fold higher concentration than 5F7. Moreover, HMW kinogen had no effect on the kinetics of factor-Xla-catalyzed factor-IX activation. Furthermore, factor XI CNBr peptide fragments that bind to the 5F7 and 3C1 antibodies were isolated. The peptides that bound to the 5F7 antibody blocked the binding of HMW kinogen to factor XI but did not inhibit factor-Xla-catalyzed factor-IX activation. However, the peptides isolated by the 3C1 antibody inhibited factor-XIa-catalyzed factor-IX activation and had no effect on factor-XI binding to HMW kinogen. Our results indicate that distinct functional domains within the heavy chain region of factor XI are important for the binding of factor XI to HMW kinogen and for activation of factor IX by factor XIa.

The initiation of intrinsic coagulation in vitro involves a complex interaction of contact proteins on negatively charged surfaces leading to the activation of factor IX. The participation of factor XI in the contact phase of blood coagulation involves intermolecular interactions with factor XIIa, high mol wt (HMW) kinogen, and factor IX. The fact that a bleeding state can result from a deficiency of factor XI strongly suggests that it has an essential role in the regulation of blood coagulation. Factor XI migrates with an apparent mol wt of 160,000 on sodium dodecyl (SDS) gels and consists of two identical disulfide-linked polypeptide chains, each of which contains a cDNA insert coding chain region of factor XI near the heavy chain region of factor XI near the HMW kinogen binding site as well as the putative substrate-binding site for factor IX.

METHODS

Purification of coagulation proteins. Factor XI was purified from human plasma by immunoaffinity chromatography using an MoAb to factor XI and had a specific activity of 250 U/mg protein. HMW kinogen was purified by the method of Kerbiriou and Griffin. Bovine factor XIa was purified by a modification of a previously published procedure and was a generous gift from Dr E.P. Kirby (Department of Biochemistry, Temple University School of Medicine, Philadelphia). Factor XIIa, which had spontaneously generated during the purification, was adsorbed to a column of insolubilized corn trypsin inhibitor and subsequently eluted with 0.1 mol/L Tris and 2 mol/L sodium thiocyanate (pH 8.0). It was then dialyzed to remove the thiocyanate. The factor XIIa concentration in solution was routinely estimated from its amidolytic activity. It hydrolyzed 3.78 × 10⁻³ mol of the chromogenic substrate S-2302/min/μg protein at pH 8.0 and 37°C. Human factor IX was purified to a specific activity of 225 U/mg by a modification of the methods described by DiScipio et al and by Miletich et al. All purified proteins appeared homogeneous by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of factor XIa. Purified factor XIa was activated by incubation at 37°C with bovine factor XIla as previously described. SDS-gel electrophoresis of the factor XIa preparation under reducing conditions showed two major bands of mol wt 50,000 and 30,000 daltons.
Radiolabeling of proteins. Purified factor XI was radiolabeled by a minor modification of the iodogen method to a specific activity of 5 x 10^6 cpm/µg. The radiolabeled protein retained >98% of its biological activity. Factor IX was labeled with tritium by a previously described modification of the method described by Silverberg et al. The tritiated factor IX had a specific radioactivity of 475 cpm/ng and retained >90% of its coagulant activity compared with unlabeled factor IX.

Coagulation and amidolytic assays. Factor XI, factor IX, and HMW kininogen were assayed by minor modification of the kaolin-activated partial thromboplastin time using appropriate congenitally deficient substrate plasmas, results of which were quantitated on double logarithmic plots of clotting times vs. concentrations of pooled normal plasma. The procoagulant activities of factor XI and factor XIIa were determined in the presence or absence of kaolin using factor XI-deficient plasma. One hundred microliters of factor XI-deficient plasma was incubated with 100 µL of kaolin (5 mg/mL in 0.85% NaCl) or 0.85% NaCl, 100 µL of 0.2% inosinit in 20 mmol/L Tris/saline, pH 7.4, and 10 µL of plasma or sample, and 90 µL of the above-mentioned buffer for five minutes at 37°C. Then 100 µL of 30 mmol/mL CaCl2 was added to initiate clot formation. The observed clotting times were converted to clotting units by comparison with the clotting activities of serial dilutions of a normal pooled plasma assayed in the presence of kaolin. Factor XIIa was assayed using the chromogenic substrate S-2302 according to the procedure previously described. The amidolytic assay of factor XIIa was carried out by a modification of the method of Scott et al. Factor XIIa generation in the experiments reported here was quantitated from a standard curve prepared using purified factor XIIa.

Assay of factor IX activation. The rate of activation of [3H]-factor IX was followed by measurement of the trichloroacetic acid-soluble activation peptide formed during activation by factor XIIa as previously described. For each time point the assay mixture consisted of 64 µL of Tris (50 mmol/L), NaCl (100 mmol/L), pH 7.5 Tris-buffered saline (TBS) containing 1 mg/mL bovine serum albumin (BSA), 8 µL of [3H]-factor IX (20 µg/mL), and 8 µL of factor XIIa (0.2 to 0.4 µg/mL). The reaction was stopped by adding 240 µL of an ice-cold mixture containing one part TBS and two parts 30 mmol/L EDTA, pH 7.5. To this mixture was added 160 µL of ice-cold 15% trichloroacetic acid. This was kept on ice and vortexed repeatedly for two minutes and then centrifuged. Then 100 µL aliquots of the supernatants were removed and counted for [3H]. The initial rate of release of the activation peptide was determined at times when less than 20% of the activation peptide had been released.

MoAbs. The production and purification of the MoAb (3C1) against factor XI has been published elsewhere. Similar procedures were used for antibody SF7, which has not been reported previously. In short, the gamma-globulin (IgG) fraction of ascites fluid was isolated by ammonium sulfate precipitation and further purified by gel filtration using Bio-Gel A-1.5 m. The MoAbs used in the present study appeared as pure heavy and light chains of IgG by SDS-PAGE. They had no protease activity, they did not cleave either factor XI or factor XIIa, and they did not affect the amidolytic activity of either factor XI or factor XIIa. Chain typing of MoAbs by immunoaffidting using rabbit antiserum antibodies (Miles Laboratories, Inc, Elkart, IN) revealed that both murine MoAbs (3C1 and SF7) were class IgG1.

Preparation of Fab' fragments. The Fab' fragment of MoAbs was prepared as previously described in the following way: 16 mg of the purified antibody in 2 mL of sodium acetate buffer (0.1 mol/L, pH 4.5) was incubated with 320 µg of pepsin for 24 hours at 37°C. Subsequently the reaction was stopped by raising the pH to 8 using saturated Tris-base solution. The F(ab')2, thus produced using these conditions did not bind to protein A-Sepharose, which was therefore used to separate the F(ab')2 from the Fc portion of the molecule. To prepare Fab' from F(ab')2, mercaptoethanol (12 mmol/L) was added and incubated for two hours at room temperature in the dark. Following reduction the fragments were alkylated with iodoacetamide (50 mmol/L) and dialyzed against 0.02 mol/L phosphate and 0.15 mol/L NaCl at pH 7.4. This preparation contained pure Fab' fragments (mol wt approximately 55,000) as judged by SDS-PAGE.

Protein analyses. Protein concentrations were determined by the Bio-Rad dye-binding assay according to the instructions provided by the manufacturer (Bio-Rad, Richmond, CA). Purified MoAbs were quantitated using an extinction coefficient of 14 for a 1% solution at 280 nmol/L.

Binding of MoAbs to factor XI. A solid phase radioimmunoassay (RIA) was used to detect antibody against factor XI. To polyvinyl chloride microtitre plates containing 96 wells was added 300 to 500 µg of factor XI in 100 µL of 0.01 mol/L sodium phosphate, 0.15 mol/L NaCl, pH 7.4 phosphate-buffered saline (PBS); the plates were incubated for 16 to 20 hours at 4°C. The wells were washed with 1% BSA in PBS to remove any unadsorbed antigen. Non-specific sites on the plates were coated with BSA by incubating with 200 µL of 0.5% BSA in PBS for two hours at room temperature. One hundred microliters of each test sample of unlabelled 3Cl or SF7 (6.7 µmol/L to 6.7 µmol/L) was added and incubated for two hours at room temperature with 1.3 mmol/L 125I-labelled SF7 in the presence of 1% BSA in the wells of microtitre plates to which purified factor XI had been bound. Thereafter the wells were washed with 1% BSA in PBS, were amputated, and were counted in a gamma counter.

To determine the affinity constants in solution of MoAb complexes with factor XI, an enzyme-linked immunosorbent assay (ELISA) was used as described by Friguet et al. Briefly, factor XI at various concentrations (125,000 to 5,000 ng/mL) was mixed with a constant amount (1 µg/mL) of 5F7 or 3Cl MoAb in 0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.4 containing 2% bovine serum albumin (TBS-BSA), previously determined to be an optimal concentration from a preliminary ELISA calibration. After incubation overnight at 4°C, 200 µL of each mixture was transferred to the wells of a microtitration plate previously coated with factor XI (200 µL per well, at 1 µg/mL in TBS-BSA, for 24 hours at 4°C). After washing with PBS supplemented with 0.5% Tween 20, the bound immunoglobulins (ie, free monoclonal antibodies) were detected and quantitated by adding goat antimeouse IgG coupled with alkaline phosphatase, the activity of which was then measured in each well. Dissociation constants (kd) for binding of each antibody to factor XI were determined as previously described.

Binding of factor XI to HMW kininogen. The binding of factor XI to HMW kininogen was studied using polyvinyl chloride microtitre plates, the wells of which were coated with HMW kininogen by incubation with 100 µL of the protein (100 µg/mL) for two hours at room temperature. This binding assay and conditions used have been previously described. Briefly, after residual binding sites on the wells were blocked, 100 µL of a mixture of 151-factor XI and either buffer or antibody were added to the wells and incubated for three to four hours at room temperature. The wells were thoroughly washed with PBS/BSA, dried, and counted in a gamma counter.

Immunoinaffinity isolation of cyanogen bromide (CNBr)-digested peptides of factor XI. We have developed procedures for the CNBr digestion of factor XI and the isolation of peptides of factor XI using MoAbs. Briefly, factor XI was dissolved in 70% formic acid (1 mg of protein/mL), and solid CNBr was added to a final concentration of approximately 100 mg/mL. After a reaction time of 20 to 24 hours in the dark at room temperature, the solution was dialyzed against distilled water for 18 hours and subsequently concentrated to dryness. MoAb affinity columns were prepared by

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incubating purified SF7 IgG or 3C1 IgG with Affigel 10 (Bio-Rad, Richmond, CA) according to the conditions described by the manufacturer. CNBr-digested factor XI was applied to the SF7 affinity column in HPLC grade water. The pass through was saved and put over a 3C1 antibody-affinity column. Both columns were washed with HPLC grade water and eluted with 4 mol/L guanidine-HCl, pH 4.0, and the eluant dialyzed with 3,500 mol wt cut off tubing (Spectropore, Spectrum Medical Industries, Los Angeles) against HPLC grade water. The dialyzed material was dried and rehydrated. The two isolated peptides were single peaks by reversed-phase high-performance liquid chromatography (HPLC) and by SDS-PAGE. The HPLC system used and the detector (Lambda-Max Model 481) were from Waters Associates (Waters 600, Milford, MA). The chromatography was performed using a Waters C8 µBondapak column that was equilibrated with 0.1% TFA and was then eluted with a linear gradient of aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 70% acetonitrile in 0.1% TFA with the detector set at a wavelength of 220 nm. The peptide bound to and eluted from the SF7 affinity column did not bind to the 3C1 column, and the peptide bound to and eluted from the 3C1 column did not bind to the SF7 column.

Materials. All chemicals were the best grade commercially available and were purchased from Sigma Chemical Co, St Louis; Fisher Chemical Co, Fairlawn, NJ; or J.T. Baker Chemical Co, Phillipsburg, NJ. Plasmas deficient in coagulation factors were purchased from George King Biomedical, Overland Park, KA. Beta-phase scintillation fluid was obtained from West Chem Products, San Diego. Tritiated sodium borohydride (75 Ci/mol) was purchased in crystalline form in aqueous solution and was obtained from New England Nuclear (Boston). The chromogenic substrates pyro-Glu-Pro-Arg-

RESULTS

Binding of MoAbs to the heavy chain of factor XI. We have previously reported on the production, characterization, and use of murine hybridoma antibodies directed against various epitopes in human coagulation factor XI.11 One of these antibodies (3C1) was shown to bind the heavy chain of reduced and alkylated factor XIa without affecting the amidolytic activity of intact factor XIa.12 Another antibody (SF7), previously not reported, also binds to the heavy chain of factor XIa as demonstrated by the following: (1) the SF7 antibody recognizes the heavy chain of reduced and alkylated factor XIa using an immunoblotting procedure; (2) when 125I-labeled reduced and alkylated factor XIa was passed over a SF7 immunoaffinity column, the light chain passed through, whereas the heavy chain was bound and then eluted by the application of 4 mol/L guanidine HCl; (3) a cyanogen bromide fragment of factor XI comprising the aminoterminal 102 amino acids was bound to a SF7 affinity column from which it was eluted by 4 mol/L guanidine HCl (data not shown). We have now used these two antibodies in a solid phase RIA to determine whether these MoAbs are directed against similar or related sites on the molecule. Figure 1 shows the effects of unlabeled monoclonal antibodies (3C1 or SF7) on binding of 125I-labeled SF7 to factor XI bound to microtiter wells. The concentration of unlabeled SF7 required to block the binding of 50% of 125I-labeled SF7 (1.3 nmol/L) to immobilized factor XI was 1.3 nmol/L (as expected), whereas the concentration of unlabeled 3C1 required to inhibit binding 50% was 1.3 µmol/L or 1,000-fold greater than that of SF7. Since these results might be explained by differing binding affinities of the two antibodies to factor XI, we determined dissociation constants in solution,22 as described under Methods. The dissociation constant (kd) for the binding of antibody SF7 to factor XI in solution

![Graph](https://via.placeholder.com/150)

**Figure 1.** Binding of 125I-labeled SF7 MoAb to factor XI. Unlabeled MoAb 3C1 or SF7 (10 pmol/L to 10 µmol/L) was incubated for 20 minutes at 20°C with 1.3 nmol/L 125I-labeled SF7 in the presence of BSA (1 mg/mL) in the wells of microtiter plates to which purified factor XI had been bound. Thereafter the wells were washed three times with PBS, amputated, and counted in a gamma counter. Results show indicate the amount of 125I-labeled SF7 bound in the presence of unlabeled antibody SF7 (O) or 3C1 (●). When factor XI was not bound to the wells of the microtiter plates, the amount of 125I-labeled SF7 was <2% of the control value. The maximum variation of cpm bound for each experiment was <3% of total cpm bound.
was \(6.2 \times 10^{-10}\) mol/L, whereas the corresponding \(k_d\) for antibody 3C1 was \(9.0 \times 10^{-10}\) mol/L. Therefore the differences in concentrations of the two antibodies required to displace \(^{125}\text{I}\)-labeled SF7 from factor XI (Fig 1) are not a consequence of different binding affinities. We conclude that 3C1 is directed at an epitope in the heavy-chain region of factor XI distinct from that recognized by the SF7 MoAb.

**Effects of MoAbs on the procoagulant activity of factor XI.** Because our previous experiments strongly implicate the heavy chain as important in the enzymatic activity of factor XIA\(^1\) and in the activation of factor XI,\(^2\) we compared the effects of antibodies 3C1 and SF7 on factor XI coagulation activity. As shown in Fig 2A, the heavy-chain–specific MoAb SF7 inhibited 100% of factor XI procoagulant activity at a concentration of 30 nmol/L, whereas the 3C1 heavy-chain–specific MoAb resulted in 75% inhibition at a similar concentration.

![Graph](chart1.png)

**Fig 2.** Neutralization of factor XI (panel A) or factor Xla (panel B) in coagulation assays by two different MoAbs, 3C1 (△) and SF7 (○). Panel A: factor XI (2.5 nmol/L) was incubated with either buffer or different antibody concentrations (2 to 42.5 nmol/L) of each antibody for 20 minutes at 37°C before measuring the coagulant activity, as described in Methods. Panel B: factor Xla (2.5 nmol/L) was incubated with either buffer or different antibody concentrations (2 to 40 nmol/L) of each antibody for 20 minutes at 37°C before measuring the clotting time in factor-XI deficient plasma in the absence of kaolin (see Methods for details).

**Effects of Fab’ fragments of MoAbs on the activation of factor XI by factor XIIa in the absence and presence of a negatively charged surface.** Because negatively charged surfaces such as kaolin, sulfatides, or dermatan sulfate are known to enhance the rate of activation of factor XI by factor XIIa,\(^7\) it was of interest to examine the effect of our antibodies on the rate of this reaction. We have previously demonstrated\(^12\) that in the presence of kaolin and HMW kininogen the initial rate of factor-XI activation was enhanced by about fivefold to sixfold compared with the fluid phase reaction. Because we were interested in studying the functions of different domains of factor XI in the surface-mediated activation of factor XI, we determined the effects of MoAb Fab’ fragments both in the absence and in the presence of kaolin and HMW kininogen. Factor XI was preincubated with antibody solution or buffer for ten minutes at 37°C and subsequently added to a mixture of kaolin, HMW kininogen, and factor XIIa. To quantitate the effects of these antibodies, the incubation mixtures were examined for factor Xla amidolytic activity (Fig 3). This shows that in the presence of kaolin and HMW kininogen (panel B), the initial rate of factor XI activation was decreased by only 30% in the presence of the heavy-chain–specific antibody (3C1) Fab’ fragments and by 65% in the presence of the SF7 MoAb Fab’ fragments. In the absence of kaolin and HMW kininogen (panel A), the initial rate of factor XI activation in the absence of antibodies was equivalent to that observed in the presence of antibody SF7, HMW kininogen, and kaolin. Both SF7, and to a lesser extent, 3C1 Fab’ fragments enhanced rates of factor-XI activation in solution, as previously reported for intact 3C1.\(^12\) Control experiments demonstrated that neither MoAb (3C1 or SF7) had any effect on the amidolytic activity of factor Xla; therefore the results in Fig 3 reflect the effects of the antibodies on factor-XI activation and not on the enzymatic activity of the factor Xla generated. Samples of identical incubation mixtures with added \(^{125}\text{I}\)-labeled factor XI were examined by SDS gel electrophoresis, and the percent cleavage of factor XI to heavy-chain (mol wt 50,000) and light-chain (mol wt 30,000) cleavage products was within 10% of the percent factor-Xla amidolytic activity (data not shown) thus confirming the effects of the MoAbs shown in Fig 3.

**Effects of MoAbs on the binding of factor XI to HMW kininogen.** It is well-documented that the heavy chain region of factor XI contains binding sites for HMW kininogen.\(^10,13\) Since the heavy-chain–specific antibodies (3C1 and SF7) inhibit the rate of factor XI activation by factor XIIa in the presence of HMW kininogen and kaolin, we hypothesized that these antibodies might inhibit the binding of factor XI to HMW kininogen, thus preventing the complex formation necessary for efficient activation by factor XIIa. Figure 4 shows the effects of intact IgG and Fab’ fragments of the MoAbs on binding of \(^{125}\text{I}\)-factor XI to HMW kininogen. In this experiment \(^{125}\text{I}\)-factor XI (0.56 nmol/L) was incubated at 20°C for 20 minutes with HMW kininogen bound to microtitre wells in the presence of various concentrations of the SF7 or 3C1 antibodies or their Fab’ fragments. It is apparent from the results that MoAb SF7 can completely
block factor XI binding to HMW kininogen and that 50% inhibition of binding occurred at approximately $5 \times 10^{-10}$ mol/L SF7 (ie, close to the $K_d$ for binding of SF7 to factor XI as determined above). However, 100-fold less Fab' fragment of SF7 (approximately $5 \times 10^{-12}$ mol/L) was required to achieve 50% inhibition of binding of factor XI to HMW kininogen than the intact SF7 MoAb. This suggests that the Fab' fragments may, by virtue of their smaller size, gain better access to the HMW kininogen-binding site. The concentration of 3C1 Fab' fragment required to inhibit factor XI binding to HMW kininogen was 1,000-fold greater ($5 \times 10^{-11}$ mol/L) than that of the SF7 Fab' fragments.

![Fig 3. Effects of Fab' fragments of MoAbs against the heavy chain, 3C1 or SF7, on the activation of factor XI by factor Xlla in the absence (A) or in the presence (B) of kaolin and HMW kininogen. The experiment was done as follows: factor XI (0.17 $\mu$mol/L) was incubated with either PBS, 3C1 Fab' fragments (2.5 $\mu$mol/L) or SF7 Fab' fragments (2.5 $\mu$mol/L) and BSA (1 mg/ml) for ten minutes at 37°C. Subsequently, factor Xlla (0.017 $\mu$mol/L), HMW kininogen (0.07 $\mu$mol/L), and kaolin (0.5 mg/ml) and BSA (1 mg/ml) were added to the mixture to obtain results shown in panel B. The experiment was carried out in panel A exactly as in panel B except that the additions of kaolin and HMW kininogen were omitted. At the times indicated, aliquots were removed into buffer containing corn trypsin inhibitor. Amidolytic assay was performed as described in Methods. Data shown are those obtained with PBS (O), with MoAb 3C1 (A), or with SF7 (△).]

![Fig 4. Effects of MoAbs and Fab' fragments on the binding of $^{125}$I-labeled factor XI to HMW kininogen. $^{125}$I-factor XI (0.56 nmol/L) was incubated with Fab' fragments of antibodies at various concentrations ($10^{-13}$ to $10^{-6}$ mol/L) at 20°C for 20 minutes in microtitre wells with bound HMW kininogen. Binding of $^{125}$I-labeled factor XI to immobilized HMW kininogen was determined as described in Methods. When HMW kininogen was not bound to the walls of the microtitre plates, the amount of $^{125}$I-labeled factor XI was <2% of the control value. The maximum variation of the cpm bound for each experimental observation was <2% of total cpm bound. Data shown are those obtained with SF7 antibody (O) or Fab' fragments (△) or with 3C1 antibody (A) or Fab' fragments (△).]
Effects of MoAbs on the procoagulant activity of factor XIa. The procoagulant activity of factor XIa, determined both in a factor XIa clotting assay and by its capacity to activate factor IX, was examined in the presence of various concentrations of heavy-chain-specific antibodies. As shown in Fig 2B, heavy-chain-specific antibody 3C1 (10 nmol/L), in the absence of kaolin, inhibited 75% of factor-XIa procoagulant activity in a coagulation assay, while heavy-chain-specific 5F7 had no effect. The effect of the antibody Fab' fragments on the rate of activation of [3H]-factor IX by factor XIa are shown in Fig 5. When the initial rates of radiopeptide release were examined, 3C1 Fab' fragments (20 nmol/L) inhibited this initial rate of [3H]-release by nearly 100%, whereas the 5F7 Fab' fragments had no effect. Normal mouse IgG did not inhibit factor XIa either in the clotting assay or in the [3H]-factor IX activation peptide-release assay.

Effects of CNBr peptides of factor XI isolated by affinity chromatography on the binding of factor XI to HMW kininogen and on factor-XI activation by factor XIa. To identify the structural domains of factor XI that bind HMW kininogen and factor IX, CNBr-digested factor XI was passed over 5F7 and 3C1 antibody-affinity columns. As shown in Table 1, CNBr peptides that bound to the 5F7 affinity column (but not the 3C1 column) inhibited binding of [3H]-factor XI to HMW kininogen but did not inhibit factor-XIa-catalyzed factor-IX activation. However, the CNBr peptides that bound to the 3C1 affinity column (but not the 5F7 column) did not inhibit binding of [3H]-factor XI to HMW kininogen but did inhibit factor-XIa-catalyzed factor-IX activation.

The effect of HMW kininogen on the kinetics of activation of factor IX by factor XIa. We have previously shown that antibody 3C1 is a competitive inhibitor of factor-XIa-catalyzed factor-IX activation, suggesting the presence of an enzyme-substrate binding site on the heavy chain of factor XIa.2 We therefore determined whether binding of HMW kininogen to factor XIa has any effect on the activation of factor IX by factor XIa. The Lineweaver-Burk plots of the reaction in the presence and absence of HMW kininogen are shown in Fig 6. The presence of HMW kininogen had virtually no effect on the kinetics of the activation of factor IX by factor XIa.

### DISCUSSION

The present study was undertaken to gain an insight into the nature of the interactions of factor XI with HMW kininogen and of factor XIa with factor IX and to determine what functional domains within the heavy-chain region of factor XI are important in these intermolecular interactions. The term functional domain is used here to denote a molecular region or site in factor XI involved in a specific intermolecular interaction (eg, with HMW kininogen or with factor IX). We have used two MoAbs directed against different epitopes of factor XI as structure-function probes for this purpose. Both antibodies are heavy-chain specific but are directed against different epitopes in the molecule (Fig 1).

It has previously been demonstrated that factor XI circulates in plasma in a noncovalent complex with HMW kininogen.5,6 Since HMW kininogen accelerates the rate of proteolytic activation of factor XI by factor XIIa in the presence of negatively charged surfaces,7,8 it has been postulated that factor XIIa, HMW kininogen, and factor XI form a ternary complex on the surface during contact activation.24 This concept is supported by our previous2 and present studies, which are consistent with the interpretation that antibody 5F7 inhibits surface-mediated factor-XI activation (Figs 2A and 3) by blocking the binding of HMW kininogen to the heavy-chain region of factor XI (Fig 4). The 3C1

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**Table 1. Effects of Isolated Peptides on HMW Kininogen Binding and Factor-IX Activation**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Inhibition of HMW Kininogen Binding*</th>
<th>% Inhibition of Factor-IX Activation*</th>
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<tbody>
<tr>
<td>5F7 Peptide (.05 μmol/L)</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>3C1 Peptide (.05 μmol/L)</td>
<td>0</td>
<td>50</td>
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*The binding of factor XI to HMW kininogen and assays of factor-IX activation were studied using procedures described in Methods.

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**Fig 6.** Effects of Fab' fragments of MoAbs, 5F7 (O) and 3C1 (△), on the activation of [3H]-labeled factor IX by factor XIa. Factor XIa (2.5 nmol/L) was incubated with various concentrations of antibody solutions (2.5 to 25 nmol/L) for 20 minutes at 20°C prior to its use in the [3H]-factor IX activation peptide release assay (see Methods). The final concentrations of factor XIa and [3H]-factor IX in the [3H]-peptide release assay were 0.04 μg/mL and 0.2 μg/mL, respectively.
antibody Fab' fragment also blocks HMW kininogen binding to factor XI but at concentrations 1,000-fold greater than the SF7 Fab' fragment (Fig 4). The 3C1 MoAb Fab' fragment inhibits the activation of factor XI by factor XIIa in the presence of kaolin and HMW kininogen, again to a lesser extent than antibody SF7 (Fig 3B). A possible interpretation of these results is that the binding of MoAbs SF7 or 3C1 to factor XI induces allosteric or conformational alterations that block the capacity of factor XI to bind HMW kininogen. However, the data presented in Table 1 indicate that the CNBr peptide that binds to antibody SF7, but not the 3C1 peptide, inhibits factor XI binding to HMW kininogen. Therefore, although the antibodies may induce allosteric effects (as suggested by their capacity to enhance rates of solution-phase factor-XI activation by factor XIIa), the experiments with isolated peptides (Table 1) support the view that the antibodies also recognize peptides that contain the binding sites in factor XI for HMW kininogen and for factor IX.

The effects of Fab' fragments of the two antibodies on factor XI activation in the fluid phase (Fig 3A) confirm our previous observation that intact 3C1 IgG increases rates of factor-XIIa-catalyzed factor-XI activation and demonstrate that the SF7 antibody has a similar influence. This interesting effect can be attributed to an alteration in conformation of the factor XI molecule when either 3C1 or SF7 binds to the heavy chain to render it a better substrate for factor XIIa.

The two heavy-chain–specific antibodies and their Fab' fragments, as well as their corresponding CNBr peptides, have distinctly different effects on the calcium-dependent, factor-XIIa-catalyzed activation of factor IX. When Fab' fragments of these two antibodies (SF7 and 3C1) were tested for their effects on factor XI in a clotting assay (Fig 2B) and in the factor-IX activation peptide release assay (Fig 5), one of these antibodies (3C1) caused 75% inhibition of factor-XIIa procoagulant activity and 85% inhibition of factor-IX activation, whereas the other (SF7) had no effect on factor-XIIa procoagulant activity or on factor-IX activation by purified factor XIa. Similarly the 3C1 peptide inhibited factor-IX activation, whereas the SF7 peptide did not (Table 1).

Thus, these studies suggest that the epitopes recognized by our two heavy-chain–specific MoAbs are separate and distinct structural sites exposed on the surface of native factor XI (XIa). These epitopes are likely to be in close proximity to functional domains within the heavy-chain region of factor XI (XIa) that are important for the activation of the zymogen and for the expression of the enzymatic activity of factor XIa. It is possible, on the basis of these studies, to formulate the hypothesis that one functional domain, the cofactor (HMW kininogen) binding site, is near the epitope recognized by the SF7 antibody, whereas another functional domain, the substrate (factor IX) binding site, is near the epitope recognized by the 3C1 antibody. Further support for this hypothesis comes from the results of the experiment presented in Fig 6, which shows that the presence of HMW kininogen at 1,000-fold molar excess relative to factor XIa and at equimolar concentration relative to factor IX, has no effect on factor-IX activation by factor XIa. This indicates that the binding of HMW kininogen to the cofactor binding site in the factor XIa heavy chain had no effect on the binding of factor IX to the substrate binding site. Conversely, the presence of factor IX has no effect on factor XI binding to HMW kininogen (unpublished observation). Our hypothesis is further supported by our studies with naturally occurring human antibodies against two distinct functional domains on the heavy chain of factor XI (XIa). One antibody (Baltimore), either as intact IgG or Fab' blocked binding of factor XI to HMW kininogen and the surface-mediated factor-XIIa–catalyzed factor-XI activation while having no effect on factor-XIa–catalyzed factor-IX activation. Another antibody (Winston-Salem), either as intact IgG or as Fab' fragments inhibited factor-XIa–catalyzed factor-IX activation without affecting factor-XI binding to HMW kininogen. The precise location and characteristics of the structural sites representing these functional domains remain to be elucidated.

ACKNOWLEDGMENT

The authors are grateful to Dr Edward P. Kirby, Department of Biochemistry, Temple University School of Medicine, for providing bovine factor XIIa and to Patricia Pileggi for typing the manuscript.

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